Doctoral Thesis

Physiological Responses of Rice to Saline-Alkaline Stress

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September 2021

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List of Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
FAO	Food and Agriculture Organization
GPX	Guaiacol peroxidase
GR	Glutathione reductase
HAs	H^+-ATPases (Adenosine triphosphate)
IRT	Iron (Fe) regulated transporter
MA	Moderate alkalinity
MDA	Malondialdehyde
mM	Millimolar
MSA	Moderate saline alkalinity
MSI	Membrane stability index
NA	Non-acclimating
NaCl	Sodium chloride
NPT	No pretreatment
NSA	Na^+ content under saline alkalinity
PT	Pre-treatment
RDW	Root dry weight
ROS	Reactive oxygen species
SA	Severe alkalinity
SDW	Shoot dry weight
SEL	Shoot electrolyte leakage
SOD	Superoxide dismutase
SPAD	Soil – plant analyses development

SSA	Severe saline alkalinity
UN	United Nations
UNESCO	United Nations Educational, Scientific and Cultural Organization
μM	Micromolar

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Chapter 1

General Introduction

1.1. Background information

Global changes in the climate scenario undeniably pose insurmountable challenge on global food supply system. Formerly agriculturally productive lands are insidiously mutating to irreparably unarable. Yet, global population is steadily increasing, projected to reach an insane 9.8 billion by 2050 (UN 2017). Therefore finding ways to sustain agricultural productivity in light of the prevailing and worsening climate to support the growing population is one of the current's and future's major global hurdles. Persistent droughts, extreme temperatures, soil salinization, and heat stresses have gradually become abiotic norms in the agricultural setting. Soil salinization for one has victimized agriculture since time immemorial, and has remained an important factor constraining worldwide crop productivity (Negrão et al. 2011). As a result, remarkable strides have been made in unmasking plants' responses and tolerance mechanisms to salinity stress. In the same way, soil pH has long been recognized as an important factor influencing plant growth, as such optimal pH ranges for plant growth have been accordingly established. For plant growth purposes, most plant species grow better in slightly acidic to neutral pH range (5.5 to 7.0), and growth is considerably downregulated at alkaline pH, this is generally referred to as "alkaline" stress.

Meanwhile, there is a growing awareness and recognition that salinity stress often co-occurs with alkaline stress (Kawanabe & Zhu 1991). Simply put, this is as a result of soil salts that are both saline (impose Na^+ toxicity) and alkaline (raise soil pH), this is referred to as saline-alkaline stress or mixed saline-alkaline stress, and its effects on crop growth and productivity are cruelly devastating (Islam et al. 2011; Zhang & Mu 2009) than neutral salinity stress. Prior, soil scientists had endeavoured to classify soils based on salt composition. While there has hardly been a consensus in nomenclature, distinctions have been made on what are considered "saline" from "sodic" soils (FAO 2019; Szabolcs & Fink 1974). Based on these, it is generally

agreed that “saline” soils are those containing a substantial amount of “neutral” soluble sodium salts with a capacity to negatively alter plant growth and productivity, in principal, these salts are sodium chlorides (NaCl) and sodium sulphates (Na_2SO_4). Conversely, soils are considered “sodic” when they contain sodium salts that are capable of undergoing “alkaline hydrolysis”, principally, these salts include sodium carbonates (Na_2CO_3) and sodium bicarbonates ($NaHCO_3$). Accordingly, salt stress arising from the former has been termed neutral salinity stress, or simply salinity stress whereas the latter has been termed “alkali” stress (Peng et al. 2008; Szabolcs & Fink 1974). Therefore, these terms will be used interchangeably in the present context. Meanwhile, the need for development of crop cultivars bearing resistance to alkali stress has never been more exigent. Attaining this therefore calls for a comprehensive understanding of plants’ responses governing resistance and adaptability to alkali stress.

1.2. Comparative outlook of salinity, alkalinity and saline-alkalinity

Salinity stress has comprehensively been studied, hence knowledge regarding its effects on plant growth is well documented. Majorly, salinity stress (1) imposes toxicity of Na^+ in plant organs at the expense of beneficial minerals particularly K^+, (2) results in osmotic stress (practically synonymous to “physiological drought”) and (3) elicits hyperaccumulation of reactive oxygen species (ROS) (Munns & Tester 2008; Shabala et al. 2010). Saline-alkaline stress exerts all the above effects, in addition to an inordinately high pH resulting from carbonates which primarily affects root properties (Peng et al. 2008; Yang et al. 2009; Zhang & Mu 2009; Zhang et al. 2019) and absorption of metal cations particularly Fe and P (Kobayashi & Nishizawa 2012; Takahashi et al. 2001). Fe deficiency manifests itself in chlorosis of younger leaves. These latter effects are also imposed by “alkaline” stress arising from non-sodic alkaline salts such as K_2CO_3, $KHCO_3$, $CaCO_3$ and $Ca(HCO_3)_2$.

Na^+ and K^+ have similar physicochemical properties, resulting into their competitive uptake systems and failure to discriminate against their identities under higher salt concentrations. This results into higher preferential Na^+ uptake at the expense of K^+. Na^+ toxicity in leaf blades is typified with premature senescence of older leaves (Munns & Tester 2008). Osmotic effect of salt stress manifests itself in failure to produce new leaves and reduced expansion of young leaves. Plants are surfeited with various strategies and mechanisms to tolerate to salinity, saline-alkaline and alkaline stress, these may be shared across the stress types while others may be distinct. A comparative outlook of salinity, alkalinity and saline-alkaline stress is shown in Table 1.1.

1.3. Growth of rice under saline-alkaline stress

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. However, despite its worldwide agronomical prominence, it is considered the most sensitive cereal crop to salinity stress (Munns & Tester 2008). Coupled with its poor Fe uptake capacities (Masuda et al. 2008 2009; Ishimaru et al. 2007) and leaky nature of its roots (Munns & Tester 2008), it is conceivable that its sensitivity to alkali stress is all the more extreme. Meanwhile, growing evidence ascertain that its response to alkali stress is poorer (Chuamnakthong et al. 2019; Zhang et al. 2017). What remains a fundamental question is whether plants bearing increased tolerance to salinity stress would potentially be better adapted to alkali stress. Lv et al. (2015) reported that a majority of japonica rice cultivars showed tolerance to salinity stress but were sensitive to saline-alkaline stress. This demonstrates either severity of alkali stress over salinity stress or involvement of different mechanisms in tolerance to the two. Earlier, similar findings were also reported (Lv et al. 2013). However, some cultivars demonstrated tolerance to both salinity and saline-alkaline stress. Chuamnakthong et al. (2019) showed that a salinity tolerant

rice cultivar exhibited higher tolerance to saline-alkaline stress by effectively excluding accumulation of Na^+ in the leaf blades.

Recently, Guo et al. (2014) developed an alkali tolerant mutant *alt1* in rice that grows well under saline-alkaline stress with less chlorosis, higher survival rate and better growth through a stable acquisition of metal ions particularly Fe. Increasing number of reports indicate considerable damage elicited by saline-alkaline stress in roots. Being directly exposed to high Na^+ and high pH, roots are particularly at higher risk of damage and injury. Marked increase in root cell injury and expression of cell death related genes have been repeatedly reported (Lv et al. 2013; Zhang et al. 2017). Consequently, preserving better root properties in rice under alkali stress has been shown to correlate with resistance to alkali stress (Guo et al. 2014; Lv et al. 2015). Zhang et al. (2017) showed that a saline-alkaline stress tolerant rice cultivar had better root properties and suffered less lipid peroxidation and cellular damage by efficiently scavenging ROS. In summary, present knowledge permits the conclusion that rice is more sensitive to saline-alkaline stress, or at least that saline-alkaline stress is more severe than neutral salinity stress. Furthermore, it is imperative to learn from plants that are naturally adapted to grow on sodic soils. Meanwhile, a number of such plants have been studied and may therefore present better prototypes for saline-alkaline stress tolerance improvement programs for agriculturally important crops such as rice.

1.4. Growth and ionic responses of other crops to saline-alkaline stress

A number of plant species, halophytes and glycophytes alike are known to have remarkable growth characteristics under salt stress. Among the cereal crops, barley (Garthwaite et al. 2005; Munns & Tester 2008; Shabala et al. 2010) and quinoa (Hariadi et al. 2011) offer excellent prototypes for studying comparative responses to salt stress.

Table 1.1. Comparative outlook of neutral salinity stress, saline-alkaline (alkali) and alkaline stress

	Salt stress		
Characteristics	**Salinity**	**Alkali**	**Alkaline**
pH	5.0 – 6.0	> 7.5	> 7.5
Dominant salts	NaCl, Na_2SO_4	Na_2CO_3, $NaHCO_3$	K_2CO_3, $KHCO_3$, $CaCO_3$, $Ca(HCO_3)_2$
Dominant ions	Na^+, Cl^+, SO_4^{2-}	Na^+, CO_3^{2-}, HCO_3^-	K^+, Ca^+, CO_3^{2-}, HCO_3^-
Main stress components	Osmotic, ion toxicity, oxidative stress	Osmotic, ion toxicity, oxidative stress, High pH induced nutrient deficiency	Oxidative stress, High pH induced nutrient deficiency
Major nutrient deficiencies	K^+, Ca^{2+}	K^+, Ca^{2+}, Fe, P	Fe, P
Symptoms	Limited leaf expansion, failure to develop new leaves, death of older leaves	Limited leaf expansion, failure to develop new leaves, death of older leaves, chlorosis in younger leaves, darker roots	chlorosis in younger leaves, darker roots
Key tolerant strategies	Na^+ exclusion, Osmotic tolerance, Tissue tolerance, ROS scavenging	Na^+ exclusion, Osmotic tolerance, Tissue tolerance, ROS scavenging, Ion balance, Induce Fe-uptake systems	ROS scavenging, Induce Fe-uptake systems

Yang et al. (2009) showed that barley plants were also more sensitive to saline-alkaline stress, but were able to tolerate up to 150 mM Na^+ at a pH above 9.0. Similar observations were shared by other studies (Shi & Yin 1993; Yang et al. 2007; Zhang et al. 2019). Consistent with observations in rice Yang et al. (2009) observed nearly ten-folds reduction in root activity under saline-alkaline stress relative to salinity stress. Similar findings were also reported in alfalfa (Peng et al. 2008), providing independent support for the fundamental role roots play under saline-alkaline stress.

On ionic relations, barley accumulates larger amounts of Na^+ in leaf blades that it effectively sequesters in vacuoles, attaining both turgor and avoidance of cytosolic Na^+ toxicity (Shabala et al. 2010). Under saline-alkaline stress, barley accumulates even more Na^+ (Yang et al. 2009) in leaf blades than that under salinity stress, consistent with observations in rice and wheat (Yang et al. 2008). Halophyte *Kochia sieversiana* is naturally adapted to saline-alkaline soils. Yang et al. (2007) studied its growth and tolerance to saline-alkaline soils, and reported that the halophyte can grow at up to 400 mM soil Na^+.

Strikingly, in *K. sieversiana* uptake and transport of Na^+ to the shoots were similar between salinity stress and saline-alkaline stress. Not only that, increases in soil Na^+ concentration under both salinity and alkali stress were associated with concomitant increases in both Na^+ and K^+ in shoots, a unique and striking feature. This was also earlier observed in another halophyte Sea buckthorn (Chen et al. 2009). These observations signal lack of competitive inhibition between Na^+ and K^+, highlighting a distinctive pathway for K^+ absorption in halophytes. While this demands further in-depth inquiry, it undeniably provides for a splendid learning and starting point for the improvement of alkali tolerance in glycophytes.

1.5. Na^+ homeostasis under saline-alkaline stress

Saline-alkaline stress is characterized by toxicity of Na^+ in plant organs, hence plants' ability to minimize uptake and transport of Na^+ to shoots is an important feature of tolerance. Comparative studies have shown that rice plants accumulate significantly higher contents of Na^+ in leaf blades under saline-alkaline stress than neutral salinity (Cao et al. 2020; Yang et al. 2009; Yang et al. 2008). These observations instigate the thinking that high pH under alkali stress either reduces plant roots' ability to control Na^+ uptake or compromises plant roots' ability for Na^+ extrusion.

In rice, extrusion of Na^+ from roots into the rhizosphere is mediated by the plasma membrane Na^+/H^+ antiporter *OsSOS1* that exchanges cytoplasmic Na^+ for external H^+ (Munns & Tester 2008; Zhu 2003). This banks on transmembrane proton gradient between cytosol and rhizosphere, generated by H^+ pumps (H^+-ATPases) (Duby & Boutry 2009; Moriyama 2015) which is otherwise deficient under high pH conditions, consequently impairing extrusion of Na^+ into the external environment. Cao et al. (2020) has identified *Zea mays* L. *Na^+ content under Saline Alkaline 1 (ZmNSA1*), a Ca^{2+} dependent gene that mediates lower shoot Na^+ accumulation by promoting H^+-ATPase (HAs) activity and consequent root Na^+ efflux. This is an important revelation for saline-alkaline studies, revealing important insights into high pH induced hyperaccumulation of Na^+ in leaf blades.

1.6. Fe uptake under saline-alkaline stress

Plants growing under high pH conditions have long been characterized by Fe deficiency chlorosis (Pushnik et al. 1984), hence dominating efforts aiming to increase tolerance of plants to alkaline soils have entailed improvement in Fe uptake (Gómez-Galera et al. 2012; Kobayashi et al. 2008 2007; Ogo et al. 2011; Takahashi et al. 2001; Ishimaru et al. 2007). Despite being

naturally abundant in the soil, Fe forms highly insoluble ferric compounds under high pH resulting into decreased bioavailability for plant uptake (Nakanishi et al. 2004). Chuamnakthong et al. (2019) reported higher differential expression of Fe deficiency responsive genes (*OsNAS1, OsNAS2, OsIRT1* and *OsIRO2*) under saline-alkaline stress in a tolerant rice cultivar. Li et al. (2016) also attributed the tolerance of a saline-alkaline tolerant rice cultivar to efficient Fe acquisition. These findings emphasize the crucial role enhanced Fe uptake plays under saline-alkaline stress hence its utilization in interventions for increasing resistance.

Roots of graminaceous plants such as rice secrete mugineic acids (MAs), a family of phytosiderophores that solubilize ferric iron (Fe^{3+}) forming Fe(III)-MA complexes that are subsequently taken up by plant roots (Ishimaru et al. 2006; Takagi 1976) using Fe(III)-MA transporter, *YS1/ YSL*, this is referred to as strategy II. Transgenic approaches have been explored by utilizing genes involved in synthesis of mugineic acids from barley into rice such as *HvNAAT-A* and *HvNAAT-B* (Takahashi et al. 2001) and *YS1/YSL* that codes for transporter of Fe(III)-MA complexes into the root cells (Gómez-Galera et al. 2012).

Other transgenic strategies to introduce genes involved in strategy I of Fe uptake have also been explored following revelations that rice plant roots can also take up Fe^{2+} directly using the *Iron regulated transporter OsIRT1* (Bughio et al. 2002; Ishimaru et al. 2006) especially under anaerobic conditions. These include introduction of a yeast chelate reductase gene, *refre1/372* (Ishimaru et al. 2007). Put together, these studies highlight the essentiality enhanced Fe uptake offers under alkaline conditions, and should thus be a fundamental attribute efforts aiming to improve rice plants' resistance to saline-alkaline stress should explore.

1.7. Abiotic stress, reactive oxygen species generation and oxidative stress in plants

An inevitable consequence of aerobic metabolism is the accumulation of ROS, extremely harmful radicals that are overproduced under environmental stresses (Apel & Hirt 2004; Kamanga et al. 2018; Mekawy et al. 2018; Sharma et al. 2012). ROS are kept in check by the synthesis of enzymatic and non-enzymatic antioxidants that detoxify them, keeping their concentrations optimal for beneficial purposes (Sharma et al. 2012). However, under stressed environments, synthesis of ROS is uncontrollably increased (Apel & Hirt 2004) past plants' detoxification capacity thereby putting the plant in a state of oxidative stress. Under such a state, ROS can potentially cause peroxidation of lipids, protein damage and programmed cell death (Apel & Hirt 2004; Sharma et al. 2012).

Under saline-alkaline stress, most studies have associated the sensitivity of plants to root damage caused by high pH environment in combination with Na^+. Being directly exposed to the saline-alkaline environment, roots are particularly at a higher risk of damage as shown by the remarkable reductions in root activity in a range of plant species (Peng et al. 2008; Yang et al. 2009; Zhang & Mu 2009; Zhang et al. 2019). Zhang et al. (2017) showed a significant accumulation of H_2O_2 and $O_2^{\bullet-}$ in roots exposed to saline-alkaline stress. Furthermore, Guo et al. (2014) found that in an alkaline tolerant rice mutant (*alt1*) 50 out of 78 abiotic stress related differentially expressed genes were involved in generation and detoxification of ROS and repair of ROS mediated DNA damage. It is reasonable therefore, that alkali stress tolerance in breeding programs should consider designing plants that are able to avoid ROS production, detoxify ROS and or repair ROS mediated damage, these three processes may translate into an oxidative stress tolerance strategy.

1.8. Study rationale

Global population is estimated to reach 9.8 billion by 2050 (UN 2017), posing a striking socio-economic challenge; to sustainably support the ever growing population. Rice is a prominent cereal crop serving as a top staple food crop for more than half of the world's population. Notwithstanding its agronomical and food value, it is also regarded as the most sensitive cereal crop to salt stress (Munns & Tester 2008). Moreover, its growth is considerably impeded by high pH, in part owing to its poor Fe uptake capacities despite being equipped with a dual Fe uptake system (Ishimaru et al. 2006; Masuda et al. 2008; Takahashi et al. 2001).

Saline-alkaline stress is supposedly more devastating, exhibiting effects of both salinity (Na^+ toxicity, osmotic stress and oxidative stress) and alkalinity (high pH). However, unlike sole salinity and alkalinity stress, rice plants responses to saline-alkaline stress have not been clearly elucidated. In order to develop rice crop cultivars tolerant to saline-alkalinity, an understanding of how saline-alkalinity stress affects survival, growth and physiology of rice plants is imperative. Present knowledge on rice plants' responses to sole salinity and sole alkaline stress is vast, hence comparative studies with saline-alkalinity may offer an optimal baseline for studying effects of saline-alkalinity.

Furthermore, it is imperative to explore quicker and cost effective options of enhancing rice plants' tolerance to saline-alkaline stress. Plants are known to develop tolerance to abiotic stress following pre-exposure to a mild level of similar abiotic stress, a process known as acclimation or acquired resistance. Furthermore, plants pre-exposed to one abiotic stress also reportedly develop tolerance to another type of stress, this is referred to as induced cross-tolerance. Hence, this study further attempts to explore an option for inducing cross-tolerance to saline-alkaline stress in rice plants by pre-exposure to salinity stress, oxidative stress and saline-alkalinity.

1.9. Study objectives

The study was conducted to achieve the following overarching objectives;

1. To investigate comparative physiology of neutral salinity, alkalinity and saline-alkaline stresses in rice.
2. To study acclimation induced cross-tolerance of rice seedlings to saline-alkaline stress pre-exposed to mild salinity, oxidative and saline-alkaline stress.
3. To investigate the role of pre-treatments in post saline-alkaline stress recovery of rice.

Chapter 2

Time-Course Study on Differential Responses of Rice Seedlings to Saline, Saline-Alkaline, and Alkaline Stress

2.1. Introduction

Land salinization and alkalinisation are widespread agricultural problems worldwide and have thus haunted mankind for centuries. Relative to neutral salinity stress, saline-alkaline stress is considerably detrimental. Meanwhile, effects of salinity stress on crop growth and productivity have been comprehensively studied in nearly all major crop plants, rice inclusive. Notwithstanding recent increases in land saline-alkalinisation and its associated aggravated effects on crop growth compared to those of neutral salinity stress, plants' responses to saline-alkaline stress are poorly understood. A large number of reports indicate that saline-alkaline stress is much more harmful than either sole salinity stress or sole alkaline stress, insinuating a cooperative effect of their co-occurrence on plants (Chan et al. 2003). As a result, plants have over the years developed sophisticated mechanisms to withstand both high Na^+ toxicity and high pH. Unlike most halophytes, glycophytes prioritize prevention of Na^+ accumulation in sensitive organs such as leaf blades and opt to partition inevitably accumulated Na^+ in the vacuoles albeit less adept. Na^+ is a toxic element in plant cells, considerably interfering with cytosolic metabolic activities, hence sequestration in the vacuole not only averts its toxicity in the cytosol but also serves as a cheap osmoticum aiding in turgor maintenance. Meanwhile, rice (*Oryza sativa* L.) has a well-known reputation for its tremendous sensitivity to salt stress (Munns & Tester 2008), hence, coupled with the leaky nature of its roots, high pH may further weaken its root properties and consequent growth and development under saline-alkaline stress.

Decreases in root activity, compromised root cell vigour, deteriorated root cell viability and aggravated cell death have often been reported under saline-alkaline stress (Guo et al. 2014; Lv et al. 2015; Lv et al. 2013; Zhang et al. 2017). Reactive oxygen species (ROS) accumulation is invariably implicated in decreased root cell viability and vigour, and altered cell membrane properties (Guo et al. 2014; Zhang et al. 2017), hence have remained central to investigations

of plants' responses to saline-alkaline stress. Moreover, high pH substantially constrains bioavailability of essential micronutrients particularly Fe that forms insoluble ferric complexes (Nakanishi et al. 2004), making it non-bioavailable for plant uptake. This is despite rice uniquely having two Fe uptake pathways; the strategy I and strategy II (Ishimaru et al. 2006; Masuda et al. 2008; Takahashi et al. 2001). Therefore, it is hypothesized that its sensitivity to mixed saline-alkaline stress would all the more be extreme. Presently, information regarding comparative performance of rice to neutral salinity, alkaline (high pH) and saline-alkaline stress is quite sketchy. This study therefore attempts to highlight key growth and physiological differences governing responses of rice to salinity stress, alkaline stress and saline-alkaline stress to inform formulation of approaches to enhance tolerance of rice to saline-alkaline stress by targeting key physiological and molecular signatures principally affected by saline-alkaline stress.

2.2. Study objectives

The study was carried out to achieve the following specific objectives;

1. To study comparative time-course changes in growth of rice seedlings to neutral salinity stress, alkalinity stress and saline-alkalinity stress.
2. To elucidate differences in Na^+ toxicity and high pH induced chlorosis under salinity stress, alkalinity stress and saline-alkalinity.
3. To elucidate comparative physiological mechanisms of damage of salinity, alkalinity and saline-alkalinity in rice plants.

2.3. Materials and methods

2.3.1. Plant material and growth conditions

Rice (*Oryza sativa* L.) seeds for the cultivar Hinohikari were incubated in tap water at 60 ^{o}C for 10 minutes and then surface sterilized with 0.1 % (v/v) Benlate solution for 30 minutes and rinsed thoroughly with distilled water. Thereafter, seeds were incubated in tap water for 24 h at 30 ^{o}C. The seeds were then transferred onto a nylon mesh wire floated in a tap water filled bucket for one week, at a water temperature of 28 ^{o}C. After one week, slightly modified Kimura B nutrient solution was added to water with the following nutrient composition in µM; 365 $(NH_4)_2SO_4$, 547 $MgSO_4$ $7H_2O$, 183 KNO_3, 365 $Ca(NO_3)_2$ $4H_2O$, 185 KH_2PO_4, 28 $FeSO_4$ $7H_2O$ was used in place of Fe-EDTA, 48.7 H_3BO_3, 9.0 $MnSO_4$ $5H_2O$, 0.3 $CuSO_4$ $7H_2O$, 0.7 $ZnSO_4$ $7H_2O$, 0.1 Na_2MoO_4. The experiment was conducted in the greenhouse at Hiroshima University's faculty of Applied Biological Sciences experimental field under hydroponic conditions. Hydroponic culture temperature was maintained at 28 ^{o}C using water heaters, average day air temperature was 30 ^{o}C. After 18 days, seedlings were transplanted from nylon mesh wire to six buckets. Treatment conditions were induced 8 days later as follows; Controls (0 mM Na^+, pH 5.5), salinity (50 mM Na^+, pH 5.5), moderate saline-alkaline (50 mM Na^+, pH 7.5), severe saline-alkaline (50 mM Na^+, pH 8.5), moderate alkalinity (0 mM Na^+, pH 7.5), and severe alkalinity (0 mM Na^+, pH 8.5). Chemical composition for the treatment conditions is shown in Table 2.1. below. pH changes were monitored on daily basis using a pH meter (AS700, As One Corp., Osaka, Japan) and were adjusted using 2 N HCl and 2 N KOH throughout the growth period. Nutrient solution was changed every 8 days, water lost through evapotranspiration was replaced by addition of tap water.

Table 2.1. Treatment conditions and chemical compositions

No.	Treatment		Supplements	pH
1	Control	0 mM Na, pH 5.5	0 mM NaCl	5.5
2	Salinity	50 mM Na, pH 5.5	50 mM NaCl	5.5
3	Moderate saline-alkaline (MSA)	50 mM Na, pH 7.5	3 mM $NaHCO_3$ + 47 mM NaCl	7.5
4	Severe saline-alkaline (SSA)	50 mM Na, pH 8.5	48 mM $NaHCO_3$ + 1.0 mM Na_2CO_3	8.5
5	Moderate alkaline (MA)	5 mM K, pH 7.5	4 mM $KHCO_3$ + 1 mM KCl	7.5
6	Severe alkaline (SA)	5 mM K, pH 8.5	0.6 mM K_2CO_3 + 3.8 mM KCl	8.5

2.3.2. Determination of fresh weight, dry weight and water content

Seedlings were harvested in replicates of four prior to imposition of stress treatments (Day 0), 24 hours after stress imposition (Day 1) and subsequently every four days up to the 16th day. Plant height and root length were measured using a measuring ruler. Plants were then dissected into leaf blades, leaf sheaths and roots and immediately measured for fresh weight (FW). Dry weights (DW) were obtained after oven drying the samples for 72 hours at 70^{o}C. Water content in leaf blades and roots was obtained as follows

$$Water\ content\ (\%) = \frac{FW - DW}{DW}\ x\ 100$$

2.3.3. Chlorophyll concentration (SPAD)

Leaf blade chlorophyll concentration was determined using a chlorophyll meter (SPAD – 502, Minolta Camera Co. Ltd., Osaka, Japan) on three fresh fully expanded leaf blades of the same positions in all plants according to Kamanga et al. (2020).

2.3.4. Determination of macro and micronutrient elements

Elemental analysis was performed on dried leaf blades and root samples after 16 days of stress imposition using inductively coupled plasma-optical emission spectrometry (ICP-OES). For this purpose, 100 mg of dried leaf blades and roots were crushed using a freeze crusher (µT – 48, Taitec, Saitama, Japan). Acid digestion was conducted using 2 mL of H_2SO_4 followed by repetitive cycles of 1 mL of H_2O_2 addition and heating in a heat block at 150–200^oC until the sample turned colourless. An extra hour of heating was performed to remove the remaining H_2O_2 after which the sample was filled to 25 mL using Milli-Q water. ICP analysis was performed using iCAP – 6300 (Thermo-Scientific, Massachusetts, USA) at wavelengths 769.8, 818.3, 422.6, 285.2, 257.6, 213.8, 324.7 and 259.9 nm for K, Na, Ca, Mg, Mn, Zn, Cu and Fe respectively. Quantification was performed using various elemental standard solutions prepared in 1 % HNO_3.

2.3.5. K^+ leakage and Na^+ efflux experiment

In order to determine plant roots' ability to retain K^+, leakage of K^+ was assessed by a method previously described in Pandolfi et al. (2016) with slight modifications. For this purpose, plants were pretreated with various pre-treatments for 7 days, at the conclusion of which, four uniform seedlings of pretreated and non-pretreated plants were transferred into 50 mL falcon tubes containing 30 mL of 50 mM Na^+, pH 8.5 (48 mM $NaHCO_3$ + 1 mM Na_2CO_3). Four more seedlings were transferred into 30 mL deionized water as control plants. After 24 hours, solution was sampled for K^+ analysis.

Efflux of Na^+ from roots was assessed by a previously outlined method (Pandolfi et al. 2016; Shabala et al. 2010), for this purpose, 7 day pretreated and non-pretreated plants were subjected to 30 mL of 50 mM Na^+, pH 8.5 for 24 hours, after which plants were transferred into bathing medium containing 0.1 mM $CaCl_2$, 0.5 mM KCl and 50 mM Na^+, pH 8.5. After 1 hour, the

solution was poured off, roots were rinsed with 10 mM $CaCl_2$ three times. Then, the roots were transferred into 10 mL bathing medium containing 0.1 mM $CaCl_2$ and 0.5 mM KCl for 4 hours. The solution was then sampled and Na^+ concentration was measured. Both K^+ and Na^+ analysis were done using flame photometry (ANA – 135, Tokyo Photoelectric, Tokyo, Japan).

2.3.6. Determination of hydrogen peroxide concentration

H_2O_2 concentration was determined using ground frozen samples based on ferrous oxidation of xylenol orange (FOX) method (Kaur et al. 2016) with slight modifications (Mekawy et al. 2018). For this purpose, 100 mg of frozen leaf blade and root samples were ground using mortar and pestle in liquid nitrogen, and homogenized with 1 mL cold acetone. The homogenate was centrifuged at 8000*g* for 15 minutes at 4°C, and 200 µL of the supernatant was added to 2 mL FOX reagent comprising 0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 125 µM xylenol orange, and 10 mM sorbitol. The reaction mixture was left at room temperature for 1 hour, afterwards, H_2O_2 levels were determined spectrophotometrically at 560 nm and quantified using H_2O_2 standard solutions.

2.3.7. Membrane integrity measurements

Membrane integrity was measured using electrolyte leakage method at the end of 16 days of main stress. Freshly harvested leaf blade disks and roots (200 mg) were cut into 2 cm pieces and fully immersed into 25 mL deionised water in 50 mL falcon tubes and kept at room temperature for 24 hours. Electrical conductivity (EC) was then obtained (EC1) using an EC meter (CM-31P, Toa DKK Co., Tokyo, Japan). Immediately, the samples were killed by incubating at 100 ^oC for 15 minutes, cooled and EC2 was obtained. Electrolyte leakage rate was calculated according to Farooq & Azam (2006).

2.3.8. Principal component analysis (PCA).

PCA was analysed using R studio (RStudio Team 2020) to identify traits associated with plants growing under salinity stress, alkalinity and saline-alkalinity.

2.3.9. Statistical analyses

The experiment was arranged in a Completely Randomized Design (CRD). Each measurement was done in biological replicates of 4. Data collected was subjected to analysis of variance (ANOVA) at 0.05 level of significance. Multiple comparison tests were performed using Tukey Test. All data was analysed using R studio (RStudio Team 2020).

2.4. Results

2.4.1. pH changes in hydroponic cultures

Prior to imposition of stress treatments, various salt supplement proportions (50 mM Na^+, 5 mM K^+) were tested by addition to 1x Kimura B nutrient solution and measuring their resultant pH for alkaline and saline-alkaline treatments. Salt supplements were considered ideal for the particular treatment if their pH just after addition to 1x Kimura B was within ± 0.10 of the required pH. Required and the obtained pH values were shown in Table 2.2. pH changes were monitored every 24 hours. The average pH values were 5.0, 5.1, 7.8, 8.7, 7.8 and 8.5 for controls, salinity stress, moderate saline-alkaline (MSA) stress, severe saline-alkaline (SSA) stress, moderate alkaline (MA) stress and severe alkaline (SA) stress, respectively (Table 2.2). The pH evolution during the growth period indicate that for MSA, SSA, and MA treatments pH increased to much severer levels than required.

2.4.2. Growth

After four days of stress treatments, growth nearly stopped under SSA stress, however differences were insignificant from all other treatments until day 8 when differences became

apparent on which MA, SA, MSA, and SSA had significantly shorter plants compared with controls and salinity stressed plants. However control plants were comparable to salinity stressed plants until day 16 (Fig. 2.1A – D and Fig. 2.2). Shoot (SFW) and root fresh weights (RFW) became significantly ($P < 0.05$) different on day 12 and day 16. On day 12, SFW and RFW were significantly lower under SSA treatment whereas no differences were shown among MA, SA and MSA, but were significantly lower than controls and salinity treatment. At day 16, SFW for both saline-alkaline treatments decreased as older leaves begun to die off. Root length for salinity stressed plants was comparable to controls throughout the treatment period whereas plant height diverged at 12^{th} day of stress imposition, decreasing significantly under salinity stress (Fig. 2.1E and F). On overall, biomass results show that the cultivar Hinohikari displayed some level of tolerance to salinity stress for up to 12 days, but was considerably sensitive to high pH treatments, particularly when combined with salinity (saline-alkaline). Typical features of all high pH treatments were darkening of roots and yellowing of younger leaves (Fig 2.2), however both of which were severer under moderate than severe treatment conditions.

2.4.3. Chlorophyll concentration

In order to distinguish chlorosis resulting from high pH – induced essential element deficiencies from that emanating from leaf Na^+ toxicity, chlorophyll concentration was measured in both younger and older leaves representing younger and older leaves respectively. From stress imposition to day 4, no visual differences were observed among treatments, and so was chlorophyll concentration in both younger and older leaves (Fig. 2.3A and B). At day 8, younger leaves of high pH treatments (MSA, SSA, MA, SA stress) became chlorotic, level of chlorosis was significantly higher in sole alkaline (MA and SA) treatments (Fig. 2.3A) indicating essential element deficiency. On day 16, chlorophyll concentration in salinity stress, MSA and SSA stress also drastically dropped. The lowest values were in MA, SA & MSA

stress (Fig. 2.3A). In SSA stress, new leaf formation had completely stopped hence their upper leaves were relatively older with less chlorosis (Fig 2.3A). In lower (older) leaves, chlorophyll concentration remained relatively stable from stress imposition until day 16, on which SSA stress had drastically lower chlorophyll concentration as the older leaves were nearly dying (Figs. 2.2 and 2.3B). The distinct patterns in chlorosis between lower and upper leaves among treatments suggest that the underlying causes was different.

2.4.4. Water content

In leaves, after stress imposition for 12 days, no significant differences were shown in water content among the treatments. Leaf blade water content became significantly lower in both saline-alkaline treatments (MSA and SSA) at day 16 dropping to 64 and 58% respectively, indicative that leaf blades were drying up and dying (Fig. 2.3C). Conversely, leaves of plants grown under high alkalinity (MA and SA) and salinity stress remained turgid and fresh comparable to controls (Figs. 2.2 and 2.3C). In roots, differences also became more apparent at day 16, however, root water content was lowest in alkaline treatments, particularly in moderate alkaline treatment (Fig. 2.3D). No differences were shown in root water content between controls and salinity stressed plants, and between MSA and SSA stress (Fig. 2.3D).

2.4.5. Macronutrient concentrations

K^+ concentration in leaf blades was significantly reduced by all Na treatments (Salinity, MSA and SSA) but was unaffected by MA stress and increased by severe alkalinity (SA) (Fig. 2.4A). However, in leaf sheaths, K concentrations were reduced regardless of treatment, but the decrease was less pronounced under salinity stress and alkaline stress, and extreme under saline-alkaline stress (Fig. 2.4B). In roots, saline-alkaline treatments significantly decreased K concentration followed by salinity stress (Fig. 4C). Na concentration was extremely high in leaf blades and sheaths under saline-alkaline treatments, whereas under sole salinity stress,

concentrations were statistically comparable to controls (Fig. 2.4D and E). In roots however, no differences were shown between salinity stress and saline-alkaline treatments (Fig. 2.4F). Ca concentration was not affected by salinity stress and SSA stress, but was increased by MSA stress and both alkaline treatments in leaf blades (Fig. 2.5A). In leaf sheaths, all treatments except salinity and severe alkaline stress reduced Ca concentration (Fig. 2.5B). In roots, Ca concentration was considerably increased by all high pH treatments, increases were more pronounced with increases in growth medium pH, accumulating to about 9 folds higher under severe saline-alkaline compared to controls (Fig. 2.5C). In leaf blades and sheaths, Mg concentration followed a similar trend as Ca (Fig. 2.5D and E), however, in roots, Mg concentration was significantly reduced by all high pH treatment by the same magnitude, whereas salinity stressed plants accumulated similar amount of Mg as did control plants (Fig. 2.5F).

2.4.6. K^+ leakage and Na^+ efflux from the roots

It was endeavoured to investigate whether the lower leaf K concentration shown in Fig 2.4A was as a result of K leakage from the roots. It was shown that both salinity and saline-alkaline stress elicited much higher leakage of K from the roots, however the leak was extreme under saline-alkaline stress affirming the K deficiency observed in leaf blades (Fig. 2.6A). Secondly, root Na^+ efflux was measured to check whether efflux of Na is perturbed by high pH under saline-alkaline stress as hypothesized by Cao et al. (2020). Results showed that while both stresses triggered significant Na efflux from the roots, the efflux was actually much higher in saline-alkaline stressed plants compared to salinity stress (Fig. 2.6B), invalidating the hypothesis that higher leaf Na was as a result of reduced root Na efflux.

2.4.7. Micronutrient concentrations

All high pH treatments significantly reduced leaf blade Fe concentrations. Under salinity stress, Fe concentration was also significantly reduced (Fig. 2.7A). In leaf sheaths, only high pH treatments suffered remarkable reductions in Fe concentration (Fig. 2.7B). In roots, all high pH treatments experienced remarkable reductions, but Fe concentrations were over 10 times higher than in leaf blades. Mn concentration in leaf blades was significantly higher in salinity, moderate alkaline and moderate saline-alkaline stressed plants than control plants. However, under severe saline-alkaline treatment, Mn concentration was significantly reduced, whereas alkaline stress was comparable to controls (Fig. 2.7D). In leaf sheaths, Mn concentration was considerably lowered by all stress treatments, and the decreases were more pronounced with increases in pH (Fig. 2.7E). However, in roots, control and salinity stressed plants had significantly lower Mn concentration, whereas concentrations were considerably increased by high pH treatments (Fig 2.7F). In leaf blades effect of the stress factors on Zn concentration depended on the severity of the medium pH. Under pH 5.5, Zn concentration was similar between control and salinity stressed plants, but was considerably increased by moderately high pH (pH 7.5) by 2.6 and 2.4, respectively, whereas under severely high pH (pH 8.5), Zn concentration was unaffected (Fig. 2.8A). In leaf sheaths, no significant changes in Zn concentration were observed except under severely high pH 8.5 (SA and SSA stress) (Fig. 2.8B). In roots, Zn concentration was unaffected by salinity but was considerably increased in all high pH treatments (Fig. 2.8C). Cu concentration was increased by salinity stress in leaf blades whereas all high pH treatments drastically decreased it, decreases were more pronounced under severe saline-alkaline stress (Fig. 2.8D). In leaf sheaths, Cu concentration was reduced by MSA, SA and SSA stress by 58.0, 66.0 and 76.8, % respectively (Fig. 2.8E). In roots, all high pH treatments considerably decreased Cu concentration, with decreases being more pronounced under alkaline treatments, whereas salinity stress did not affect it (Fig. 2.8F).

2.4.8. Correlation of SPAD values with Fe and Na concentration

In order to clarify the causes of leaf blade chlorophyll concentration loss in plants subjected to different stresses, Pearson correlation analysis was performed. It was shown that chlorophyll concentration ($R = 0.73$, $P < 0.00084$) in younger leaf blades significantly correlated with leaf blade Fe concentration (Fig. 2.9A) signifying that in younger leaf blades, Fe deficiency was the prime cause of chlorosis. In older leaf blades, there was a significant negative correlation ($R = -0.72$, $P < 0.00072$) between chlorophyll concentration and Na concentration (Fig. 2.9B). Therefore, in older leaf blades, Na accumulation in leaf blades contributed significantly to chlorosis.

2.4.9. Membrane integrity parameters

Saline-alkaline treatments (MSA and SSA) caused remarkable leakage of electrolytes in leaf blades, significantly higher than alkaline treatments (MA and SA) and salinity stress (Fig. 2.10A). Electrolyte leakage in salinity stressed plants was comparable to controls. In roots, leakage of electrolytes was generally higher than in leaves, except for saline-alkaline treatments (Fig. 2.10B). Nonetheless, root electrolyte leakage was highest in SSA, however differences were not as pronounced. Membrane injury in leaves showed significantly higher injury in saline-alkaline treatments but were significantly lower under salinity stress and alkalinity stress (Fig. 2.10C). In roots, severe saline-alkalinity also caused considerable membrane damage whereas the rest of the treatments showed insignificant differences (Fig. 2.10D).

2.4.10. H_2O_2 concentration

H_2O_2 concentration was much higher in leaves than in roots (Fig. 2.11A and B). In leaves, severe saline-alkaline treatment caused significantly higher H_2O_2 accumulation followed by moderate saline-alkaline and salinity treatments. Alkaline treatments did not induce any

significant H_2O_2 accumulation in leaf blades wherein concentration was similar to controls (Fig. 2.11A). In roots however, moderate alkalinity significantly induced H_2O_2 accumulation followed by severe saline-alkaline stress (Fig. 2.11B).

2.4.11. Principal component analysis

Principal component analysis was performed using several traits of plants growing under all treatment conditions. It was shown that plants growing under saline-alkaline conditions (MSA and SSA stress) were associated with higher values of Na^+ concentration in both leaf blades and roots, H_2O_2 concentration in leaf blades and membrane damage in shoots and roots (Fig. 2.12) and lower values of SDW, K concentration and chlorophyll concentration.

Table 2.2. Chemical composition of various treatment conditions and their respective actual pH, targeted pH and average daily pH values.

No.	Treatment	Chemical composition	pH		
			pH*	pH†	pH‡
1	Control	-		5.5	5.0
2	Saline	50 mM NaCl		5.5	5.1
3	Moderate saline-alkaline (MSA)	3 mM $NaHCO_3$ + 47 mM NaCl	7.32	7.5	7.8
4	Severe saline-alkaline (SSA)	48 mM $NaHCO_3$ + 1.0 mM Na_2CO_3	8.50	8.5	8.7
5	Moderate alkaline (MA)	4 mM $KHCO_3$ + 1 mM KCl	7.51	7.5	7.8
6	Severe alkaline (SA)	0.6 mM K_2CO_3 + 3.8 mM KCl	8.55	8.5	8.5

* The actual pH after adding chemicals to 1x Kimura B.

† The required/ target pH.

‡ Average pH at 24 hours after adjusting to required range (before adjusting)

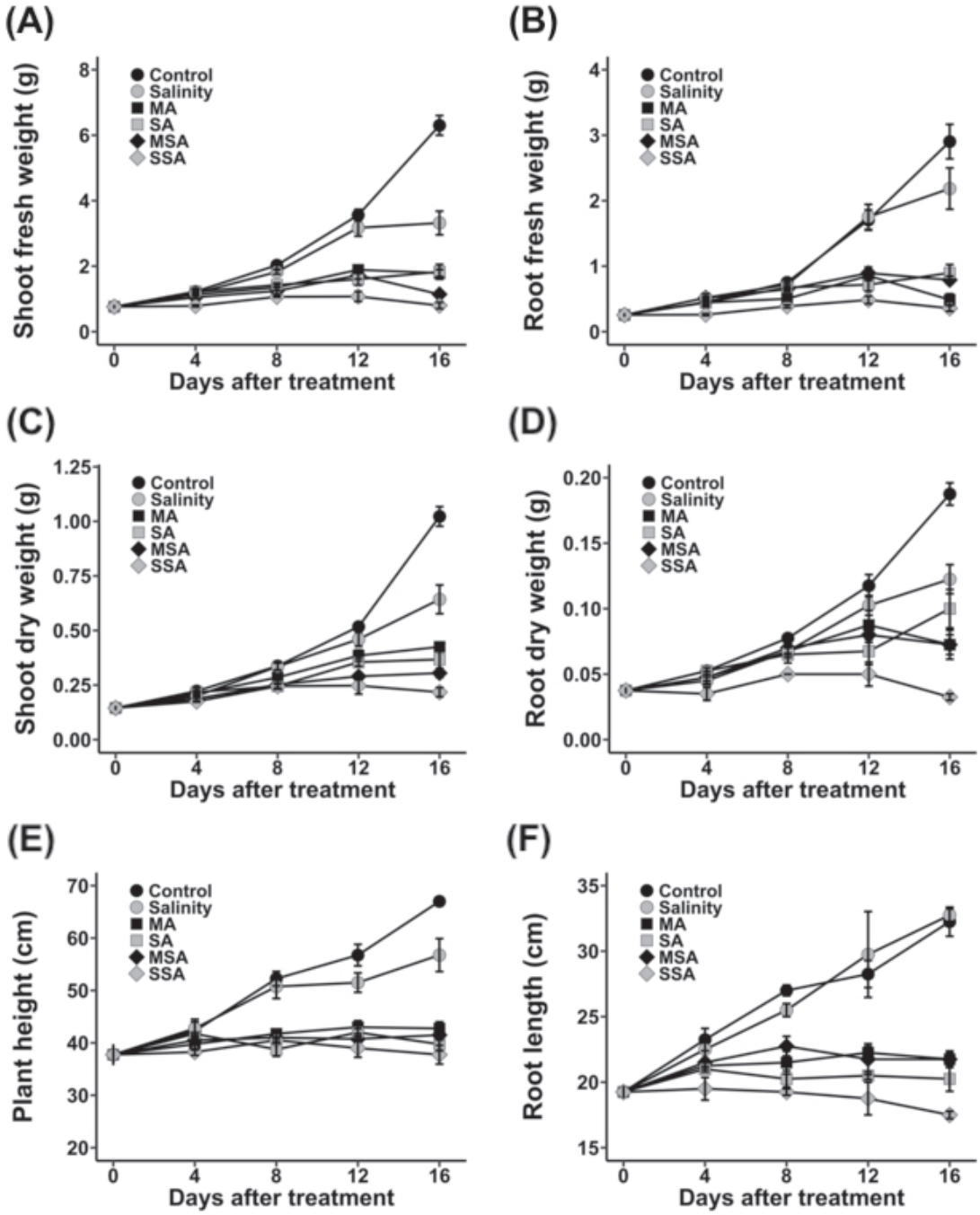

Fig. 2.1. Time course changes in growth of rice plants to salinity, saline-alkalinity and alkalinity showing shoot fresh weight (A), root fresh weight (B), shoot dry weight (C), root dry weight (D), plant height (E) and root length (F). Plants were exposed to salinity stress, moderate alkalinity (MA), severe alkalinity (SA), moderate saline-alkalinity (MSA) and severe saline-alkalinity (SSA) for 16 days. Data shown was taken every four days and represents means of 6 biological replicates.

Fig. 2.2. Comparative shoot and root growth of rice plants showing photograph of plants after 16 days of being exposed to neutral salinity stress, moderate saline-alkalinity (MSA), severe saline-alkalinity (SSA), moderate alkalinity (MA) and severe alkalinity (SA).

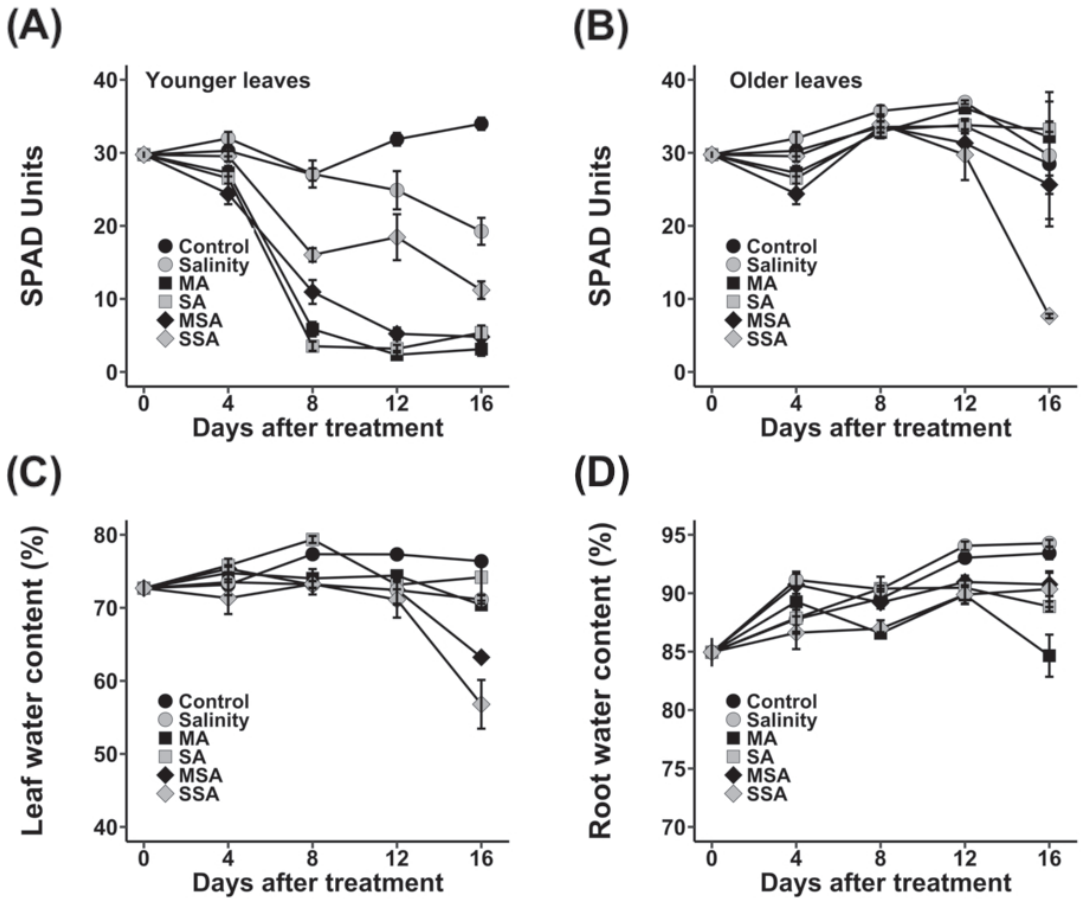

Fig. 2.3. Time-course physiological responses of rice plants to salinity, saline-alkalinity and alkalinity showing leaf chlorophyll concentration measured as SPAD units in younger upper leaves (A) and in older lower leaves (B); leaf water content (C) and root water content (D). Plants were exposed to salinity stress, moderate alkalinity (MA), severe alkalinity (SA), moderate saline-alkalinity (MSA) and severe saline-alkalinity (SSA) for 16 days. Data shown was taken every four days and represents means of 6 biological replicates.

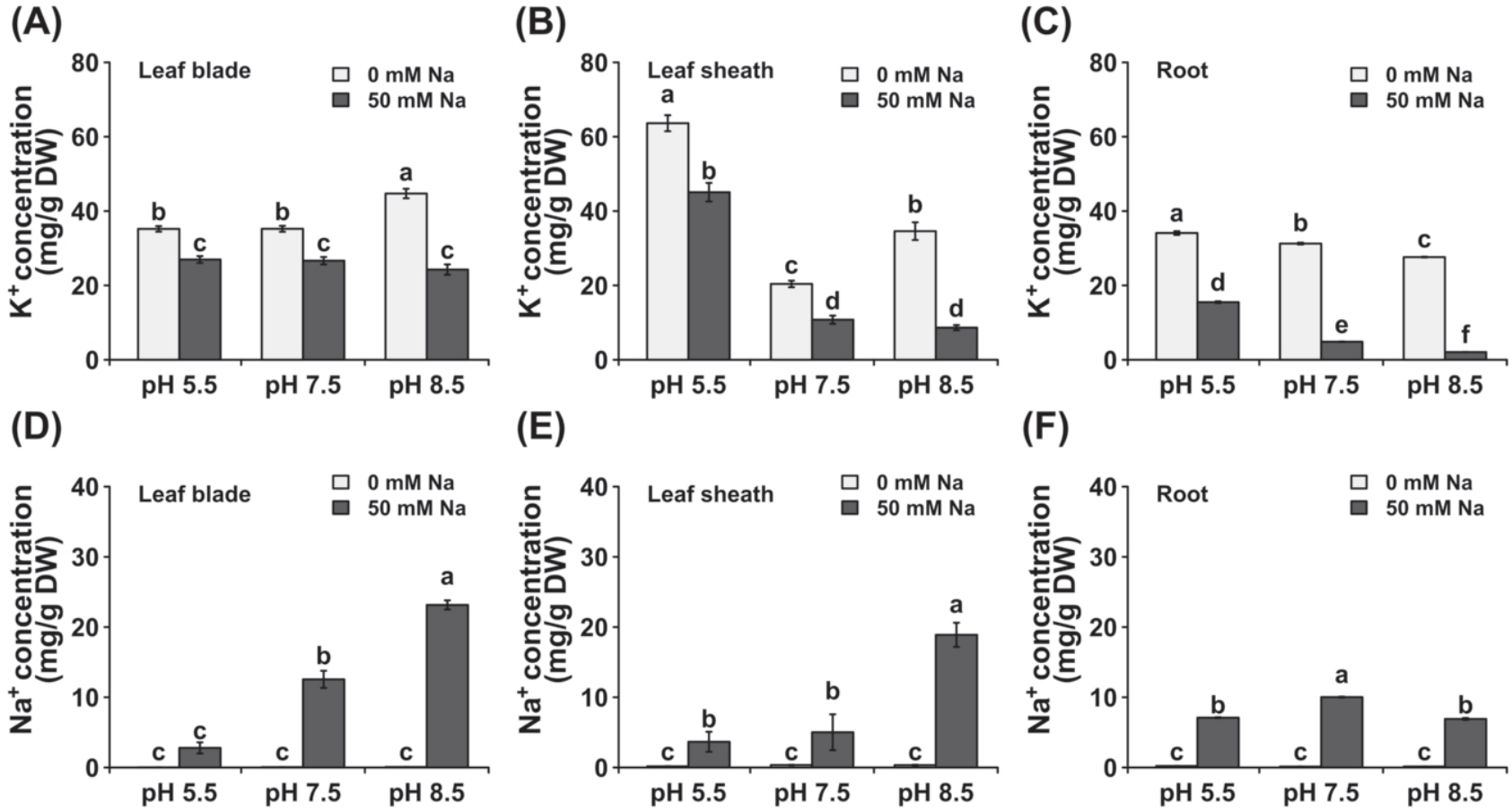

Fig. 2.4. Macronutrient concentration in rice plants showing K^+ concentration in leaf blades (A), leaf sheaths (B), and roots (C); and Na^+ concentration in leaf blades (D), leaf sheaths (E), and roots (F). Plants were exposed to control conditions (0 mM Na^+, pH 5.5), salinity stress (50 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

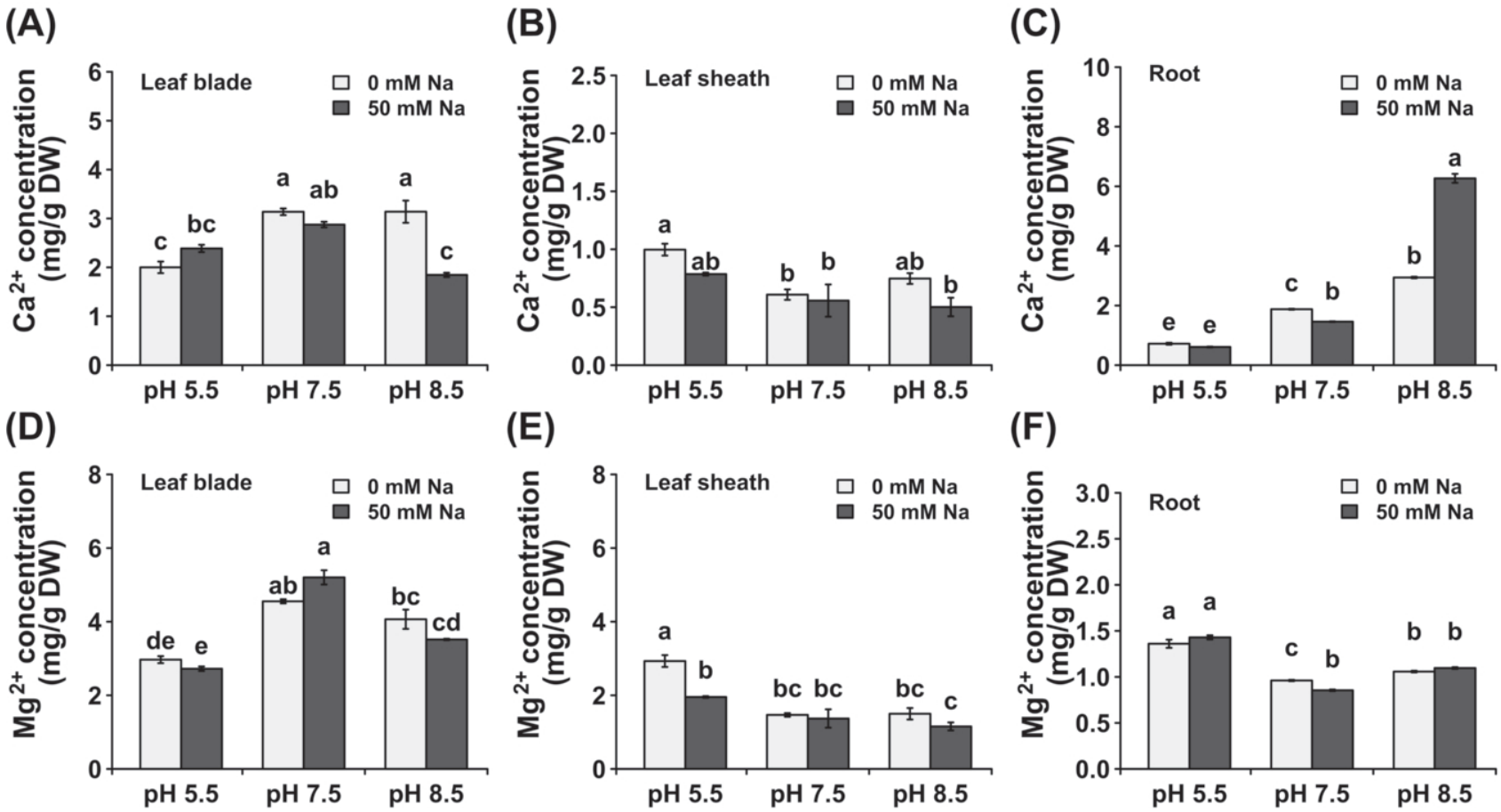

Fig. 2.5. Macronutrient concentration in rice plants showing Ca^{2+} concentration in leaf blades (A), leaf sheaths (B), and roots (C); and Mg^{2+} concentration in leaf blades (D), leaf sheaths (E), and roots (F). Plants were exposed to control conditions (0 mM Na^+, pH 5.5), salinity stress (50 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

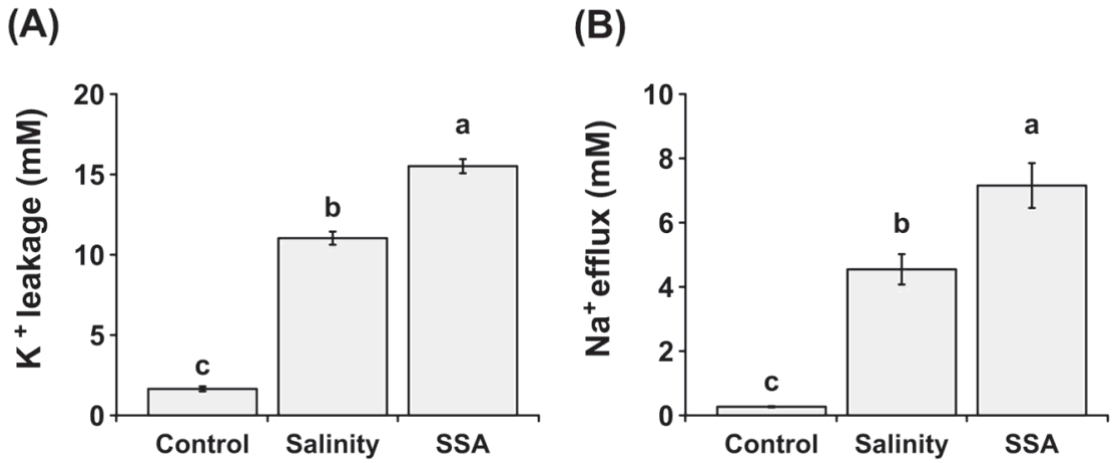

Fig. 2.6. Na^+ and K^+ concentrations in rice plants after 16 days of exposure to different treatments showing leakage of K^+ in plant roots subjected to 50 mM Na^+, pH 5.5 (Salinity stress) and 50 mM Na^+ pH 8.50 (SSA stress) for 24 hours (A) and efflux of Na^+ from roots of rice seedlings subjected to 50 mM Na^+, pH 5.5 (Salinity stress) and 50 mM Na^+ pH 8.50 (SSA stress) for 24 hours, and deionized water for 2 hours. Data shown represents means and standard errors of 6 biological replicates.

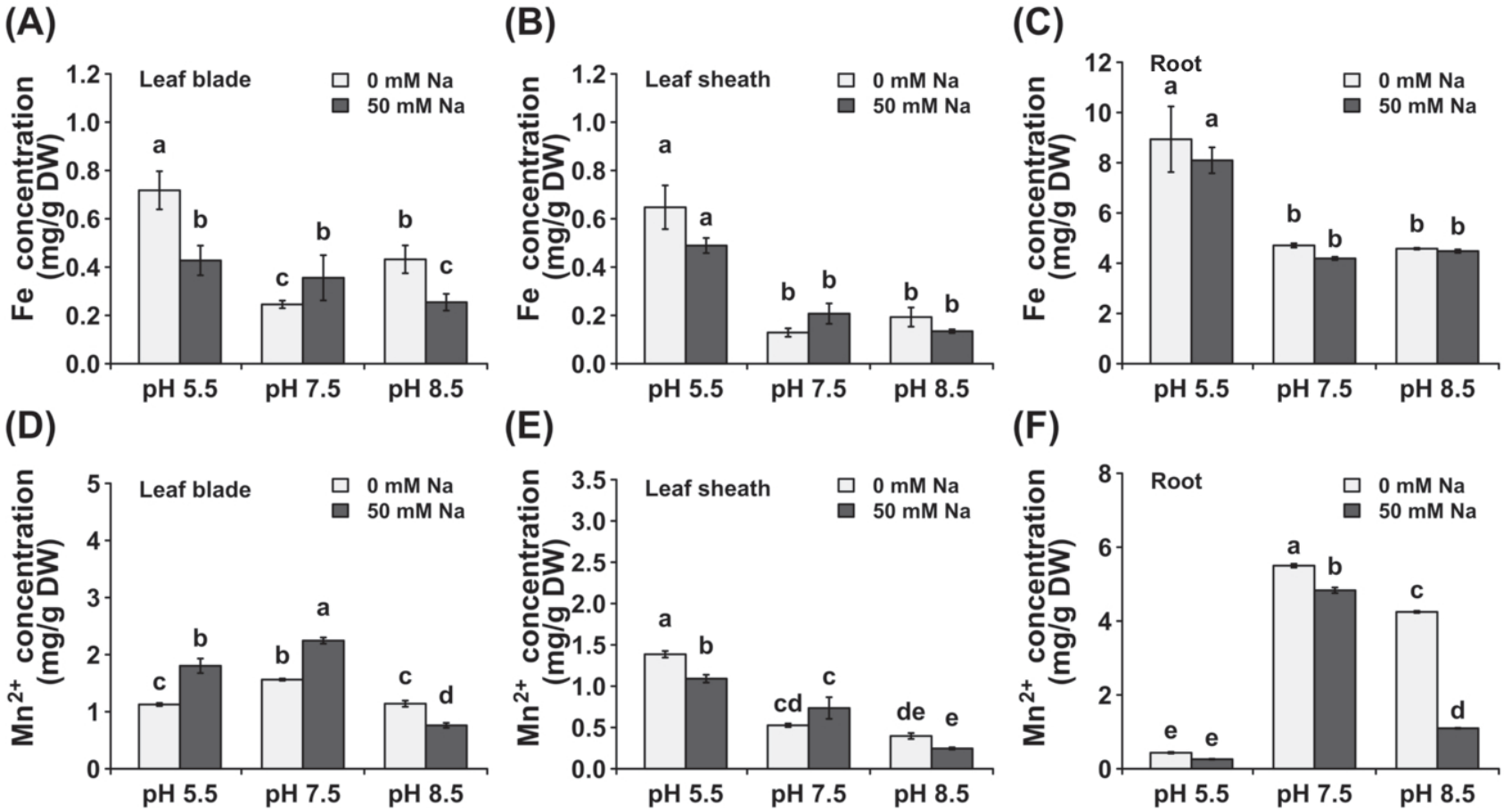

Fig. 2.7. Micronutrient concentration in rice plants showing Fe concentration in leaf blades (A), leaf sheaths (B), and roots (C); and Mn concentration in leaf blades (D), leaf sheaths (E), and roots (F). Plants were exposed to control conditions (0 mM Na^+, pH 5.5), salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

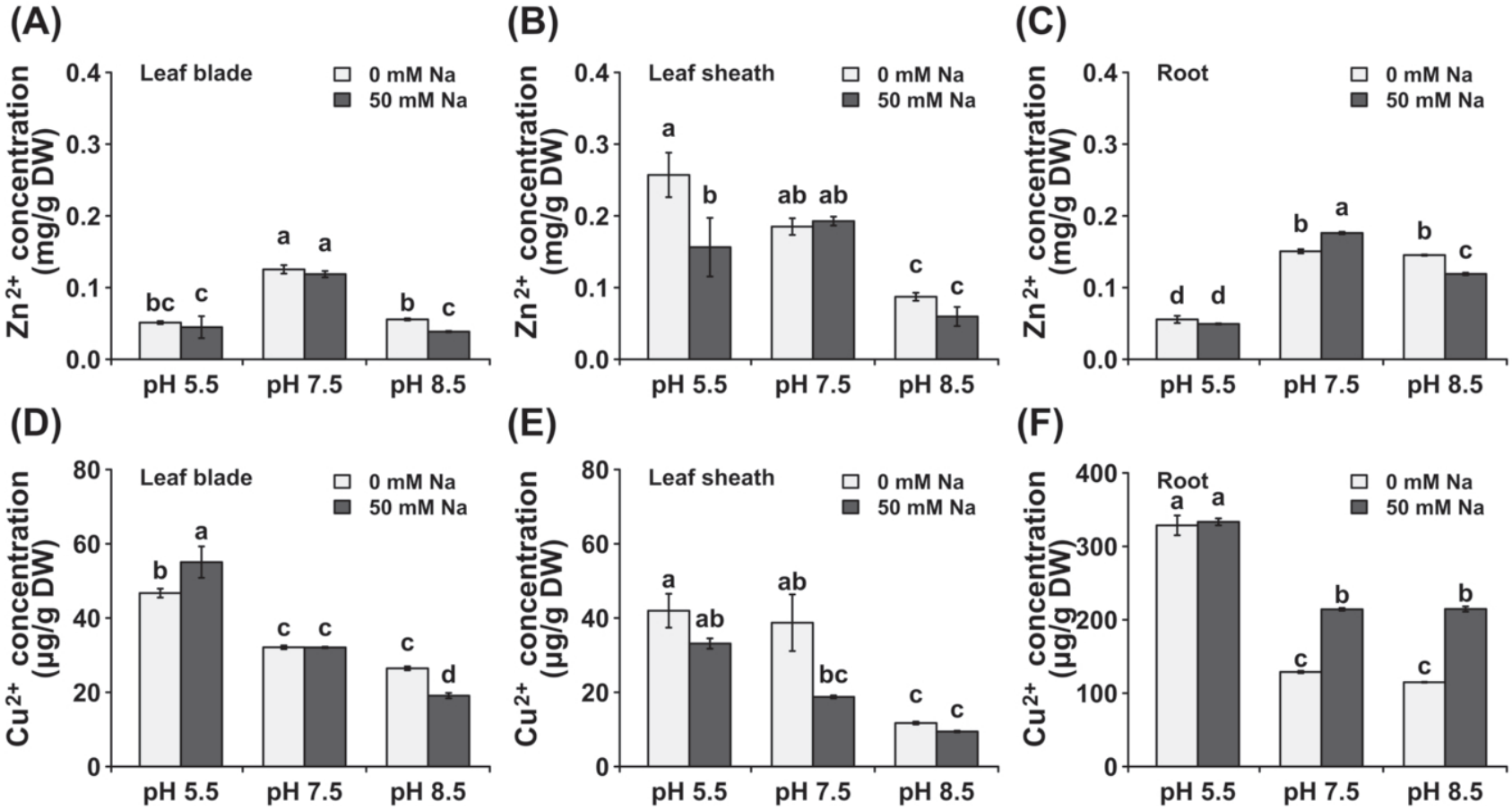

Fig. 2.8. Micronutrient concentration rice plants showing Zn^{2+} concentration in leaf blades (A), leaf sheaths (B), and roots (C); and Cu^{2+} concentration in leaf blades (D), leaf sheaths (E), and roots (F). Plants were exposed to control conditions (0 mM Na^+, pH 5.5), salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

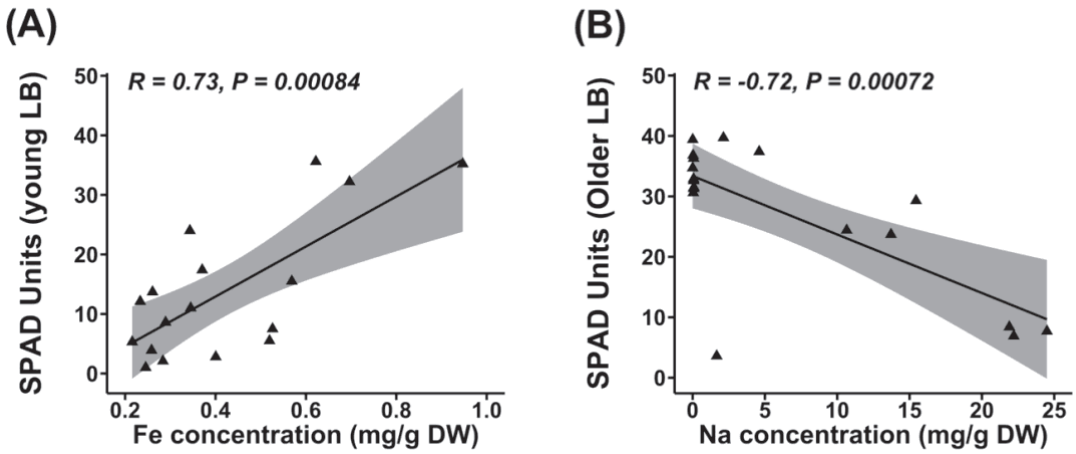

Fig. 2.9. Correlation plot showing Pearson correlation between chlorophyll concentration (SPAD values) in younger leaf blades with leaf blade Fe concentration (A) and correlation of chlorophyll concentration in older leaf blades (LB) with leaf blade Na concentration (B). Plants were exposed to salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days.

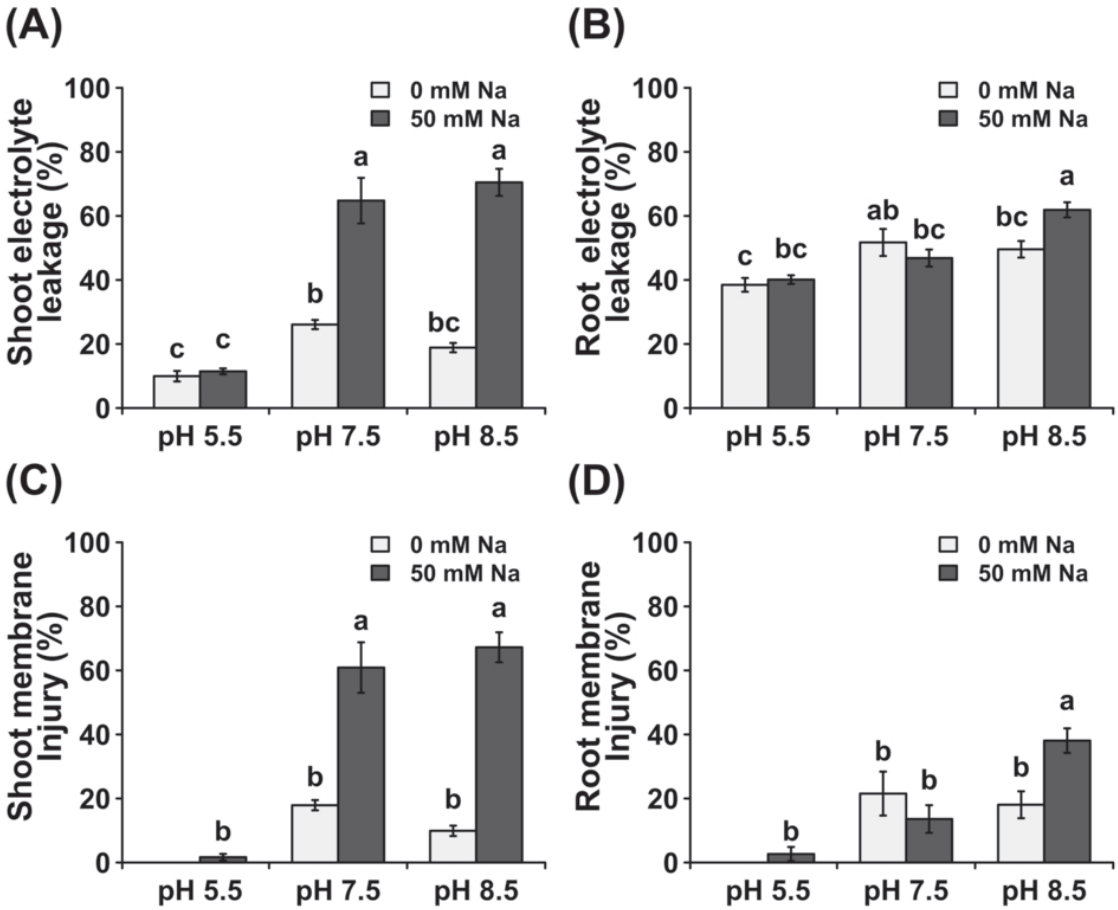

Fig. 2.10. Membrane damage parameters in rice plants after 16 days of exposure to different treatments showing electrolyte leakage ratio in shoots (A) and roots (B); and membrane injury in shoots (C) and roots (D) concentration in leaf blades (A) and roots (B). Plants were exposed to salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

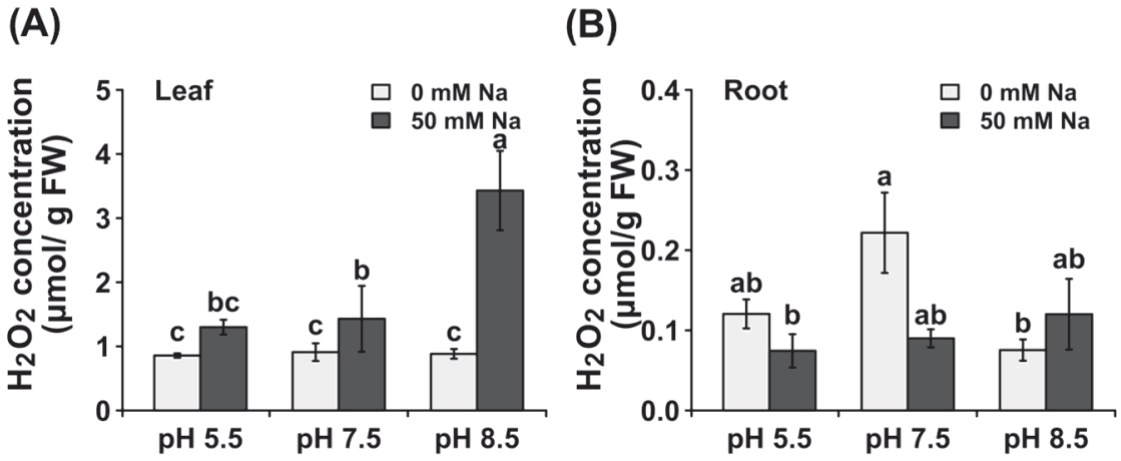

Fig. 2.11. Reactive oxygen species accumulation in rice plants after 16 days of exposure to different treatments showing hydrogen peroxide (H_2O_2) concentration in leaf blades (A) and roots (B). Plants were exposed to salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5), severe alkalinity (0 mM Na^+, pH 8.5), moderate saline-alkalinity (50 mM Na^+, pH 7.5) and severe saline-alkalinity (50 mM Na^+, pH 8.5) for 16 days. Data shown represents means and standard errors of 6 biological replicates.

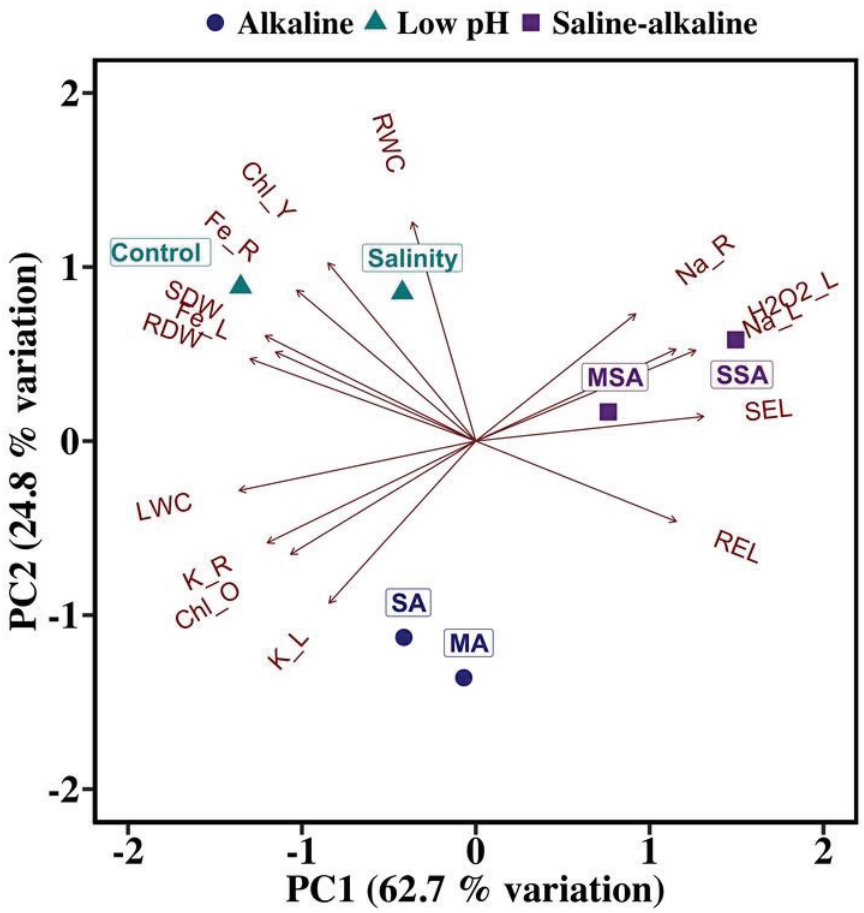

Fig. 2.12. A summary of key traits useful for studying comparative responses to saline-alkaline stress, salinity and alkalinity stress using Principal Component Analysis (PCA). Traits used were relative water content (RWC), SPAD units in young and old leaves (Chl_Y and Chl_O, respectively), Fe, K, Na in leaf blades (Fe_L, K_L, Na_L) and roots (Fe_R, K_R, and Na_R, respectively), shoot dry weight (SDW), root dry weight (RDW), leaf water content (LWC), shoot electrolyte leakage (SEL), root electrolyte leakage (REL) and leaf blade hydrogen peroxide concentration (H2O2_L). All values were measured after exposure to salinity stress (0 mM Na^+, pH 5.5), moderate alkalinity (0 mM Na^+, pH 7.5, MA), severe alkalinity (0 mM Na^+, pH 8.5, SA), moderate saline-alkalinity (50 mM Na^+, pH 7.5, MSA) and severe saline-alkalinity (50 mM Na^+, pH 8.5, SSA) for 16 days.

2.5. Discussion

2.5.1. Interactive effects of high pH and Na^+ toxicity are more detrimental than their sole effects

Saline-alkaline stress is a major detriment to plant growth. Rice is a glycophytic cereal crop characterized as extremely sensitive to a range of abiotic stresses including salinity stress and high pH. This study finds that the interactive effects of high pH and salinity stress are far much more devastating than sole salinity stress and sole alkaline stress. Here, rice cultivar Hinohikari was able to grow normally under salinity stress for up to 12 days of stress imposition, considerable growth reductions were only noticeable on 16^{th} day of stress imposition, whereas under high pH treatments, growth reductions were apparent even four days after stress treatment. Munns & Tester (2008) distinguished between the osmotic and ionic effects of salinity stress based on speed of onset and primary visible site of effect. Based on that and the present results, it is inferable that under salinity stress treatment, the slower nature of visible effect on plant growth (16 days) and sustained production of younger leaves present more of an ionic response than osmotic, albeit not severe enough to cause remarkable leaf senescence. Root growth was the most sensitive to severe saline-alkaline stress (Fig. 2.1B; Fig. 2.2D and F), which might have severely constrained water uptake ability of plants. Previous studies have shown that saline-alkaline stress severely affects root growth (Guo et al. 2014; Lv et al. 2013; Zhang et al. 2017), hence plants with intact root structure and morphology are well capacitated for absorptive functions such as water uptake and metal ion acquisition under saline-alkaline stress. The dramatically reduced leaf water content in this study (Fig. 2.3C) in part reflects incapacitated roots as a result of both Na^+ toxic and a high pH rhizosphere, generating hyperosmotic stress and causing premature leaf senescence due to hyperaccumulation of Na^+ in the leaf blades (Fig. 2.4A). In total, it is inferable that at the present experimental conditions, high rhizosphere pH interferes with growth and some essential physiological processes more

than sole salinity, and furthermore, the toxicity of Na^+ is inordinately aggravated in an alkaline rhizosphere than a neutral one. The physiological rationale for these observations will be detailed in the subsequent sections.

2.5.2. High pH boosts shoot Na accumulation at the expense of root K

Plants growing under saline-alkaline stress are not only confronted with a high pH rhizosphere but also toxicity of Na^+, an element that plants do not need in excess. In this study, it was observed that under the same molar concentration of Na^+, saline-alkaline treated plants accumulated 4.8 and 8.2 times more Na in the leaf blades than sole salinity stressed plants under moderate and severe saline-alkaline stress respectively. This high pH induced Na^+ hyperaccumulation in leaf blades has been repeatedly reported (Cao et al. 2020; Chen et al. 2009; Paz et al. 2012; Yang et al. 2009; Yang et al. 2008), yet no consensus is reached on the proposed mechanisms for the high pH induced hyperaccumulation of Na^+ in the shoots. A recent study in maize identified and characterized a Ca^{2+} dependent *Zea mays* Na^+ *Content under Saline-Alkaline condition* (*ZmNSA1*), a gene responsible for natural variation in shoot Na^+ contents and saline-alkaline tolerance (Cao et al. 2020). Plants lacking *ZmNSA1* promote *ZmSOS1* mediated root Na^+ efflux by increasing plasma membrane H^+-ATPases (MHA2 and MHA4). SOS1 mediated root Na^+ efflux exchanges cytoplasmic Na^+ for external H^+. However, high rhizospheric and cytosolic pH weakens function of H^+ gradient dependent transporters (Haruta & Sussman 2012; Yang et al. 2019) such as SOS1 (Duby & Boutry 2009; Moriyama 2015). Contrarily, measurements of root Na^+ efflux showed considerably higher root Na^+ efflux under saline-alkaline stress relative to plants exposed to salinity stress (Fig. 2.6B), annulling the hypothesis that root Na^+ efflux was upset by high pH. Therefore, in this study, the mechanism of high shoot Na^+ accumulation may likely relate to processes regarding uptake of Na^+ by roots and its subsequent transport to the shoots. Strikingly, this ionic burst was

associated with considerable reductions in K^+ accumulation particularly in roots under saline-alkaline stress (MA and SA, Fig. 2.4A). Measurement of K^+ leakage revealed that while both salinity stress and saline-alkalinity induced significant leakage of K^+ in the roots, leakage was intensified by saline-alkalinity (Fig. 2.6A). Therefore, the observed K deficiency under saline-alkalinity was primarily as a result of root K^+ leakage. The present study did not record any decreases in leaf blade Ca^{2+} concentration under all stress treatments but was enhanced by some high pH treatments, particularly in roots, recording a 9-fold increase under SSA. That notwithstanding, the failure for the root increase in Ca^{2+} uptake to translate similarly in shoots reveal that it is Ca^{2+} transport to the shoot other than uptake by the roots that is perturbed by high pH under saline-alkaline stress. Also, this observed increase in root Ca^{2+} uptake reflects more of a high pH specific response, than a salinity one, but is tremendously provoked by their interaction. The physiological rationale behind this observation remains a mystery.

2.5.3. Chlorosis is distinct among salinity, saline-alkalinity and alkalinity

Chlorosis is an apparent feature of a high pH rhizosphere (Gómez-Galera et al. 2012; Guo et al. 2014), a chlorophyll deficiency symptom that impacts on plants' photosynthetic competency. It is known that high pH reduces plant roots' ability for Fe uptake (Ishimaru et al. 2006; Takahashi et al. 2001). Fe is crucial for chlorophyll biosynthesis, the deficiency of which manifests itself through consequent chlorosis particularly in younger leaves due to Fe's immobile nature (Taiz et al. 2015). Under salinity stress, Na^+ toxicity in leaf blades coupled with failure to compartmentalize it in the vacuoles degrades chlorophyll, resulting into somewhat similar symptoms. According to Munns & Tester (2008), Na^+ toxicity manifests itself through premature senescence of older leaves that are no longer expanding hence are incapable of diluting incoming Na^+. Therefore, under saline-alkaline stress, toxic presence of Na^+ in the rhizosphere and inordinately high pH may produce somewhat physiologically

confusing symptoms of chlorosis and senescence, both of which entail lack of chlorophyll. The observation that in younger leaves, all high pH treatments induced severe chlorosis (Fig. 2.3A), coupled with a significant positive correlation ($R = 0.73$, $P < 0.00073$) between Fe concentration and SPAD units in young leaf blades (Fig. 2.9A) suggests that Fe deficiency was the prime cause of chlorosis. It is crucial to note that following onset of severe saline-alkaline stress, literally no new leaves were formed, hence the supposedly newer leaves did not essentially represent younger leaves, with Fe already partitioned to them, consequently having relatively higher chlorophyll. Conversely, in older leaves, sole alkaline and salinity treatments had much turgid and greener leaves (Figs. 2.2, 2.3B), whilst severe saline-alkaline plants experienced 71.4 % reduction in leaf chlorophyll concentration. These leaves represent older leaves undergoing senescence due to Na^+ toxicity, this is justifiable noting that leaf blades accumulated 4.8 and 8.1 times more Na^+ than salinity stress (Fig. 2.4D). This was further supported by a significant and high negative correlation ($R = -0.72$, $P < 0.00072$) between chlorophyll concentration in older leaf blades and Na concentration (Fig. 2.9B). This implies that in older leaf blades under saline-alkaline stress, lower chlorophyll concentration was primarily due to leaf blade Na toxicity. This result not only reflects leaf Na^+ toxicity but also inability to sequester Na^+ away from the cytosol, indicative that these concentrations were clearly above its vacuolar sequestration capacity (Pandolfi et al. 2012; Shabala et al. 2010). It is therefore unsurprising that severe saline-alkaline treatments experienced such an agonizing chlorosis, representing an overwhelming photosynthetic incompetency that in part validates its poor growth. Therefore, while both Na^+ toxicity and high pH stress reduce chlorophyll concentration, the former does so by damaging chlorophyll whereas the latter reduces its biosynthesis, hence under saline-alkaline stress, the co-existence of these dual factors and their resultant chlorosis is distinguishable by leaf age.

2.5.4. Saline-alkaline stress elicits oxidative stress in leaves

Generation of reactive oxygen species (ROS) is an inevitable fate of organisms undergoing aerobic metabolism (Apel & Hirt 2004; Sharma et al. 2012). Abiotic stresses such as salinity and high pH upset plants' inherent abilities to tightly regulate ROS generation, throwing plants into a state of "oxidative stress" that may potentially cause lipid peroxidation, protein and nucleic acid damage, and programmed cell death (Kamanga et al. 2018). In the present study, saline-alkaline stress caused considerably higher electrolyte leakage and membrane injury (Fig. 2.10A – D), indices indicative of membrane damage (Elsawy et al. 2018; Nyoka et al. 2018; Ueda et al. 2013). This was particularly more pronounced in leaves than roots. In roots, all high pH treatments considerably elicited root membrane injury (Fig. 2.10D), producing visually darker roots with conspicuous symptoms of damage (Fig. 2.2). A number of reports indicate that roots are the primary physiological sites damaged by saline-alkaline stress (Peng et al. 2008; Yang et al. 2009; Yang et al. 2008; Zhang & Mu 2009; Zhang et al. 2019). This study was consistent with previous reports, but further report high membrane injury in leaves, this may be primarily attributed to high pH, whether through accelerated Na^+ uptake and accumulation of ROS. However, measurements of H_2O_2 concentration in leaves showed that only saline-alkaline treatments considerably increased it, whereas in roots it was elicited by moderate alkalinity. Therefore, while growth media pH alone could be implicated in root oxidative stress and resultant membrane damage, it certainly wasn't an important cause in leaf blades, neither was the sole growth media Na^+, but the interactive effects of both and their resultant ionic burst in the leaves. It has been suggested that oxidative stress is the prime cause of lethality under saline-alkaline stress (Zhang et al. 2017) such that its amelioration improves tolerance to saline-alkalinity. Guo et al. (2014) showed that an *alkaline tolerance mutant* (*alt1*) rice had lower H_2O_2 production, with 64 % of abiotic stress differentially expressed genes under saline-alkaline stress were involved in generation and detoxification of ROS and repair

of ROS mediated damage, highlighting the role that ROS homeostasis may play under saline-alkaline stress.

2.6. Conclusion

From this study, it is derivable that the rice cultivar Hinohikari is extremely sensitive to high pH, and that its sensitivity is aggravated under saline-alkalinity than sole salinity stress. Essentially, saline-alkalinity promotes Fe deficiency chlorosis in younger leaf blades and Na^+ toxicity induced senescence of older leaf blades. Furthermore, high pH rhizosphere boosts shoot Na^+ accumulation at the expense of K^+ that leaks excessively in the roots. A secondary consequence of Na^+ toxicity and high pH under saline-alkalinity is compromised membrane integrity particularly in plant leaves as a result of excessive accumulation of ROS. Therefore, efforts attempting to enhance tolerance of rice plants to saline-alkalinity should consider incorporating these priority traits in breeding programs.

Chapter 3

Study of Acclimation and Induced Cross-Tolerance of Rice Seedlings to Saline-Alkaline Stress

3.1. Introduction

Plants are known to have remarkable abilities to acclimate to lethal stress events when subjected to a prior mild stress. This is referred to as acclimation, describing plants' physiological ability to adapt to a stress event (Djanaguiraman et al. 2006; Pandolfi et al. 2012). Acclimation has been demonstrated as an effective and quicker way to improve plants' tolerance to abiotic stresses such as drought (Bruce et al. 2007; Conrath et al. 2006; Crisp et al. 2016; Ding et al. 2012, 2013; Walter et al. 2011) and salinity stress (Kamanga et al. 2020; Pandolfi et al. 2012, 2016; Sriskantharajah et al. 2020). In a primed state, plants are typified with faster and stronger responses triggered by systemic defence systems that confer enhanced resistance. Under alkali stress, Na^+ toxicity, osmotic stress, oxidative stress, and high pH are key known stress factors responsible for growth inhibition and plant death (Yan et al. 2005; Yang et al. 2008, 2009; Zhang et al. 2019), hence choice of acclimating agents should aim at those that may enhance defence responses that counteract these stress factors. It is speculated that high pH reduces plant roots' SOS1 mediated Na^+ efflux as a result of deficiency in transmembrane proton gradient required for SOS1 activity (Haruta & Sussman 2012; Yang et al. 2019). Therefore, in order to address the latter, ability to maintain a lower pH through H^+-ATPases mediated proton secretion is a prerequisite for regulation of leaf Na^+ toxicity through root Na^+ extrusion. Moreover, rhizospheric acidification is also necessary for Fe uptake in plants utilizing strategy I, solubilizing ferric iron (Fe^{3+}) to utilizable ferrous form (Fe^{2+}) for uptake by plant roots. Rice employs both strategy I and strategy II of Fe uptake, hence maintenance of a lower rhizospheric pH cannot be overlooked.

Reactive oxygen species (ROS) are unstable molecules inevitably produced by plants both as a result of normal aerobic metabolism and in response to abiotic stresses (Kamanga et al. 2018; Sharma et al. 2012), and also participate in cellular signalling. It is alleged that an important

overlap exists in signalling and response pathways to different abiotic stresses, involving ROS, hormones, protein kinase cascades and calcium gradients as common elements (Atkinson & Urwin 2012). This crosstalk may promote tolerance to a range of abiotic stresses, illustrating a concept of cross-tolerance, in which exposure to one stress type enhances tolerance to another type (Foyer et al. 2016; Mittler 2006; Perez & Brown 2014), offering a prospect for breeding crop plants resistant to multiple forms of stress. The recognition that a crosstalk to multiple stressors in part involves ROS (Chamnongpol et al. 1998; Pastori & Foyer 2002; Perez & Brown 2014) has lured agronomists to pursue chemical means to activate ROS induced systemic resistance. This has included direct application of ROS particularly H_2O_2 (Fedina et al. 2009; Gondim et al. 2013; He et al. 2009; Wahid et al. 2007) and application of salts such as NaCl (Kamanga et al. 2020; Sriskantharajah et al. 2020) and $NaHCO_3$ (Liu & Saneoka 2019) which are hypothesized to activate antioxidant defence systems. Nevertheless, little is known regarding the physiological and molecular mechanisms governing cross-tolerance between oxidative stress and saline-alkaline stress, and whether acclimation to salinity stress would increase resistance to alkali stress. It is hypothesized here, that acclimation to either or a combination of the four key stress factors under alkali stress i.e. Na^+ toxicity, osmotic stress, oxidative stress and high pH may trigger an inter-stress crosstalk that may upsurge resistance of rice seedlings to alkali stress.

3.2. Study objectives

The study was conducted to achieve the following objectives;

1. To determine optimal pretreatment concentrations that promote acclimation to saline-alkaline stress in rice
2. To elucidate the physiological processes underpinning acclimation and cross-tolerance of rice to saline-alkaline stress

3. To investigate the effect of pretreatments in post saline-alkaline stress recovery of rice

3.3. Materials and methods

3.3.1. Plant materials and growth conditions

Seeds of rice (*Oryza sativa* L.) cultivar Hinohikari were incubated, surface-sterilized, and germinated according to Sriskantharajah et al. (2020). After one week, a slightly modified Kimura B nutrient solution was added to the hydroponic culture following Chuamnakthong et al. (2019). The nutrient solution was provided by immersing the plant roots directly in the hydroponic solution. The experiment was conducted in a greenhouse at Hiroshima University's Faculty of Biological Sciences Experimental Field. The hydroponic culture temperature was maintained at 28 ºC, and the average day air temperature was 30 ºC. After 18 days, seedlings were transplanted from a nylon mesh wire to 20 L buckets.

3.3.2. Treatment conditions

Twenty-six (26) day-old seedlings were subjected to three concentrations of each of the following three sets (N = 20) of pre-treatments for 7 days: (1) NaCl (S) (2.5, 5.0, and 10 mM), (2) H_2O_2 (OX) (1.0, 5.0, and 10 µM), and (3) $NaHCO_3$ (SA) (0.5, 1.0, and 2.5 mM). A fourth (4) set of plants was non-pretreated and maintained as controls (N = 40). Out of these, after 7 days acclimation period, half were subjected to saline-alkalinity (NPT) while the remaining were maintained as control plants (no pre-treatment and no saline-alkaline stress). Except for set 3, all sets were maintained at a pH of 5.0–5.5 using 2.0 N HCl and 2.0 N KOH. After seven days, pretreated (PT) and non-pretreated (NPT) plants were subjected to main SAS treatment (50 mM Na, pH 8.25) using a mixture of 0.75 mM Na_2CO_3, 25 mM $NaHCO_3$, and 23.5 mM NaCl. Growth medium pH was monitored every 24 hours but was left unadjusted except for control conditions adjusted to 5.0–5.5 daily. To evaluate whether pre-treatments facilitate a

quicker recovery after the disappearance of SAS conditions, all pretreated plants were subjected to 50 mM Na, pH 8.25 for 14 days, at the conclusion of that all stressed plants were recovered when exposed to 0 mM Na (1x Kimura B), pH 5.0–5.5 for 10 days. Shoot and root dry weight, chlorophyll concentration, and membrane integrity were measured.

Table 3.1. Pre-treatment conditions used. pH for PT No. 1 – 8 was being adjusted daily to the indicated range. pH for PT No. 9 – 11 was left unadjusted, indicated values are round average values during the 7 day PT period.

No.	Pre-treatment (PT)	Treatment	Designation	Chemical composition	pH
1	None	Control	Control	-	5.0 – 5.5
2	None	50 mM Na^+, pH 8.25) Saline-alkaline stress (SAS,	NPT	-	5.0 – 5.5
3	Salinity		$S_{2.5}$	2.5 mM NaCl	5.0 – 5.5
4			$S_{5.0}$	5.0 mM NaCl	
5			S_{10}	10.0 mM NaCl	
6	Oxidative		Ox_1	1 µM H_2O_2	5.0 – 5.5
7			Ox_5	5 µM H_2O_2	
8			Ox_{10}	10 µM H_2O_2	
9	Saline-alkalinity		$SA_{0.5}$	0.5 mM $NaHCO_3$	~ 6.5
10			$SA_{1.0}$	1.0 mM $NaHCO_3$	~ 7.0
11			$SA_{2.5}$	2.5 mM $NaHCO_3$	~ 7.5

3.3.3. Determination of plant fresh and dry weight

Seedlings were harvested in replicates of 4 – 5 after 7 days of pre-treatment period, after 14 days of main stress and after 10 days of recovery period. Plant height and root length were measured using a measuring ruler. Plants were then dissected into leaf blades, leaf sheaths and roots and immediately measured for fresh weight (FW). Dry weights (DW) were obtained after oven drying the samples for at least 72 hours at 70 ^{o}C.

3.3.4. Chlorophyll concentration (SPAD)

Leaf blade chlorophyll concentration was determined using a chlorophyll meter (SPAD – 502, Minolta Camera Co. Ltd., Osaka, Japan) on three fresh fully expanded leaf blades of the same positions in all plants according to Kamanga et al. (2020).

3.3.5. Determination of macro and micronutrient elements

Elemental analysis was performed in dried leaf blades and root samples after 14 days of stress imposition using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). iCAP – 6300 (Thermo-Scientific, Massachusetts, USA). Plant samples were digested using H_2SO_4 and H_2O_2 following a procedure outlined in section 2.2.4. Quantification was done using various elemental standard solutions prepared in 1 % HNO_3.

Table 3.2. Wavelengths used for ICP analysis

Element	Wavelength (nm)	Element	Wavelength (nm)
Potassium (K)	766.4	Iron (Fe)	259.9
Sodium (Na)	818.3	Boron (B)	249.7
Phosphorus (P)	177.4	Zinc (Zn)	213.8

3.3.6. K^+ leakage and Na^+ efflux experiment

In order to determine plant roots' ability to retain K^+, leakage of K^+ was assessed by a method previously described in Pandolfi et al. (2016) with slight modifications. For this purpose, plants were pretreated with various pre-treatments for 7 days, at the conclusion of which, four uniform seedlings of pretreated and non-pretreated plants were transferred into falcon tubes containing 30 mL of 50 mM Na^+, pH 8.5 (48 mM $NaHCO_3$ + 1 mM Na_2CO_3). Four more seedlings were transferred into 30 mL deionized water as control plants. After 24 hours, solution was sampled for K^+ analysis.

Efflux of Na^+ from roots was assessed by a previously outlined method (Pandolfi et al. 2016; Shabala et al. 2010), for this purpose, 7 day pretreated and non-pretreated plants were subjected to 30 mL of 50 mM Na^+, pH 8.5 for 24 hours, after which plants were transferred into bathing medium containing 0.1 mM $CaCl_2$, 0.5 mM KCl and 50 mM Na^+, pH 8.5. After 1 hour, the solution was poured off, roots were rinsed with 10 mM $CaCl_2$ three times. Then, the roots were transferred into 10 mL bathing medium containing 0.1 mM $CaCl_2$ and 0.5 mM KCl for 4 hours. The solution was then sampled and Na^+ concentration was measured. Both K^+ and Na^+ analysis were done using flame photometry (ANA – 135, Tokyo Photoelectric, Tokyo, Japan).

3.3.7. Na^+ uptake in excised leaves

In order to exclude contribution of Na^+ exclusion from uptake by roots, rice seedlings were pretreated with various pre-treatments for 7 days, thereafter uniform leaf blades were excised under running water, and immersed into 30 mM Na^+, pH 8.0 at about the same depth (30 mm) for 3 days following a method described in Shabala et al. (2010). Thereafter, immersed cut ends of leaf blades were thoroughly washed using deionized water, and dried at 65 ^oC for 72

hours. Then, 50 mg of leaf blades was agitated on a shaker in 1.0 N HCl for 24 hours and measured for Na^+ concentration using flame photometry according to Kamanga et al. (2020).

3.3.8. Determination of membrane integrity

Membrane integrity was measured using electrolyte leakage method and lipid peroxidation at the end of 14 days of main stress and 7 days of recovery. Freshly harvested leaf blade disks and roots (200 mg) were cut into 2 cm pieces and fully immersed into 25 mL deionised water in 50 mL falcon tubes and kept at room temperature for 24 hours. Electrical conductivity (EC) was then obtained (EC1) using an EC meter (CM-31P, Toa DKK Co., Tokyo, Japan). Immediately, the samples were killed by incubating at 100 ^oC for 15 minutes, cooled and EC2 was obtained. Electrolyte leakage rate was calculated according to Farooq & Azam (2006).

Lipid peroxidation was determined by obtaining malondialdehyde (MDA) concentration using the improved thiorbarbituric acid (TBA) reaction previously described in Hodges et al. (1999). For this purpose, 100 mg of fresh leaf and root samples were ground in presence of liquid nitrogen using mortar and pestle and extracted with 3 mL 80 % ethanol after incubating under room temperature for 20 minutes and centrifuging at 3,000 x *g* for 10 minutes. Thereafter, two sets of 1 mL of the supernatant were transferred into two glass tubes, respectively, one containing 1 mL TBA (-) solution (20 % trichloro acetic acid and 0.01 % Butyl Hydroxy Toluene, BHT) and another containing 1 mL TBA (+) solution (20 % trichloro acetic acid, 0.01 % BHT and 0.65 % TBA). MDA concentration was calculated from the absorbances of the samples, which were measured spectrophotometrically (U-3310, Hitachi, Tokyo, Japan) at 440, 532, and 600 nm after incubating the samples at 95 ^oC for 30 minutes, cooled at room temperature for 5 minutes followed by ice for 5 minutes, and centrifuging the samples at 3,000

x *g* for 10 minutes. MDA concentration was calculated using a 155 mM^{-1} cm^{-1} extinction coefficient.

3.3.9. Measurement of root and leaf cell viability

Root and leaf cell viability were assessed using Evan's blue staining procedure as previously described (Rodriguez-Serrano et al. 2006; Romero-Puertas et al. 2004; Zhang et al. 2017). In brief, fresh root tips and leaf blades were cut into 2 cm long pieces, 100 mg of which were infiltrated with 1.5 mL of 0.25 % aqueous solution of Evan's blue for 30 min under room temperature. Upon absorption of the stain, leaf and root samples were rinsed with deionized water until no further blue dye was eluted from the samples. To quantify the Evan's blue stain absorbed, the stain was solubilized by incubating in 4 mL of 1 % (w/v) sodium dodecyl sulfate (SDS) prepared in 50 % (v/v) methanol at 50 °C for 30 min, and measure absorbance at 600 nm using SDS as a blank. Stain absorption was then quantified using Evan's blue standard solutions.

3.3.10. Determination of hydrogen peroxide concentration

H_2O_2 concentration was determined using ground frozen samples based on ferrous oxidation of xylenol orange (FOX) method (Kaur et al. 2016) with slight modifications (Mekawy et al. 2018) similar to section 2.2.6. The reaction mixture was left at room temperature for 1 hour and H_2O_2 concentrations were determined spectrophotometrically at 560 nm and quantified using H_2O_2 standard solutions.

3.3.11. Antioxidant enzyme activity essays

In order to analyze antioxidant enzyme activities, 300 mg frozen leaf blade and root samples were ground in presence of liquid nitrogen using mortar and pestle and extracted using 3 mL

potassium phosphate (KPB) buffer (pH 7.0) and centrifuged at 10,000 x *g* for 15 min at 4 °C. Catalase activity was obtained as previously described (Mekawy et al. 2018; Takagi and Yamada 2013). The 700 µL reaction mixture for catalase (CAT, EC 1.11.1.6) activity comprised of 665 µL of 10 mM H_2O_2 in 50 mM KPB, pH 7.0, and 35 µL crude extract. Catalase activity was measured by following decrease in absorbance at 240 nm for 1 minute. CAT activity was calculated using H_2O_2 extinction coefficient of 0.0436 mM^{-1} cm^{-1}. One unit of CAT activity was determined as mmol of H_2O_2 consumed min^{-1} mg^{-1} protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) was essayed according to Mekawy et al. (2018). For this purpose, 700 µL reaction mixture contained 630 µL APX reaction mixture containing 0.2 mM H_2O_2, 0.15 mM EDTA, 50 mM ascorbic acid and 70 µL crude extract in 50 mM KPB, pH 7.0. APX activity was obtained by recording decrease in absorbance at 290 nm for 1 minute, and calculated using extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX activity was determined as µmol of ascorbate oxidized min^{-1} mg^{-1} protein.

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was investigated as described previously (Jebara et al. 2005; Uarrota et al. 2016). In brief, the 700 µL GPX reaction mixture contained 665 µL reaction buffer containing 20 mM H_2O_2, 10 mM guaiacol, and 35 µL crude extract in 50 mM KPB, pH 7.0. GPX activity was obtained by recording increase in absorbance at 470 nm for 1 minute, and calculated using extinction coefficient of 26.6 mM^{-1} cm^{-1}. One unit of GPX activity was determined as µmol tetraguaiacol formed min^{-1} mg^{-1} protein.

Glutathione reductase (GR, EC 1.8.1.7) was essayed according to outlined methods in Liu & Saneoka (2019) with slight modifications. For this purpose 200 µL crude extract was added to 1,800 µL GR reaction mixture, containing 1 mM oxidized glutathione (GSSG), 0.5 mM EDTA,

0.04 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH). GR activity was calculated by following decrease in absorbance at 340 nm using extinction coefficient of 6.22 mM^{-1} cm^{-1}. One unit of GR was determined as µmol NADPH oxidized min^{-1} mg^{-1} protein.

Activity of superoxide dismutase (SOD, EC.1.15.1.1) was essayed based on its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) as outlined in Beauchamp & Fridovich (1971) and Dhindsa et al. (1980). Here, SOD activity was estimated by recording the decrease in absorbance of superoxide-nitro blue tetrazolium complex by the enzyme. The complete 3,200 µL SOD reaction mixture contained 100 µL of 1.5 M sodium carbonate 200 µL of 200 mM methionine, 100 µL of 2.25 mM NBT, 100 µL of 3 mM EDTA, 1,500 µL of 100 mM potassium phosphate buffer, 1,000 µL distilled water and 100 µL of enzyme. Four tubes without enzyme extract were taken as control. The reaction was started by adding 100 µL of 1 mM riboflavin and placing the tubes below a light source of two 15 W florescent lamps for 15 min. Reaction was stopped by switching off the light and covering the tubes with aluminium foil. Tubes without enzyme developed maximal colour. Another set with complete reaction mixture but without the enzyme was nonirradiated, did not develop colour hence served as blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme which caused 50 % inhibition of NBT photoreduction. Protein concentration in the enzyme extract was measured using Bradford reagent (Bradford 1976) using bovine serum albumin as standards.

3.3.12. Statistical analyses

The experiment was arranged in a completely randomized design (CRD). Each measurement was performed using four to five biological replicates. Inferential statistics were performed using analysis of variance (ANOVA) at a significance level of 0.05. Multiple comparison tests

were performed using Tukey's test. All data were analyzed using R Studio (RStudio team 2020).

3.4. Results

3.4.1. Effectiveness of PTs on growth

After 7 days of PTs, no significant reductions in growth were recorded by PTs, instead, some PTs, notably 1 µM H_2O_2 slightly increased shoot and root growth (Fig. 3.1A). At the end of 14 days of main stress, shoot and root dry weight were considerably decreased in non-pretreated plants (Fig. 3.1B). However, pre-treatment with 5 mM and 10 mM NaCl, and all concentrations of H_2O_2 (1, 5, 10 µM) significantly increased shoot dry weight. Acclimation to saline-alkaline stress (SAS) using mild SA PTs was not as effective (Fig. 3.1B), despite no significant reductions in growth during the acclimation period (Fig. 3.1A). Nonetheless, 0.5 mM $NaHCO_3$ proved relatively effective, slightly increasing both shoot and root growth compared with non-pretreated (NPT) plants. Plant height and root length were not significantly affected under saline-alkaline pre-treatments (data not shown), however, the tendencies for 10 mM NaCl PT favoured shoot growth whereas H_2O_2 PTs favoured root growth. Overall, 10 mM NaCl, 10 µM H_2O_2 and 0.5 mM $NaHCO_3$ PTs were the most effective in their respective groups, with the former two being the best, hence were chosen for subsequent experiments, and will be the main focus subsequently.

3.4.2. Chlorophyll concentration

At the conclusion of the pre-treatment period, chlorophyll concentration was significantly reduced by $NaHCO_3$ PTs whereas no differences were shown between controls and NaCl and H_2O_2 PTs (Fig. 3.1C). After 14 days of main stress, chlorophyll concentration was significantly

reduced in non-pretreated and $NaHCO_3$ pretreated seedlings, whereas NaCl and H_2O_2 PTs maintained it (Fig. 3.1C).

3.4.3. Changes in growth medium pH

Growth medium pH was monitored on daily basis and showed that during the onset of stress, pH generally progressively increased. On the first day of stress for example, medium pH was set at 8.0 for all plants, but increased to around 8.45 by the end of 14 days of stress for non-pretreated plants (Fig. 3.2). In comparison to non-pretreated plants, PTs with NaCl were the most effective in reducing growth medium pH (Fig. 3.2A) followed by H_2O_2 PTs (Fig. 3.2B). It was hypothesized that acclimating rice with saline-alkaline PT ($NaHCO_3$) would reduce medium pH under high saline-alkaline stress, however, it was striking to observe that 1.0 and 2.5 mM $NaHCO_3$ considerably increased growth medium pH to about 8.7 by the end of 14 day stress period, whereas 0.5 mM did not significantly change it (Fig. 3.2C).

3.4.4. Accumulation of K, Na and P in roots and leaf blades

Significant reductions in K concentration were observed in both leaf blades and roots, particularly pronounced in roots (Fig. 3.3A). However, in leaf blades, PT with 10 µM H_2O_2 and 0.5 mM $NaHCO_3$ significantly increased K concentration while in roots all PTs significantly increased it (Fig. 3.3A). Content of K per plant was significantly reduced by saline-alkaline treatment, however, all PTs significantly increased K content per plant (Fig. 3.3B). This was coincidental with significant increases in Na concentration especially in the roots. In leaf blades, PT with 10 mM NaCl significantly reduced Na accumulation, and so did $NaHCO_3$, but H_2O_2 PT accumulated the highest Na concentration (Fig. 3.3C). In roots however, 10 mM NaCl and 10 µM H_2O_2 PTs significantly reduced Na concentration. However, content of Na^+ was significantly higher in all pretreated plants compared to NPT under saline-alkaline

conditions (Fig. 3.3D). P concentration was significantly reduced in both leaves and roots, whereas PTs with 0.5 mM $NaHCO_3$ and 10 μM H_2O_2 significantly increased it especially in roots (Fig. 3.3E).

3.4.5. Effectiveness of PTs on micronutrient acquisition

All three PTs significantly increased Fe concentration in both organs, this was however most pronounced in roots (Fig. 3.4A). Likewise, content of Fe was considerably reduced under saline-alkaline stress especially in NPT plants (Fig. 3.4B), however, all PTs were able to significantly increase Fe content per plant. The same was observed for boron (B) in both leaf blades and roots (Fig. 3.4C). In leaf blades, Zn concentration was significantly reduced by saline-alkaline stress, except for plants acclimated using 0.5 mM $NaHCO_3$ that accumulated nearly two-folds higher Zn than NPT, NaCl and H_2O_2 pretreated plants (Fig. 3.4D), whereas in roots, Zn concentration was significantly increased by saline-alkaline stress in all PTs (Fig. 3D) and so was Mn (data not shown). Overall, 0.5 mM $NaHCO_3$ was the most effective at increasing micronutrient concentration, implying that acclimation to saline-alkaline stress increases the efficiency for uptake of micronutrients in rice.

3.4.6. Effect of PTs on root K^+ retention and root Na^+ efflux

In the previous result (Fig. 3.3A and B), rice plants had significantly lower concentrations and content of K^+, but this was somehow ameliorated by PTs. Plant roots' K^+ leakage showed extremely high leakage of K^+ regardless of PT (> 60 folds higher), implying that lower root K concentrations under saline-alkaline stress were chiefly as a result of leakage of K^+ from roots (Fig. 3.5A), and that PTs were not effective enough to reduce it.

Then, efflux of Na^+ was studied in a 4 hour period, and showed that saline-alkaline stressed plants exhibited significantly higher Na^+ efflux (Fig. 3.5B), however this was more pronounced in pretreated plants, particularly by 10 mM NaCl, in part explaining its lower leaf blade and root Na^+ concentrations.

3.4.7. Na^+ in excised leaves

In order to eliminate contribution of plant roots' Na^+ exclusion and efflux, Na^+ uptake was measured in excised leaves and showed that devoid of these factors, 10 mM NaCl and 10 µM H_2O_2 significantly reduced Na^+ concentration accumulating in the leaf blades, whereas non-pretreated and 0.5 mM $NaHCO_3$ pretreated plants accumulated significantly higher amounts (Fig. 3.5C). Also, there was significantly lower chlorophyll concentration in non-pretreated and 0.5 mM $NaHCO_3$ pretreated leaf blades (Fig. 3.5D), which could be due to the higher Na^+ accumulation and inefficient sequestration of Na^+ away from the cytosol.

3.4.8. Effectives of PTs on membrane integrity and cell viability

Leaves of non-pretreated plants exhibited considerably higher leakage of electrolytes (Fig. 3.6A), signifying loss of membrane integrity, whereas in roots, despite having much higher leakage of electrolytes than leaf blades, no significant differences were detected among all treatments (Fig. 3.6A). This was further verified by measurement of MDA, a marker of lipid peroxidation that showed significantly lower concentration in leaf blades of NaCl and H_2O_2 pretreated plants, whereas in roots, only NaCl reduced MDA accumulation (Fig. 3.6B).

Cell viability using Evan's blue staining method was significantly enhanced in leaf blades by all PTs (Fig. 3.6C). In roots, cell viability was only significantly increased by NaCl and H_2O_2 PT (Fig. 3.6C). Finally, it was checked whether changes in membrane integrity and cell

viability among various PTs could be attributed to oxidative stress. For this purpose, H_2O_2 concentration was analyzed in both leaf and root organs. It was shown that in leaves, 10 μM H_2O_2 and 0.5 mM $NaHCO_2$ PTs significantly reduced H_2O_2 accumulation whereas in roots all PTs significantly reduced oxidative stress (Fig. 3.6D).

3.4.9. Oxidative stress and antioxidant enzyme activity

In order to elucidate the biochemical basis in which pre-treatments ameliorate oxidative stress, antioxidant enzyme activities were essayed in leaf blades and roots. Catalase activity was significantly increased in leaf blades of NaCl and H_2O_2 PTs, whereas in roots, only NaCl and $NaHCO_3$ PTs significantly increased its activity (Fig. 3.7A). Guaiacol peroxidase (GPX) was not enhanced by any of the PTs in both leaf blades and roots (data not shown). However, ascorbate peroxidase (APX) was considerably increased in both leaves and roots of NaCl and H_2O_2 PTs (Fig. 3.7B). Next, antioxidant activity of glutathione reductase (GR) was also essayed, and showed that its activity was significantly induced in leaf blades, however, only pretreated plants were able to significantly induce GR activity (Fig. 3.7C). In roots, no GR activity was detected in all samples. Finally, superoxide dismutase (SOD), an important enzyme for detoxification of superoxide radicles was essayed. No any significant differences were shown in leaf blades among PTs, except for H_2O_2 pre-treatment that considerably decreased its activity (Fig. 3.7D). On the other hand, in roots, SOD activity was significantly increased by all PTs whereas in non-pretreated plants it was considerably supressed (Fig. 3.7D). Protein concentration was measured for purposes of expressing specific enzyme activity, and was highest in leaf blades than roots (Fig. 3.7E).

3.4.10. Effect of PTs on post – stress recovery

In order to investigate if acclimation and cross-tolerance would influence recovery of rice plants after conclusion of stress, saline-alkaline stressed plants were recovered by cultivating under normal growth conditions (0 mM Na^+, pH 5.5) for 10 days. It was observed that during recovery, plants under control conditions grew about 3 times faster than during the stress period (Fig. 3.8A and B). However, in previously stressed plants, despite subjecting them to similar recovery conditions, growth rates did not significantly change, except for H_2O_2 pretreated plants that considerably increased both shoot and root mean growth rates (Fig. 3.8A and B). Therefore 10 μM H_2O_2 PT facilitates a quicker recovery and resumption of growth when stress is concluded. Strikingly, in roots, $NaHCO_3$ PT significantly reduced root mean growth rate during recovery (Fig. 3.8B), suggesting that here and in NPT plants, irreparable damage was inflicted. Despite the 10 day recovery, non pretreated plants were unable to recover chlorophyll concentration, but other PTs were able to accumulate chlorophyll concentration comparable to control plants (Fig. 3.8C). All plants suffered considerably less membrane damage as measured by electrolyte leakage, and no differences were detected among the pretreated seedlings (Fig. 3.8D). These observations imply that during the recovery period, some plants were able to physiologically recover, despite the inability of some to resume rapid growth.

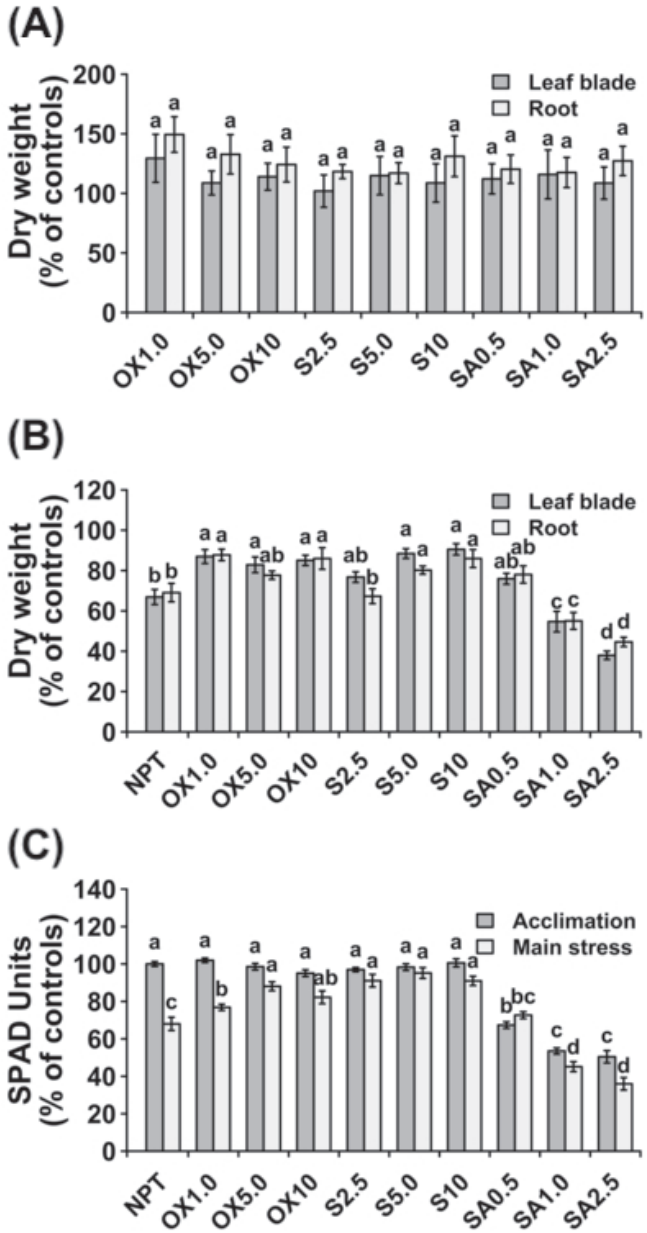

Fig. 3.1. Vegetative growth and physiological data showing dry weight after 7 days acclimation period (A) and dry weight (B) and leaf chlorophyll concentration shown as SPAD units (C) after 14 days main saline-alkaline treatment. Plants were pretreated with various concentrations of NaCl (S) (2.5, 5.0, 10 mM), H_2O_2 (OX) (1.0, 5.0, 10.0 µM) and $NaHCO_3$ (SA) (0.5, 1.0, 2.5 mM) for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days. The data represents means ± standard errors from five biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance, same/ no letters indicate no significant differences.

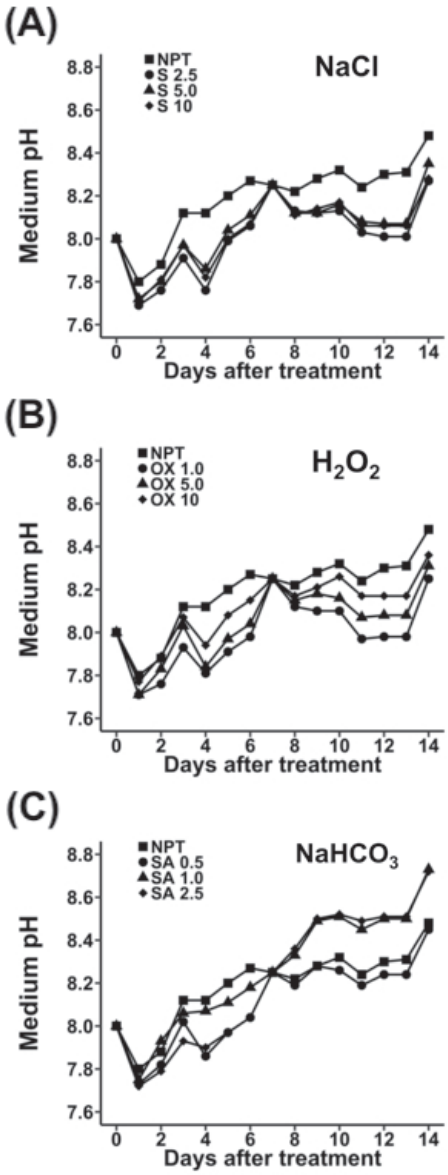

Fig. 3.2. Rhizosphere acidification data showing daily growth medium pH changes during a 14 day period of main saline-alkaline treatment in plants pretreated with various concentrations of NaCl (A), H_2O_2 (B) and $NaHCO_3$ (C) versus non-pretreated seedlings. Plants were pretreated with various concentrations of NaCl (S) (2.5, 5.0, 10 mM), H_2O_2 (OX) (1.0, 5.0, 10.0 μM) and $NaHCO_3$ (SA) (0.5, 1.0, 2.5 mM) for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days.

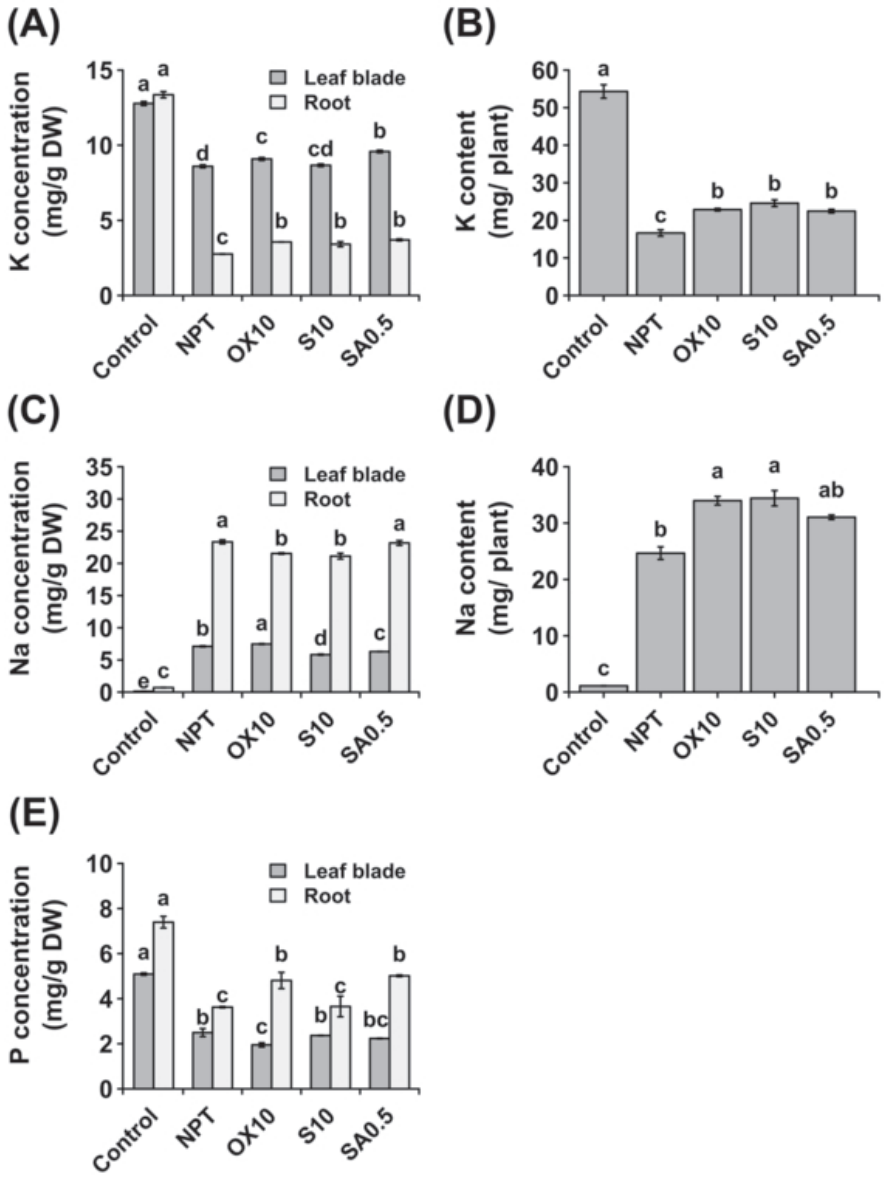

Fig. 3.3. Macronutrient analysis showing K concentration (A) and content (B), Na concentration (C) and content (D), and P concentration (E) in leaf blades, roots and whole plant after 14 days of main saline-alkaline treatment. Plants were pretreated with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5), for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days. The data represents means ± standard errors from four biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance.

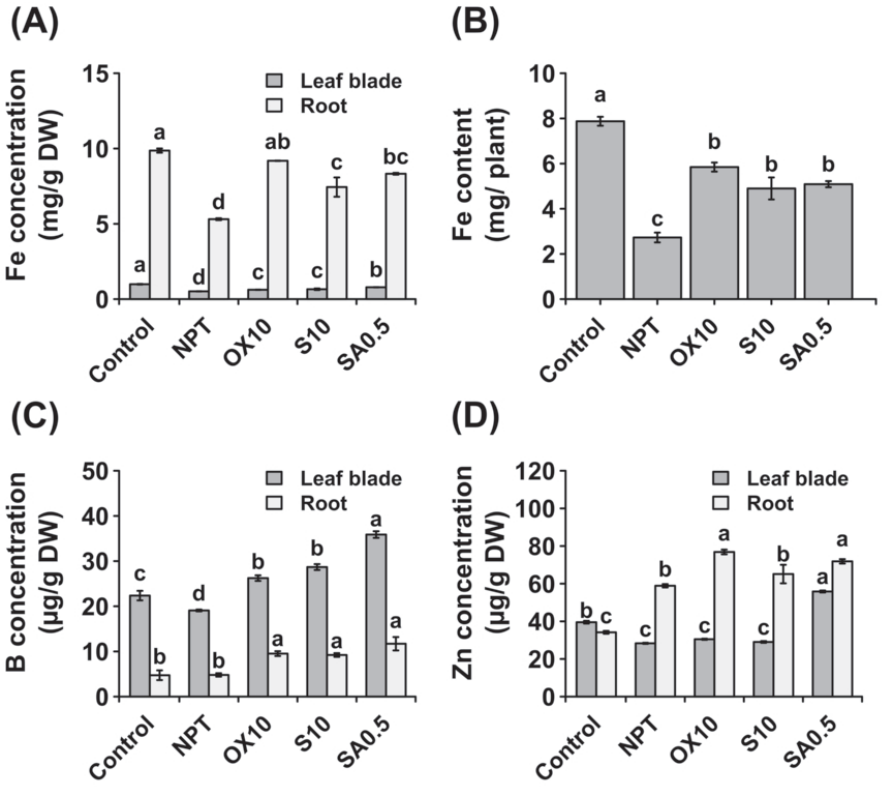

Fig. 3.4. Micronutrient analysis showing Fe concentration in leaf blades and roots (A) and content in the whole plant (B), and concentrations of B (C) and Zn (D) in leaf blades and roots after 14 days of main saline-alkaline treatment. Plants were pretreated with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5), for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days. The data represents means ± standard errors from four biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance.

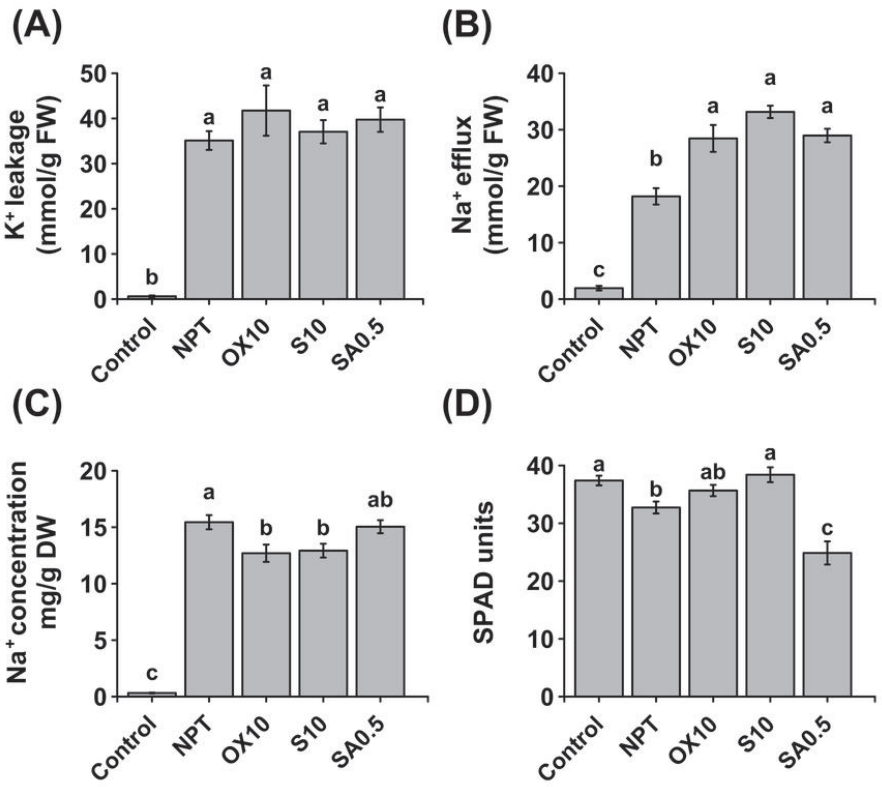

Fig. 3.5. Physiological experiments for K^+ and Na^+ homeostasis showing K^+ leakage in roots of 7 day pretreated and non-pretreated seedlings subjected to 50 mM Na, pH 8.50 for 24 hours (A), efflux of Na^+ from roots of 7 day pretreated and non-pretreated seedlings, subjected to 50 mM Na, pH 8.50 for 24 hours, and deionized water for 2 hours (B), and concentrations of Na^+ (C) and chlorophyll (D) in excised leaves from 7 day pretreated and non-pretreated plants subjected to 30 mM Na, pH 8.0 for 3 days. Plants were pretreated with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5), for 7 days and subjected to the above experiments. The data represents means ± standard errors from four biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance.

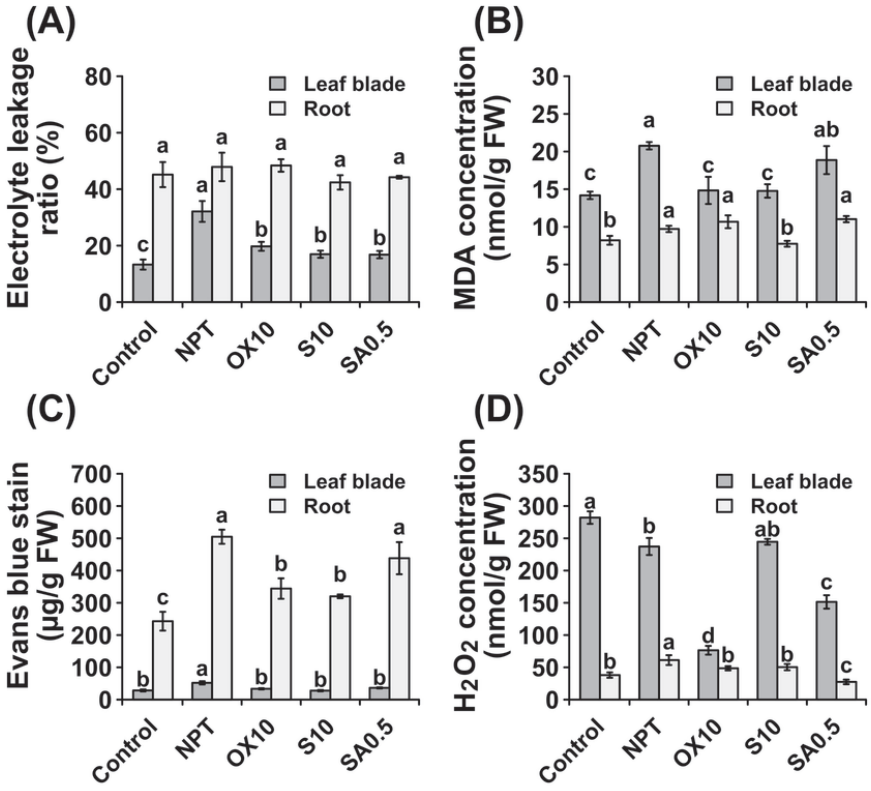

Fig. 3.6. Oxidative stress, membrane integrity and cell viability parameters showing leakage of electrolytes (A), malondialdehyde concentrations (B), Evans Blue staining (C) and concentrations of hydrogen peroxide (D), in leaf blades and roots after 14 days of main saline-alkaline treatment. Plants were pretreated with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5), for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days. The data represents means ± standard errors from four biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance.

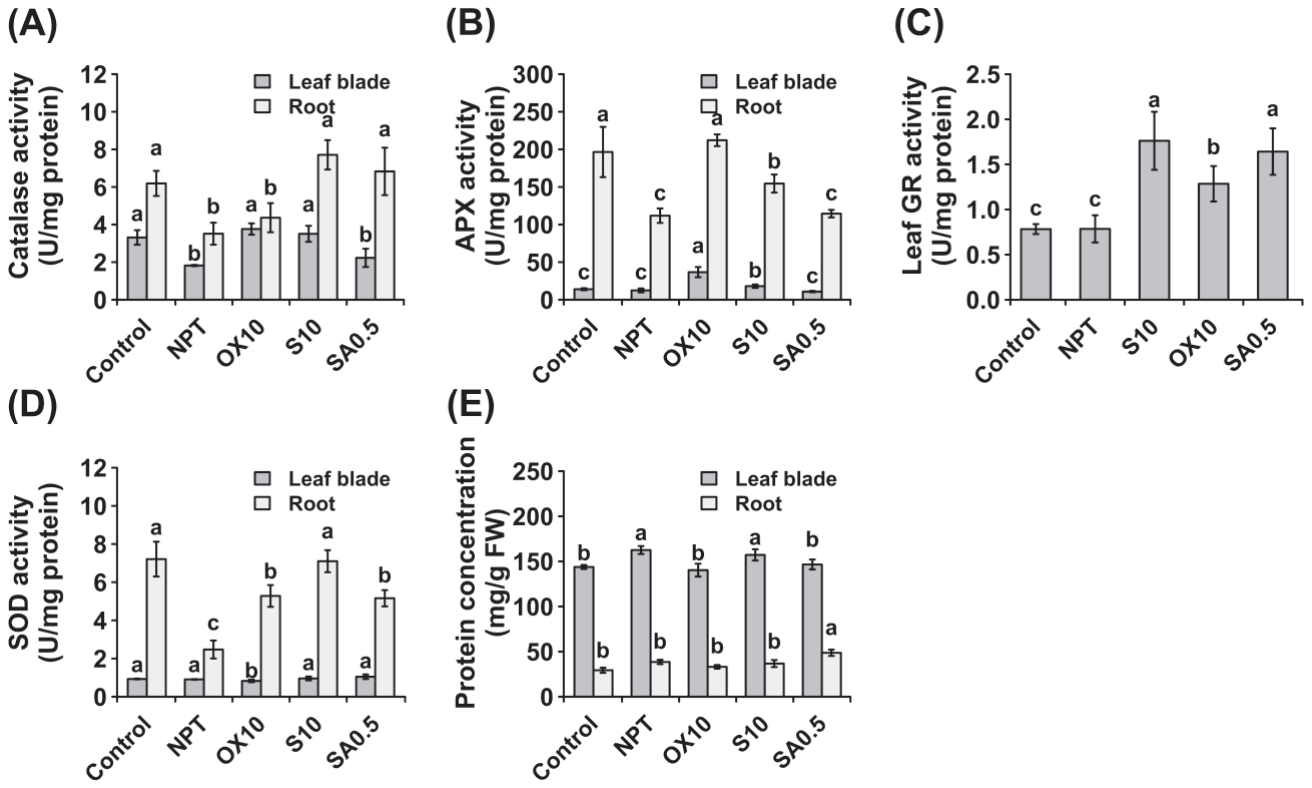

Fig. 3.7. Antioxidative enzyme activities of catalase (A), ascorbate peroxidase (B), glutathione reductase (C), and superoxide dismutase (D) and protein concentration (E) after 14 days of main saline-alkaline treatment. Plants were pretreated with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5), for 7 days and subjected to 50 mM Na, pH 8.25 for 14 days. The data represents means ± standard errors from four biological replicates. Different letters indicate significant differences by Tukey multiple comparison tests at 0.05 level of significance

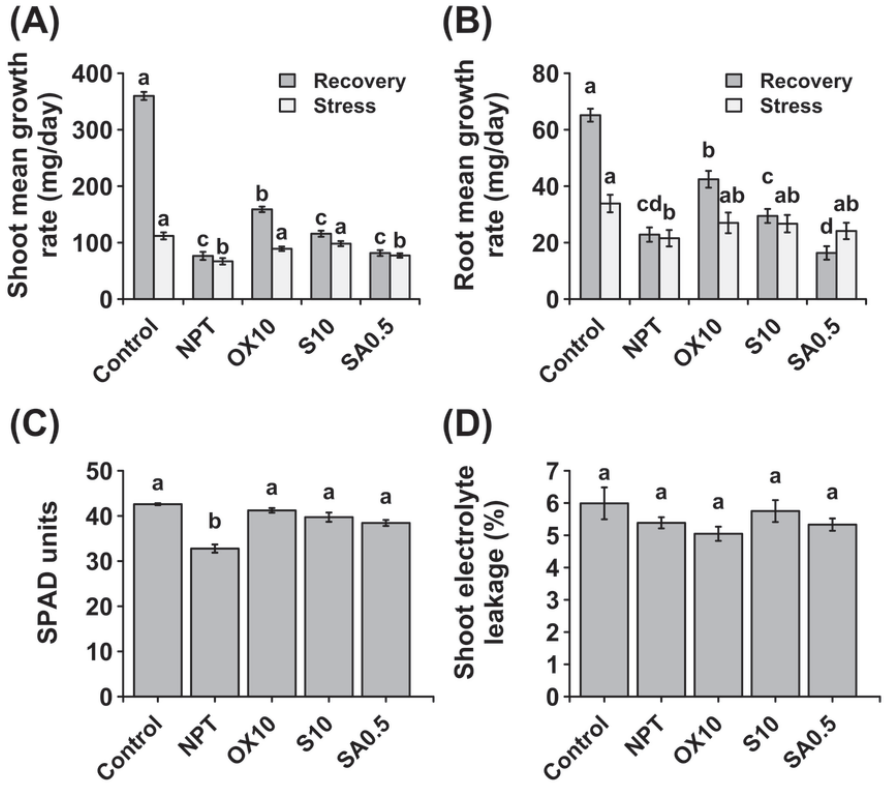

Fig. 3.8. Effect of pre-treatments on recovery of plants after exposure to saline-alkaline stress. Comparative mean growth rates during and after stress in shoots (A) and roots (B). Chlorophyll concentration (C) and membrane integrity assessed by the electrolyte leakage method in shoots (D) at the end of the 10-day recovery period. "Stress" group represents mean growth rate of plants during the 14 day growth period under main saline-alkaline stress (SAS) (50 mM Na^+, pH 8.25) after pre-treatment with 0 (NPT), 10 mM NaCl (S10), 10 µM H_2O_2 (OX10), and 0.5 mM $NaHCO_3$ (SA0.5) for 7 days. "Recovery" group represents the growth rate of the same plants upon completion of main saline-alkaline stress (SAS) and subjecting them to recovery under normal conditions (0 mM Na^+, pH 5.5) for 10 days. The data represents means ± standard errors from five biological replicates. Different letters indicate significant differences by Tukey's multiple comparison tests at $P < 0.05$.

3.5. Discussion

Rice is extremely sensitive to salt stress, although it is one of the prominent staples in the world. Hence, it remains imperative to identify quicker, yet effective means to increase rice growth and yield in such environments. Plants are adept at acclimating to salt stress, characterized by quicker and stronger responses to subsequent lethal exposure to the same stress (Kamanga et al. 2020; Liu & Saneoka 2019; Sriskantharajah et al. 2020). While acclimation studies are widespread, the physiological processes governing responses of plants to multiple exposures of different stresses are unclear. Here, we have shown that plants pre-exposed to lower concentrations of NaCl and H_2O_2 show improved growth and physiological characteristics under SAS conditions. Contrary to the hypothesis, the failure of $NaHCO_3$ pre-treatment to enhance growth suggests that the concentrations used were too high for pre-treatment in rice, likely causing physiological injuries. However, a similar concentration of 1 mM $NaHCO_3$ in Rye (*Secale cereale*) effectively improved tolerance to SAS conditions (Liu & Saneoka 2019). Overall, 10 mM NaCl and 10 µM H_2O_2 were the most effective (Fig. 3.1B). These latter results illustrate the concept of cross-tolerance, wherein pre-exposure to one stress, such as salinity or oxidative stress, promotes tolerance to another stress, such as saline-alkaline stress, as previously described {Formatting Citation}. In light of a myriad of abiotic stresses as currently faced, this critical attribute offers a prospect for generating plants tolerant to multiple abiotic stresses.

Under SAS conditions, the first line of defence entails acidification of the rhizosphere through H^+-ATPase-mediated proton efflux by plant roots (Li et al. 2016). In the present study, the failure of $NaHCO_3$ pretreated plants to suppress rise in growth medium pH (Fig. 3.2C) may partly elucidate their inability to effectively promote tolerance to SAS conditions (Fig. 3.1B). In contrast, plants pretreated with NaCl and H_2O_2 maintained a much lower growth medium

pH throughout the growth period compared to the NPT plants, indicative of higher root activity. Lower rhizospheric pH is also advantageous for maintaining the transmembrane proton gradient required for SOS-mediated efflux of Na^+ by roots SOS1 (Cao et al. 2020; Moriyama 2015). Experiments on Na^+ efflux showed that all pretreated plants had higher root Na^+ efflux than non-pretreated (Fig. 3.5B) including $NaHCO_3$. Therefore, whether higher root Na^+ efflux and resultant lower leaf blade Na accumulation (Fig. 3.3C) were as a result of lower growth medium pH are difficult to untangle. Furthermore, when the contribution of Na^+ uptake by roots was eliminated using experiments on Na^+ accumulation in excised leaf blades, both 10 mM NaCl and 10 µM H_2O_2 pretreated plants accumulated significantly lower Na in the leaf blades (Fig. 3.5C). In H_2O_2 pretreated plants, notwithstanding higher leaf blade Na^+ accumulation, the deleterious effects of Na^+ were ameliorated and leaf photosynthetic competency (Fig. 3.1C) was preserved. Moreover, despite the "statistical" significance of leaf blade Na^+ accumulation among PTs, this result suggests that the observed differences may not have been "biologically significant".

Na and K have similar physicochemical properties; hence, they exhibit competitive uptake systems (Marschner & Marschner 2012). Moreover, rice plant roots are leaky (Munns & Tester 2008), potentially losing significant amounts of K^+ under SAS conditions. Retention of root K^+ has been cited as a principal trait for salt tolerance (Chen et al. 2007). In the present study, pre-treatment with NaCl and H_2O_2 maintained a higher root K concentration (Fig. 3.3A) and K content (Fig. 3.3B). Separate experiments revealed extremely high root K^+ leakage under SAS (Fig. 3.5A), which could however not be alleviated by pre-treatments. Therefore, while root K^+ leakage was the primary cause of K deficiency under SAS, its contribution to the observed differences among various pre-treatments could not be verified. Nonetheless, considering the extreme loss of K^+ in roots under SAS conditions, these results suggest that even the slightest

improvements in root K^+ retention may be beneficial under saline-alkaline stress, primarily for osmotic adjustments and maintenance of root growth as also suggested by Chen et al. (2007). Additional benefits of acclimation included increased P uptake and accumulation especially in roots (Fig. 3.3E and D). Uptake of P is driven by H^+ gradient across the plasma membrane, such that when the rhizospheric pH is lower than root cytosolic pH, P uptake is promoted (Cao et al. 2020). Hence, under saline-alkaline stress, maintenance of P uptake is an extremely important attribute for plant growth and development, and also dependant on plant roots ability to lower rhizospheric pH through H^+-ATPases mediated proton efflux. However, further studies are required to elucidate the physiological mechanisms underlying this enhanced P uptake.

Fe deficiency, which is a typical feature of plants growing under high pH conditions, is characterized by chlorosis. Although Fe is a naturally abundant element, it forms insoluble ferric compounds that are non-bioavailable (Marschner 1995; Nakanishi et al. 2004) for uptake under high pH conditions. As shown in Fig. 3.1C, NPT plant leaves were chlorotic, which is a typical sign of Fe deficiency (Marschner & Marschner 2012). However, in NaCl and H_2O_2 pretreated plants, leaves were healthier (Fig. 3.1C) and accumulated higher Fe concentration and content (Fig. 3.4A and B). Therefore, it is tempting to attribute the high Fe concentration to the ability of plant roots to suppress rise in growth medium pH, as observed in Fig. 3.2A and B. Lower growth medium pH is thought to increase the solubility of ferric ions or support the reducing capacity of ferric Fe on the root surface (Kobayashi & Nishizawa 2012). However, similar increases in Fe concentration and content were also observed in $NaHCO_3$ pretreated plants (Fig. 3.4A and B, respectively) which otherwise did not show different growth medium pH from NPT plants (Fig. 3.2C), raising doubts on the causality between Fe concentration and lower rhizospheric pH. Alternative causes for the variations in Fe uptake could relate to

differences in key enzyme activities involved in Fe uptake (Nakanishi et al. 2004; Kobayashi & Nishizawa 2012), however, further studies to elucidate the physiological and molecular bases of this acclimation induced increase in accumulation are imperative. In $NaHCO_3$ pretreated plants (SA0.5), despite having higher leaf blade and root Fe concentration (Fig. 3.4A), leaf blades were relatively chlorotic (Fig. 3.1C), similar to non-pretreated plants, inconsistent with our expectations. This might have been as a result of considerably reduced chlorophyll concentration during the 7-day acclimation period (Fig. 3.1C), such that enhanced Fe uptake and accumulation during the main stress could not fully recover chlorophyll concentration by the sampling time.

Several reports have implicated the lethality of SAS to ROS-mediated damage. In an alkaline tolerant (*Alt1*) rice mutant, Guo et al. (2014) credited tolerance to enhanced defence against oxidative stress. Oxidative damage to membrane lipids causes peroxidation, which is analytically deduced from the concentration of malondialdehyde (MDA), an aldehyde by-product of lipid peroxidation (Ayala et al. 2014). In the present study, NaCl and H_2O_2 pre-treatments considerably alleviated SAS-induced membrane damage and cell death (Fig. 3.6A – C). These results, in part, indicate mitigated oxidative stress, owing to the enhanced ROS scavenging capacity in pretreated plants. ROS generation in plants is an inevitable consequence of aerobic metabolism. Until recently, the traditional paradigm that considers ROS as deleterious to plant cells is gradually fading owing to recent revelations of their critical role as secondary messengers (Apel and Hirt 2004; Mittler 2017). We hypothesized that pre-treatments would spark a mild generation of ROS, which would eventually activate systemic antioxidant defence responses. In agreement, H_2O_2 generation was significantly reduced in roots in all pretreated plants, whereas only H_2O_2 and $NaHCO_3$ pre-treatments were effective in leaf blades. In NaCl pretreated plants, despite having exceptional growth and lower Na

concentration in leaf blades, the higher H_2O_2 in tandem cautions against the inevitable expectation that ROS results in poor growth, harmonizing with notions earlier expressed in Mittler (2017). Perhaps the crucial aspect is maintaining a delicate balance between ROS generation and detoxification. Given that NaCl pretreated plants had higher antioxidant activities of CAT, APX, and GR (Fig. 3.7A – C), this balance might have been attained, cushioning against vicious functions of ROS (Apel and Hirt 2004). Better antioxidant enzyme activities were also observed in H_2O_2 pretreated plants, in agreement with earlier observations (Fedina et al. 2009; Wahid et al. 2007). Moreover, all pre-treatments considerably increased the SOD activity (Fig. 3.7D). Since H_2O_2 and hydroxyl radicals are superoxide radical derivatives, SOD offers a first line of defence against oxidative stress; hence, acclimation provided nearly complete protection against oxidative stress. Besides, H_2O_2 concentration (Fig. 3.6A) did not essentially correspond to the trends of electrolyte leakage, MDA concentration, and cell viability (Fig. 3.6B – C) in some PTs, in part cementing the above notions. Apparently, this may be ascribed to different antioxidant enzyme capacities (Fig. 7A – C) or capacities to repair of ROS mediated damage. Strategies adopted by plants to confront oxidative stress mediated damage engage three lines of steps; avoidance of ROS generation, detoxification of ROS (scavenging) and repair of ROS mediated damage (Guo et al. 2014; Mittler 2017). Hence, measurement of ROS generation alone may not sufficiently predict resultant ROS mediated damage. Furthermore, this result may point to involvement of other ROS, such as superoxide and hydroxyl radicles that were otherwise not assayed in the present study.

3.6. Conclusion

The present study strengthens the role of ROS in stress tolerance, and reports that acclimation to salinity stress, or oxidative stress promotes cross-tolerance to saline-alkaline stress by fostering stable Fe acquisition and ROS homeostasis. These processes are interlinked to plant

roots' ability to resist excessive changes in growth media pH, such that maintaining a lower pH is the first line of defence against saline-alkaline stress. These findings further suggest a crosstalk between salinity stress, oxidative stress and saline-alkaline stress, likely engaging common signals and pathways that develop cross resistance. While these signalling elements need to be fully investigated, results suggest involvement of ROS such as hydrogen peroxide, but calls for exploration of molecular signatures underlying these responses. These results demonstrate that acclimation to salinity stress and oxidative stress triggers systemic defence in rice plants, that aids stronger and quicker responses to saline-alkaline stress. Moreover, except for $NaHCO_2$ pretreated plants, during recovery period, plants quickly physiologically recover and resume rapid growth. This may be related to lower accumulation of H_2O_2 in the leaf blades (Fig. 3.6D), as a result of higher antioxidant enzyme activities specifically CAT, APX and GR (Fig. 3.7A – C) that detoxify cellular H_2O_2.This is a fundamental attribute for recurrent stresses with prolonged recovery periods, therefore, pretreatment with optimal NaCl and H_2O_2 may be a recommended option for quick improvements in tolerance to saline-alkaline stress in rice. This trait offers a prospect for developing plants tolerant to multiple stressors.

Chapter 4

General Discussion

Notwithstanding the worldwide agronomic prominence of rice, its growth and productivity remains considerably affected by a range of abiotic stresses. This study (1) comparatively investigated the effects of salinity stress, saline-alkalinity and alkaline stress on growth and physiology of rice seedlings; and (2) elucidated physiological mechanisms of acclimation-induced cross-tolerance to saline-alkalinity. This study finds that rice plants are far much sensitive to saline-alkaline stress than to either sole salinity or sole alkalinity. Furthermore, relative to salinity stress, at the concentrations used in the study, rice plants are more sensitive to high growth media pH than salinity stress. These results highlight the gravity of saline-alkaline stress to rice plants. Similar conclusions have been derived at in a number of studies (Lv et al. 2013; Shi & Wang 2005; Yang et al. 2007; Yang et al. 2008; Zhang et al. 2019).

Plants have a unique ability to tolerate to one stress following previous exposure to another stress, this is referred to as cross-tolerance (Foyer et al. 2016; Pastori & Foyer 2002; Perez & Brown 2014). However, this phenomenon has never been reported under saline-alkaline stress. In order to explore this option in rice, this study endeavoured to investigate the physiological mechanisms in which saline-alkaline stress confronts growth of rice to understand key stress components of saline-alkaline stress that can be harnessed as pre-treatments to elicit systemic defence responses. Consistent with previous reports, plants grown under high pH conditions, both saline-alkalinity and sole alkalinity exhibited clear signs of chlorosis (Fig 2.3) in both younger and older leaf blades as a result of Fe deficiency and Na toxicity, respectively (Li et al. 2016; Munns & Tester 2008). Saline-alkaline stressed plants accumulated multiple folds more Na in leaf blades than salinity stressed plants (Fig. 2.4D). This finding harmonized with pre-existing reports and further revealed chronic K deficiency in plant roots grown under saline-alkalinity (Fig. 2.4C), as a result of excessive K^+ leakage in the roots (Fig. 2.6A). Furthermore, this study observed excessive accumulation of H_2O_2 in leaf blades, that may have

exceeded plants' inherent antioxidative capacity causing membrane damage. Oxidative stress has been implicated in lethality of saline-alkalinity, particularly in plant roots of many species (Fu et al. 2017; Peng et al. 2008; Zhang et al. 2017; Zhang & Mu 2009).

Premised on the above results, the study acclimated rice seedlings to mild stress components; Na^+ toxicity (NaCl), oxidative stress (H_2O_2) and high pH ($NaHCO_3$). Direct application of ROS by oxidative agents such as H_2O_2 has been investigated as a potential means of inducing cross-tolerance in plants (Perez & Brown 2014). H_2O_2 is a recognized inducer of antioxidant responses, hence has been utilized as a seed pretreatment for plants growing under abiotic stresses (He et al. 2009; Wahid et al. 2007). Besides, we had previously shown that plants pre-exposed to mild NaCl develops tolerance upon subsequent exposure to a higher NaCl stress (Kamanga et al. 2020; Sriskantharajah et al. 2020). Hence, this study has shown that rice seedlings pre-exposed to 10 mM NaCl and 10 µM H_2O_2 showed greater tolerance to and better photosynthetic properties under saline-alkalinity (Fig. 3.1A – C). Moreover, upon conclusion of the stress treatment, H_2O_2 pretreated plants showed better recovery compared to non-pretreated plants, in part attributed to lower H_2O_2 accumulation, an essential attribute for recurrent stresses with prolonged recovery periods. In total, these results reveal that rice seedlings pre-exposed to salinity stress and oxidative stress develop cross-tolerance to saline-alkalinity, pointing to a likelihood for involvement of common pathways and signalling elements among these stresses.

In this study, it was shown that NaCl and H_2O_2 pretreatments (PTs) were able to supress excessive rise in growth medium pH (Fig. 3.2A and B), a rhizosphere acidification strategy that is dubbed a first line of defence under alkaline conditions (Li et al. 2016). This crucial attribute also plays a critical role in increasing Fe solubility, facilitating rice plants' Fe uptake

(Ishimaru et al. 2006; Kobayashi & Nishizawa 2012). As a consequence, plants pretreated with 10 mM NaCl and 10 μM H_2O_2 exhibited higher leaf blade Fe concentration and content in the plant (Fig. 3.4 A and B) and accordingly averted leaf Fe deficiency chlorosis (Fig. 3.1C). Lower rhizospheric pH is also crucial for SOS root Na^+ efflux, to avoid excessive accumulation of Na^+ in shoots. Consequently, plants pretreated with NaCl and H_2O_2 showed considerably higher efflux of Na^+ (Fig. 3.5B) and lower leaf blade Na^+ concentration (Fig. 3.3C) but it did not translate into any decreases in leaf blade Na^+ content (Fig 3.3D). That notwithstanding, deleterious effects of Na^+ were ameliorated and leaf photosynthetic competency was preserved by both H_2O_2 and NaCl PTs. Besides, the PTs enhanced K and P uptake capacities, elements critical for plant growth under saline-alkaline conditions, however mechanisms behind the enhanced uptake need to be further investigated.

Considering the high oxidative stress mediated membrane damage observed in chapter 2 (Fig. 2.10 A – D, Fig. 2.11 A and B), I investigated whether acclimation to saline-alkaline stress would ameliorate oxidative stress induced damage as previously reported (He et al. 2009; Wahid et al. 2007; Kamanga et al. 2020; Liu & Saneoka 2019). Accordingly, leaf and root membrane integrity (Fig. 3.6A and B) and cell viability (Fig. 3.6C) were considerably enhanced in H_2O_2 pretreated plants by tremendously reducing leaf and root H_2O_2 accumulation (Fig. 3.6D). While NaCl PT also enhanced membrane properties and cell viability, oxidative stress was only significantly reduced in roots (Fig. 3.6A – D). Plants are surfeited with a battery of antioxidants to detoxify generated ROS under abiotic stresses. Here, NaCl and H_2O_2 pretreated plants exhibited higher CAT and APX activities in both leaf blades and roots, and GR activity in leaves (Fig. 3.7A – C), these enzymes are crucial for detoxification of H_2O_2. Superoxide dismutase (SOD) offers the first line of defence against oxidative stress, since H_2O_2 and hydroxyl radicles are derivatives of superoxide radicles. SOD antioxidant activity was also

considerably increased in root tissues (Fig. 3.7D) of all pretreated plants. Being directly exposed to Na^+ toxic and alkaline rhizosphere, efficient antioxidant capacity in roots is crucial. These results suggest that a nearly complete shield against oxidative stress was instituted in H_2O_2 and NaCl pretreated plants. Albeit having higher H_2O_2 concentration in the leaf blades in NaCl pretreated plants, efficient antioxidant capacities cushioned against their harmful effects attaining an optimal equilibrium between their generation and scavenging.

In conclusion, this study reports that saline-alkaline stress is a highly lethal abiotic stress relative to sole salinity and sole alkaline stress. Plants exposed to saline-alkaline stress experience severe growth reductions due to leaf blade Na^+ toxicity, chronic Fe deficiencies particularly in younger leaves, aggravated root K^+ deficiency as a result of tremendous root K^+ leakage and oxidative stress mediated damage in leaf and root tissues. However, these effects were ameliorated by pre-exposure to mild ionic (NaCl) and oxidative (H_2O_2) stress components that developed cross-tolerance to saline-alkalinity. While mechanisms of acquired resistance differed across PTs, an aggregated consensus suggests the crucial role played by the following physiological mechanisms; (1) ability to maintain a lower growth medium pH (2) maintenance of a stable acquisition of Fe, P and K and (3) regulated detoxification of reaction oxygen species (ROS) via enhanced antioxidant enzyme activities. These results suggest that mechanisms which protect against salinity stress or oxidative stress may also provide protection against saline-alkalinity. Furthermore, they also suggest and strengthen the possibility that a crosstalk exists between salinity stress, oxidative stress and saline-alkalinity, likely engaging some overlapping signalling elements. This is an important finding, offering a prospect to develop plants tolerant to multiple forms of stress, an important attribute and a major breeding goal in light of a myriad of abiotic stresses.

Chapter 5

Summary

Salt stress is a major abiotic stress globally, affecting over 800 million ha of land. Depending on soil pH, salt stress can be categorized into neutral salt stress, popularly known as "salinity stress" and saline-alkaline stress, also known as "alkali" stress. Over the years, salinity stress has been extensively studied, hence physiological and molecular mechanisms underpinning plants' responses to salinity stress have been thoroughly elucidated. Contrarily, relatively less is known regarding how saline-alkalinity confronts plant growth. Meanwhile, physiological mechanisms regulating plants' resistance under saline-alkaline stress remain hugely conjectural. Rice (*Oryza sativa* L.) represents a crop of prominent worldwide food value in addition to being a key model research crop, yet known for its reputation of extreme sensitivity to salt stress. This study was aimed at unravelling comparative physiological mechanisms in which saline-alkalinity confronts growth and physiology of rice plants. Furthermore, the study explored the potential of acclimation to different stress components in developing cross-tolerance to saline-alkaline stress.

In order to evaluate comparative physiological responses of rice to saline-alkalinity, 21 day old seedlings of rice cultivar Hinohikari were subjected to control conditions (0 mM Na^+, pH 5.5) neutral salinity stress (50 mM Na^+, pH 5.5), moderate and severe alkalinity (0 mM Na^+, pH 7.5 and 8.5 respectively) and moderate and severe saline-alkalinity (50 mM Na^+, pH 7.5 and 8.5 respectively) for 16 days. Time course changes in growth were monitored every 4 days. The study revealed that saline-alkaline stress was the most growth limiting, with growth nearly ceasing on the fourth day of stress imposition, whereas under salinity stress growth decreases were only apparent from the 12^{th} day. Furthermore, at the same growth media pH, sole alkalinity was less detrimental relative to saline-alkalinity, but more lethal than salinity stress. Therefore, effects of rhizospheric Na^+ are aggravated by growth media pH, rendering saline-alkalinity more harmful on growth than sole salinity and sole alkalinity.

Next, comparative physiology underlying saline-alkaline damage on rice seedlings was investigated. Plant grown under both alkaline and saline-alkaline conditions was characterized by chlorosis, which was confirmed to have resulted from Fe deficiency, particularly in younger leaves owing to immobile nature of Fe. In older leaves, alkaline stressed plants exhibited healthier leaves, whereas saline-alkaline stressed plants were severely chlorotic and nearly dying off. This chlorosis was ascribed to leaf blade Na^+ toxicity, which was inordinately high in saline-alkaline stressed plants, resulting into premature senescence of older leaves. Additionally, saline-alkaline stress incited K^+ deficiency predominantly in roots, due to tremendous root K^+ leakage, thus, while resistance to saline-alkaline stress may be complex and multigenetic, ability to regulate root K^+ loss may be a valuable trait. Furthermore, it was observed that under saline-alkaline stress, leaf blade membrane integrity was substantially compromised, due to toxic accumulation of Na^+ in leaf blades, and resultant oxidative stress as shown by high accumulation of H_2O_2 in leaf blades. Hence, it was concluded that at the used experimental conditions, rice plants are more sensitive to high growth media pH, and this sensitivity is aggravated under mixed saline-alkaline conditions. Primarily, hyperaccumulation of Na^+ in leaf blades, accelerated root K^+ deficiency due to root K^+ leakage, Fe deficiency in younger leaves, and oxidative stress mediated membrane damage were crucial causes of severity under saline-alkalinity.

Premised on the these findings, the study further explored the potential of acclimation in developing cross-tolerance to saline-alkalinity. Rice plants were pretreated (PT) with three concentrations of each of NaCl, $NaHCO_2$, and H_2O_2 for one week, then subjected to 50 mM Na^+ at pH 8.25 for 14 days and investigated growth and physiological responses relative to non-pretreated (NPT) plants. Acclimation to 10 mM NaCl (salinity) and 10 µM H_2O_2

(oxidative stress) were the most effective, hence were the focus for further investigation. These PTs developed cross-tolerance to saline-alkaline stress, in addition to promoting quicker recovery and resumption of growth upon conclusion of stress. Physiologically, both PTs noticeably maintained a lower growth medium pH, employing a growth medium acidification strategy which was not further investigated. The lower growth medium pH also facilitated Fe acquisition, whose uptake is else constrained under high pH. On ionic relations, NaCl pretreated plants accumulated much lower Na^+ in leaf blades by enhancing root Na^+ efflux. In H_2O_2 pretreated plants, despite higher root Na^+ efflux, leaf blade Na^+ accumulation was not reduced. However, experiments on Na^+ uptake in excised leaf blades showed lower accumulation of Na^+. These results may suggest that the high leaf blade Na^+ accumulation in H_2O_2 pretreated plants emanated from processes relating to either uptake by roots and/ or resultant transport to the shoots. That notwithstanding, deleterious effects of Na^+ were ameliorated and leaf photosynthetic competency was preserved by both H_2O_2 and NaCl PTs. Additionally, both PTs were unable to lower saline-alkaline – triggered root K^+ leakage, but efficiently reduced oxidative stress – triggered membrane damage and lipid peroxidation, and improved cell viability by constituting a complete shield of antioxidant enzymes SOD, CAT, APX and GR, establishing an ultimate equilibrium between ROS generation and scavenging.

These findings propose a possible crosstalk between salinity stress, oxidative stress and saline-alkaline stress, such that exposure to salinity stress or oxidative stress develops cross-tolerance to saline-alkaline stress. This is an important finding particularly in light of a myriad of abiotic stresses, offering a prospect for breeding plants resistant to a range of abiotic stresses. Besides, upon elimination of stress and resumption of normal growth conditions, pretreated plants, particularly those pretreated with H_2O_2 quickly resumed growth and physiologically recovered, a crucial attribute especially under recurrent abiotic stresses with some prolonged inter stress

recovery periods. In order to provide a fairly broad picture of the mechanism of cross-tolerance, this study recommends a further exploration of molecular and perhaps epigenetic marks underpinning cross-tolerance, and an investigation of overlapping signalling elements between salinity, oxidative and saline-alkaline stress.

Box 5.1. Key summary points of the study

Key summary points of the study

• The rice cultivar Hinohikari is extremely sensitive to high pH, and its sensitivity is aggravated under saline-alkalinity than sole salinity stress.
• Saline-alkalinity promotes Fe deficiency chlorosis in younger leaf blades and Na^+ toxicity induced senescence in older leaf blades.
• High pH rhizosphere accelerates above ground organ Na^+ accumulation relative to same molar growth medium Na^+ under neutral rhizospheric pH.
• Saline-alkaline stress incites root K^+ deficiency through excessive root K^+ leakage
• Membrane damage in plant leaves through excessive accumulation of ROS was also an important effect of saline-alkaline stress in rice plants.
• Acclimation to 10 mM NaCl and 10 µM H_2O_2 develops cross-tolerance to saline-alkalinity by;
  Supressing rise in growth medium pH
  Sustaining a stable acquisition of Fe and other micronutrients
  Enhancing uptake of K and P
  Regulating generation of ROS via enhanced antioxidant enzyme activities of SOD, CAT, APX and GR

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Acknowledgements

I thank the Almighty God for the gift of healthy life, wisdom and talents. To my academic supervisor, Associate Prof. Dr. Akihiro Ueda of Plant Nutritional Physiology Laboratory, thank you so much for offering me with the opportunity to study under your guidance. Your mentoring, advices, teaching and guidance have shaped me into a better scientist. Further gratitude goes to my co-advisors Prof. Rumi Tominaga, Prof. Jun Wasaki, Associate Prof. Toshinori Nagaoka, and Prof. Sakae Agarie for always being there to offer their academic guidance towards my doctoral thesis. To Prof. Hirofumi Saneoka, thank you for your academic mentoring and guidance and always making my stay in the laboratory more comfortable. Also, I sincerely thank Ms. Himiko Koi of the student support office of graduate school of Integrated Sciences for Life, and all members of the Laboratory of Plant Nutritional Physiology for their support in various day to day laboratory and personal activities. Further gratitude goes to my family, my late father Mr. Maganizo Phillip Kamanga, to whom this thesis is dedicated, my mother Mrs. Gertrude Thabu Kamanga, my wife Ethel N. Kamanga, and all the family and friends. Finally, to the People of Japan, through the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for funding my doctoral studies.