Germination, Survival and Growth of the Coleoptile of Rice under Combined NaCl and Anoxia when Submerged

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ABSTRACT

In many tidal lowland and coastal areas, direct seeded rice is often exposed to flooding in saline water. Understanding the physiological adaptations of submerged (non-transpiring) rice seedlings in these unfavorable conditions will provide new information on salt tolerance mechanism under submergence.

In aerated salinity, submerged rice seeds germinated and tolerated at least 200 mM NaCl. Na^+ and Cl^- were used by seedlings as osmotica, by maintaining tissue concentrations approximately similar to those in the external medium during the quasi-steady state. With less organic solutes available and decreases of Na^+ and Cl^- concentrations with time, osmotic pressure in roots was probably maintained by restricted growth (i.e. less volume expansion). At 200 mM NaCl, supplemental Ca^{2+} at 5 and 10 mM alleviated ~ 24% of the growth inhibition in roots, but had no effects in shoots. The different responses of shoots and roots to the different addition of Ca^{2+} might be due to the increase of K^+/Na^+ ratio in roots but not in shoots.

In a combination of NaCl and anoxia, intact rice seedlings tolerated at least 100 mM, whereas excised coleoptile tips tolerated only 50 mM NaCl. In this energy starvation, Na^+ and Cl^- concentrations in shoots of intact seedlings were ~ 30% less than concentrations in shoots of seedlings submerged in aerated solutions. In anoxic intact seedlings, an increase of ethanol production at 50 mM NaCl compared to that in 0.3 mM NaCl indicated that more energy was required for cell maintenance. Despite a ~ 25% shoot growth inhibition, anoxic rice seedlings were able to survive at 100 mM NaCl by maintaining K^+/Na^+ ratio and also turgor pressure. Excised coleoptile tips failed to survive at 100 mM NaCl in anoxia due to K^+ loss and drop in turgor pressure. However, measurement of K^+/Na^+ ratio and ethanol formation in excised coleoptile tips at 50 mM NaCl gave a relatively similar value as the assessed value for the coleoptile of intact seedlings, and therefore those excised tips provide a convenient system for evaluation of anoxia tolerance (i.e. ion uptake and ethanol production) of rice under salinity up to ~ 50 mM NaCl. Despite 50-75% reduction in growth, ethanol produced by coleoptile tips at 50 mM NaCl was 10 – 16% higher than that at 0.3 mM NaCl. This energy was presumably consumed during Na^+ exclusion when Na^+ and Cl^- net uptakes increased prior to reaching a quasi-steady state. However, calculation of the energy expenditure using the assumption that Na^+ was transported via Na^+-H^+ antiporter showed that fermentation could never meet these requirements entirely, indicating other

types of transport, involving less energy expenditure must be involved during initial phase of acclimation to 50 mM NaCl in anoxia.

After return to non-saline aerated solutions, the rapid resumption of growth and K^+ uptake in intact seedlings at 100 mM NaCl, and a very low leakage of inorganic P and vigorous K^+ and Cl^- net uptakes in coleoptile tips at 50 mM NaCl, indicated that rice seedlings survived without substantial injuries under those combinations of NaCl and anoxia. Overall, the survival of the coleoptile of rice in combined anoxia and 50 mM NaCl was associated with management of energy production and consumption required for solute transport, which is likely to have priority for available energy to enhance survival in these unfavorable conditions.

THESIS DECLARATION

This thesis was completed during the course of my enrolement in a PhD degree in the School of Plant Biology at The University of Western Australia. This thesis contains no experimental material that has been previously presented for any degree at this or any other institution. The thesis contains published work which has been co-authored. The detail of this work is outlined below.

The design of the experiments, experimental work and the preparation of this thesis were done by myself, under the supervision of Winthrop Professor Timothy D Colmer and Honorary Associate Professor Hank Greenway.

Chapter 3.

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The version in this thesis is the original chapter submitted for examination, with revisions made as suggested by the examiners. The chapter was more extensively re-ordered and revised for the manuscript submitted to, and now published in, *Physiologia Plantarum*.

Budiastuti Kurniasih

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LIST OF ABBREVIATIONS

AA	:	amino acids
At	:	*Arabidopsis thaliana*
ADH	:	alcohol dehydrogenase
ASPs	:	anaerobic stress polypeptides
ATP	:	adenosine triphosphate
CBLs	:	calcineurin B-like
CCC	:	cation chloride co-transporter
CHX	:	cation/H^+ exchanger
CIPKs	:	CBL-interacting protein kinases
CLC	:	voltage gated Cl^- channel
EDTA	:	ethylenediaminetetraacetic acid
EIDW	:	ethanol insoluble dry weight
FW	:	fresh weight
GABA	:	gamma-aminobutyric acid
HAK	:	high-affinity K^+ transporter
HKT	:	high-affinity K^+ transporter
H^+-PPiase	:	proton pumping pyrophosphatase
HPLC	:	high-performance liquid chromatography
ICPMS	:	inductively coupled plasma-mass spectrometry
LOD	:	limit of detection
MES	:	2-(N-morpholino) ethanesulfonic acid
MPa	:	megapascal
NaCl	:	sodium chloride
NAD	:	nicotinamide adenine dinucleotide
NHX	:	Na^+/H^+ exchanger in tonoplast
NKCC	:	Na^+, K^+ and Cl^- cotransporter
NSCCs	:	nonselective cation channels
Os	:	*Oryza sativa*
OA	:	organic acids
P	:	total phosphorus
PCA	:	perchloric acid
PCD	:	program cell death

PDA	:	photodiode array detector
PDC	:	pyruvate decarboxylase
PPDK	:	pyruvate orthophosphate dikinase
Pi	:	inorganic phosphate
PPi	:	inorganic pyrophosphate
REL	:	relative electrolyte leakage
RGR	:	relative growth rate
ROS	:	reactive oxygen species
SE	:	standard error
SOD	:	superoxide dismutase
SOS	:	salt overly sensitive
Ta	:	*Triticum aestivum*
TCA	:	tricarboxylic acid

Symbol annotation:

Ψ_w	:	water potential
π_{sap}	:	sap osmotic potential
π_{sol}	:	solution osmotic potential
ΔG	:	free energy gradient
P	:	turgor pressure
µmol	:	micromol
Nmol	:	nanomol
d^{-1}	:	per day
g^{-1}	:	per gram
h^{-1}	:	per hour
(X)in	:	internal ion concentration
(X)out	:	external ion concentration

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CHAPTER 1

General Introduction

Introduction

Rice is an important source of calories for over 3 billion people and rice cultivation is the principal activity and source of income for more than 100 million households in developing countries in Asia, Africa and Latin America (Nguyen, 2004). Rice seedlings in the field are often exposed to salinity. This often occurs immediately after planting in saline soil or fields irrigated with saline water or in areas inundated by sea water (Flowers and Yeo, 1995; Hakim et al., 2010). The detrimental effects of salinity on the growth and yield of salt-sensitive crops, including rice, are well documented (Maas and Hoffman, 1977). In addition, paddy fields with flood irrigation and also other areas of poorly drained land will have soil O_2 deficiency (Drew et al., 1988; Ram et al., 2002; Ismail et al., 2009). Since O_2 deficiency alone is harmful to the growth of most crops, although rice is tolerant (Ismail et al., 2009), the combined effects of excess salt and O_2 shortage can be especially damaging to crops (Drew et al., 1988; Barrett-Lennard, 2003). Understanding the physiology of rice germination adaptation to combined salinity and anoxia will help to improve rice seedling establishment under these adverse conditions.

Rice germination and growth under salinity

Rice genotypes have different germination abilities in saline conditions and a high variation is found amongst the different varieties of the same crop (Maas and Hoffman, 1977; Khan et al., 1997). Based on the classification of salinity tolerance in crops, rice is classified as sensitive, so that its tolerance falls in the range up to 8 dS m^{-1} (~ 77 mM) (Maas et al., 1986). Although rice is salt-sensitive in vegetative and generative growth stages, rice is relatively tolerant during germination stage (Pearson et al., 1966; Heenan et al., 1988; Khan et al., 1997; Lutts et al., 1995).

High internal concentrations of Na^+ and Cl^- can inhibit the metabolism of dividing and expanding cells (Neumann, 1997), retarding germination and leading to seedling death (Zhang et al., 2010). On the other hand, using salt as an osmoticum in mild to moderately saline conditions appears to allow some cereal seeds to germinate more rapidly, even at the moderately lower osmotic potential in these NaCl levels (Zhang et al., 2010). Salt ions are metabolically cheaper than sugars for generating osmotic potential in plant cells (Raven, 1985). The net uptake of Na^+ into plant cells is due to a balance between influx through ion channels and efflux through a Na^+/H^+ antiporter (Blumwald et al., 2000; Tester and Davenport, 2003). The energy expensive

mechanism of Na^+ extrusion has been suggested in some studies on cereals, e.g. the increase in respiration that far exceeds the theoretical cost of Na^+ extrusion in rice (Malagoli et al., 2008).

Rice growth responses in waterlogging/ flooding

Waterlogging is defined as a condition of the soil in which excess water limits gas diffusion (Setter and Waters, 2003). O_2 diffusivity in water is approximately 10,000 times slower than in air (Grable, 1966) and therefore the primary cause of impairment to plants grown in waterlogged soil is inadequate supply of O_2 to the submerged tissues.

Unlike the growth and development of other crop plants which are largely inhibited under waterlogged conditions, rice (*Oryza sativa* L.) has some adaptive traits for tolerance of waterlogging and even complete submergence which enables it to grow well under waterlogged and flooded conditions (Amstrong, 1971; Colmer, 2003; Hattori et al., 2011). A rice plant may survive 10-14 days of complete submergence and renew its growth when the water subsides (Nishiuchi et al., 2012). In the absence of O_2, however, cell metabolic activity has to be restricted to save ATP and thus energy consumption is decreased (Geigenberger, 2003; Greenway and Gibbs, 2003). During this energy crisis in anoxic tissues, the decrease of energy requirement for maintenance includes reduction of protein turnover and ion fluxes (Greenway and Gibbs, 2003).

Significant decreases in K^+ and Cl^- fluxes have been studied in anoxic rice coleoptiles (Zhang and Greenway, 1995; Colmer et al., 2001) as well as K^+ translocation from endosperm to the coleoptile of seedlings in anoxia (Huang et al., 2003). This inhibition in solute transport may be associated with direction of limited amounts of energy to transport of more important solutes such as sugars via down-regulation of some of the transport systems (Greenway and Gibbs, 2003). The assessed energy requirement for maintenance of some plant tissues and cells during anoxia were 58–88% of those required for the maintenance of leaves in air (Huang et al., 2005).

Plant growth under a combination of salinity and anoxia

Stress resistance mechanisms in plants rely on energy supplied by respiration. Therefore, when environmental stresses occur under O_2-deficient conditions, plants may be too energy limited to cope with the stress. Research has been conducted to

study how salinity affects growth, ion selectivity of roots, ion transportation, accumulation and exclusion, as well as to separate the effect of ion toxicity and osmotic stresses caused by salinity in many plants (e.g. reviewed by Munns and Tester, 2008), but only a few studies were concerned about the energy requirement to cope with salinity stress under low O_2 (Drew et al., 1988; Barrett-Lennard, 2003). This question of energy requirements in saline conditions is particularly interesting to approach by studying responses to salinity at low O_2 concentrations, when there is severely curtailed energy production. Even in the coleoptile of anoxia tolerant rice, ATP production is much lower in anoxia than in air. With the combined effect of anoxia and salinity, any energy deficit may be aggravated, since plants have to cope with ion excess and osmotic stress. This thesis will contribute to fill the gap in the available information on physiological responses of rice to combined salinity and anoxia, as related to acclimation to these conditions.

Previous studies on the combined salinity and anoxia were conducted on transpiring plants, with only roots which were submerged in nutrient solution. There is lack of information on the mechanism of plant growth response under non-transpiring saline conditions, since all the information on growth, ion regulation, osmotic relations as well as energy budgets was discussed based on transpiring conditions and may not be relevant to non-transpiring plants. In fact, during flooding in tidal lowland and coastal areas, direct seeded rice will be exposed to salinity in non-transpiring anoxic condition. Understanding a metabolic adaptation of rice to a combined salinity and anoxia may contribute to minimizing yield losses by tidal flooding or excessive saline irrigation in lowland and coastal areas, and provide new insight on plant adaptation to the interaction of salinity and anoxia.

Thesis outline and research objectives

The tolerance of rice seedlings to waterlogging/anoxic condition has been summarized elsewhere (Menegus et al., 1991; Perata et al., 1992, 1997; Nishiuchi et al., 2012), and rice tolerance to salinity during germination has also been assessed (Pearson et al., 1966; Heenan et al., 1988; Khan et al., 1997) and yet our understanding of rice responses to the combination of these unfavorable environments is limited. It is also widely acknowledged that in the absence of O_2, rice is able to perform anaerobic fermentation to supply ATP for cell maintenance during the energy crisis. Therefore, the overall aim of the studies described in this thesis was to elucidate rice growth,

survival and how rice coped with the high Na^+ and Cl^- concentrations during NaCl exposure, with a very limited energy supply during anoxia.

More specifically the research objectives were:

1. Consolidate the literature on rice tolerance to salinity and anoxia at germination and emergence of the coleoptile, and contextualize it with some literature on the combined effects of salinity and waterlogging commonly found for transpiring plants (Chapter 2).
2. Examine NaCl dose response of rice (cv. Amaroo) in terms of germination and growth to obtain the broad response of rice to then define the appropriate range of NaCl to be tested in the next experiments that involved a combination of high NaCl and anoxia (Chapter 3).
3. Determine the effect of Ca^{2+} in alleviating growth inhibition by high and low NaCl, and thus the appropriate Ca^{2+} concentration to be applied in experiments (Chapter 3).
4. Examine the responses of rice seedlings to combined NaCl and anoxia as well as determine whether excised coleoptile tips can be used for studying the interaction between anoxia and high NaCl (Chapter 4).
5. Investigate the responses of excised coleoptile tips to combined salinity and anoxia (Chapter 5):
   a. Quantify the Na^+ and Cl^- net uptakes, ethanol production and contribution of ions and organic solutes to π_{sap}.
   b. Evaluate the energy production and estimated energy consumed for Na^+ and Cl^- fluxes during the energy crisis in combined NaCl exposure and anoxia.
6. In a Concluding Discussion, compare the different responses of rice germination and growth to NaCl exposure in non-transpiring condition and the responses previously described for transpiring plants, and examine the responses of rice to a combination NaCl and anoxia as a compilation from the preceding three experimental chapters (Chapter 6).

The work described in this thesis found that rice seedlings are able to germinate and grow in at least 200 mM NaCl without substantial injury, by using Na^+ and Cl^- as efficient osmoticum. When exposed to anoxia, rice seedlings could grow and tolerated at least 100 mM NaCl. However the tolerance of excised coleoptile tips was lower (~50 mM NaCl) than that of intact seedling shoots. Survival of the rice coleoptile tips in a

combination of anoxia and 50 mM NaCl was associated with management of energy production and consumption needed for transport of solutes which is likely to have priority for energy available to enhance survival in this energy crisis.

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CHAPTER 2

Rice Germination and Growth under the Combination of High NaCl and Anoxia

Introduction

The principal aim of this thesis was to study the tolerance of germinating rice seedlings to a combination of high NaCl and anoxia, i.e. an energy deficit. Seed germination is an important stage in the life cycle of plants. Despite the importance of seed germination under high salinity (Zhang et al., 2010), the mechanism of salt tolerance in germinating seeds is relatively poorly understood, compared with the more information available for salt tolerance of vegetative plants (Greenway and Munns, 1980; Flowers, 2004; Munns and Tester, 2008) and reviewed in this section.

Most of the previous studies of plant response to salinity described in this review used transpiring plants. Since 50 times more water is transpired than is retained in leaves and only a small portion of Na^+ is retranslocated from shoot to root, therefore mitigating high Na^+ concentration in shoots is the main problem for transpiring plants to survive in saline soil (Munns and Tester, 2008). On the other hand, little information is available on salt tolerance mechanism of non-transpiring plants. With the absence of transpiration stream, shoots of non-transpiring plants which are surrounded by solution are therefore faced with a condition more similar to that of roots than in shoots in air of transpiring plants, i.e. in terms of the possibility to exchange ions with the medium. However, salt tolerance mechanism in roots of transpiring plants are also given less attention due to the less Na^+ accumulation in roots than in shoots. Hence, the current study provides some information to help fill the gap in understanding salt tolerance mechanisms of non-transpiring plants.

In contrast to the lack of information on salt tolerance of non transpiring plants, many studies have been conducted to investigate anoxia tolerance in rice at germination and early seedling growth (Bertani et al., 1980; Menegus et al., 1991; Guglielminetti et al., 1995; Colmer et al., 2001; Greenway and Gibbs, 2003; Huang et al., 2003, 2005). Therefore, tolerance to anoxia during rice germination will be first reviewed, whereas the general themes of tolerance to salinity will be considered more broadly to provide an overview, followed by some detail on response of rice to salinity in the germination stage.

Rice tolerance to anoxia

A single rice cultivar (Amaroo) was chosen for all experiments in this study. Amaroo is a commercially-grown cultivar and has been used for studies on anoxia

tolerance. It was well-known for its anoxia tolerance at the seedling stage (Huang et al., 2003; 2005). The salt-resistance level of this cultivar was, however, not reported previously, so work in this thesis helps to fill that knowledge gap, as well as to elucidate the interactive effects of salinity combined with anoxia.

During anoxia, most cereals such as barley and wheat are unable to grow or even germinate, whilst rice (Perata et al., 1997) shows exceptional tolerance to germinate and elongate the coleoptile. Anoxia tolerance is important to direct seeded rice when pre-germinated seed is sown in rain-fed lowlands and rainfall may result in complete submergence of seeds or seedlings (Setter and Ella, 1994). Understanding the mechanisms of adverse effects of submergence on seed germination, early growth and survival could contribute to improving crop establishment in flood-prone environments. This section will review plant adaptation, ion transport, osmotic regulation and energy expenditure in germinating rice during the energy crisis in anoxia.

Energy crisis and Pasteur Effect

Some plants can survive without O_2 for prolonged periods. Example of anoxia tolerance in plants are rice coleoptiles (Gibbs and Greenway, 2003) and aged storage tissue of beetroot (Zhang and Greenway, 1995). During an energy crisis, plant cells need to reduce and direct the limited amount of energy produced to the energy-consuming processes that are critical to survival (Greenway and Gibbs, 2003). One example of the energy saving is an efficient use of pyrophosphate (PPi) produced in amino acid activation during protein synthesis, rather than its hydrolysis by pyrophosphatases (Greenway and Gibbs, 2003), as reported in shoots and roots of rice, pyruvate orthophosphate dikinase (PPDK) activity increased 1.4-2 fold during anaerobiosis (Huang et al., 2005).

In some plants exposed to anoxia, the large reduction of energy produced by fermentation compared with respiration is alleviated by accelerating glycolysis, i.e. known as ‘Pasteur Effect’. This Pasteur Effect is normally expressed as the ratio of carbon flow in glycolysis linked to ethanol production in anoxia, to carbon flow of glycolysis linked to TCA cycle in aeration. This Pasteur Effect occurs in the coleoptile of rice (Menegus et al., 1991; Greenway and Gibbs, 2003; Huang et al., 2005). The ratio for Pasteur Effect was 1.6 in excised rice shoots (Menegus et al., 1991) and 1.4-1.7 (Gibbs et al., 2000) and 1.25 (Huang et al., 2005) in excised coleoptiles of 3 d old rice seedlings. Despite the high rates of glycolysis, the ATP produced under anoxia is

much less than the rate under aerated condition. The high rates of ethanol or ATP production in anoxic rice coleoptile at 50 mol m^{-3} exogenous glucose were still 7-10 fold lower than the assessed ATP production in aerated coleoptile tips (Huang et al., 2005).

Morphological and metabolic adaptations

The tolerance of rice seedlings to submergence is associated with morphological and metabolic adaptations (Greenway and Setter, 1996; Ram et al., 2002). During complete submergence in short floods, survival of rice is negatively correlated with underwater elongation of the shoot (Jackson et al., 1985; Setter and Laureles, 1996). During submergence, rapid growth or elongation of leaves competes with maintenance respiration for carbon source, leaving less resource available for survival (Greenway and Setter, 1996). The elongating organs need to allocate their limited energy production to growth, i.e. energy for solute uptake to generate osmotic pressure, turgor pressure for cell expansion, as well as energy for protein, lipid and cell wall synthesis (Greenway and Gibbs, 2003). It was shown in rice coleoptiles that 3 mm growing basal segments had 3-fold faster ethanol production (~21 μmol g^{-1} FW h^{-1}) than in 3-mm non-growing tips and this difference was also evident on a protein basis (Setter and Ella, 1994).

In low O_2 supply, cell metabolic activity has been restricted and some biosynthesis activities are inhibited to save ATP and to allow O_2 consumption to be decreased (Geigenberger, 2003; Greenway and Gibbs, 2003). Metabolic acclimation to anoxia with respect to energy production is promoted via activation of fermentation. The metabolic responses to O_2 deprivation have been assessed elsewhere (Drew, 1997; Greenway and Gibbs, 2003; Vartapetian and Jackson, 1997 (for plants in general); Perata, 1992; 1997 (in cereal seeds); Menegus et al., 1991; Huang et al., 2005 (in rice)). Submergence tolerance is associated with high carbohydrate supply for cell maintenance (Ram et al., 2002). In rice, less tolerant cultivar had less substrate availability for embryo and coleoptile which was due to slow starch hydrolysis in endosperm (Huang et al., 2003). This continued supply of substrate and the two key enzymes, alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) determine the efficiency of anaerobic fermentation pathway, which is the main source of energy production under anoxia (Alpi and Beevers, 1983; Setter et al., 1994; Gibbs et al., 2000; Greenway and Gibbs, 2003).

During energy crisis, the decrease of energy requirement for maintenance included reduction of protein turnover and ion fluxes (Greenway and Gibbs, 2003). Since protein synthesis has a high energy cost (Mocquot et al., 1981), so unless a particular protein is of acclimative value during anoxia, the protein synthesis is unlikely to continue (Greenway and Gibbs, 2003). During anoxia, a number of anaerobic stress polypeptides (ASPs) are induced and these may differ among species or between tolerant and sensitive cultivars (Ram et al., 2002; Huang et al., 2005). Most of those ASPs are identified as enzymes involved in sugar breakdown, glycolysis and fermentation (Huang et al., 2005). The identified protein synthesis in anoxic rice coleoptile includes PPDK which has possible role in regulation of glycolysis and production of PPi for function of tonoplast H^+-PPiase and sucrose breakdown (Huang et al., 2005). The protein synthesis in anoxic rice coleoptile shows a higher rate and more complex pattern of newly synthesized protein than in anoxic maize root tips (Mocquot et al., 1981; Ricard et al., 1991). Protein synthesis in anoxic rice coleoptiles consumed the largest proportion of ATP synthesized under aeration, hypoxia and anoxia with the proportion of ATP allocated to protein synthesis in anoxia (52%) more than two-fold of that in aerated coleoptiles (19%) (Edwards et al., 2012).

Solute transport in anoxia

During O_2 deprivation, cell survival has a high priority for the scarce energy production (Greenway and Gibbs, 2003). Energy flow during anoxia must be directed towards essential solute transport. Energy savings are also indicated by significant decrease in K^+ efflux (Colmer et al., 2001).

Anoxic rice coleoptiles took up Cl^- at 10-15% of the rates during aeration at 0.25 mM NaCl (Zhang and Greenway, 1995), and Cl^- radial transport to the xylem of maize roots also decrease in low O_2 supply (Gibbs et al., 1998). K^+, P and NH_4^+ net uptake were also inhibited during anoxia (Kronzucker et al., 1998; Colmer et al., 2001; Huang et al., 2005). K^+ translocation from endosperm to the coleoptile was less inhibited than K^+ uptake by the coleoptile from the external medium (Huang et al., 2003). These low ion uptakes during anoxia might be associated with some possibilities. (i) Cells direct their restricted amounts of energy to transport of more important solutes/ ions, such as in pH regulation and sugars for anaerobic catabolism (Greenway and Gibbs, 2003). (ii) Closure of membrane channels as reported in animal cells (Hochachka, 1986) during low anaerobic catabolism to save energy might also cause a reduction in ion effluxes.

In rice coleoptiles, a 16 to 18-fold lower permeability for K^+ efflux in anoxia than in aeration is suggested to be associated with the closure of K^+ out channels (Colmer et al., 2001). The very low Cl^- and K^+ uptake under anoxia might be associated with down-regulation of the transport system (Greenway and Gibbs, 2003; S. Shabala, personal communication), and possibly related with the generation of ROS in hypoxic tissue. In human cells, ROS is required in hypoxia-induced NSCCs down-regulation by decreasing the number of active Na^+ transporters at the plasma membrane (Gusarova et al., 2011).

In anoxia, inhibition of glucose uptake ranged between 35-95% (Greenway and Gibbs, 2003). Glucose uptake by anoxic tissue might not require energy at high (~ 50 mM) external glucose concentrations since entry is possibly maintained by a concentration gradient across the plasma membrane. While in intermediate (~ 20 mM) exogenous glucose concentrations, a combination between uptake via free energy gradient and transport involving an H^+-sugar symport may occur (Greenway and Gibbs, 2003). Sugar transportation from endosperm to the coleoptile in rice seedlings is adequate to maintain anaerobic fermentation (Atwell and Greenway, 1987; Greenway and Gibbs, 2003). However, with excised coleoptiles, at least 50 mM exogenous sugar is required to enable maximum ethanol fermentation (Huang et al., 2005).

Osmotic pressure

Cell expansion of rice coleoptile under anoxia depends on the ability to develop turgor pressure and maintain cell wall extensibility. There is no appreciable difference in turgor pressure in coleoptile tissues under aerated, hypoxic, stagnant and anoxic conditions, which had different rates of extension and so indicating the role of cell wall properties in elongation rates (Atwell et al., 1982). The contribution of K^+ to osmotic pressure was larger than that of sugar, but together K^+ and sugars gave to about 50-100% of the osmotic pressure (Atwell et al., 1982).

As in aerated solutions, Pi in anoxia contributes ~ 10% to the total osmotic pressure in the rice coleoptile (Menegus et al., 1984). Further free amino acids increased by more than 10-fold to contribute ~ 26% to total osmotic pressure after 2 d in anoxia. Among those amino acids, alanine predominates by contributing more than 50% of the osmotic pressure generated by total amino acids (Menegus et al., 1984).

Ethanol production and energy requirement for cell maintenance

In the absence of O_2, there is a shift in energy production and NAD recycling from TCA cycle and respiration chain to fermentative pathways. This anaerobic fermentation, however, has reduced efficiency of ATP production expressed as mol ATP per mol hexose unit, i.e. only 2 mol ATP is produced via glycolysis linked to fermentation, compared with 24-36 mol ATP via respiration in aerated conditions (Huang et al., 2005; Lambers et al., 2008). Ethanol is the major fermentation end product in rice seedlings (Bertani et al., 1980; Menegus et al., 1991). The importance of alcoholic fermentation in rice growth and survival in the absence of O_2 has been reviewed in many studies (Alpi and Beevers, 1983; Setter et al., 1994; Gibbs et al., 2000; Greenway and Gibbs, 2003; Huang et al., 2005).

The estimate of energy requirement for maintenance based on ethanol production, i.e. estimated from the rates of ethanol production by coleoptile tips at the lowest exogenous glucose (2.5 mM) that can be used for survival, without growth, was 3.3 μmol ATP g^{-1} FW h^{-1} (Huang et al., 2005). The assessed energy requirement for maintenance of some plant tissues and cells during anoxia are 3.8-5.0 μmol ATP g^{-1} FW h^{-1} in whole rice coleoptiles (Colmer et al., 2001), 12.0 μmol ATP g^{-1} FW h^{-1} in cultured rice cells (Mohanty et al., 1993) and 14.5 μmol ATP g^{-1} FW h^{-1} in maize root tips (Xia and Saglio, 1995). The energy requirement for maintenance in aerated leaves was assessed by Greenway and Gibbs (2003) from data in de Visser et al. (1992), and showed that in anoxia, the assessed energy requirements were 58–88% of those required for the maintenance of leaves in air (Huang et al., 2005).

Tolerance to salinity

Ion uptake

Initial entry of Na^+ from the soil solution into the root is passive (Cheeseman, 1982) driven by free energy gradient consisting of the sum of concentration and electrical gradients. Net accumulation of Na^+ in plants is the balance between passive influx and active efflux (Tester and Davenport, 2003). Unidirectional Na^+ influx that has been recorded in some plant species, i.e. rice, wheat and arabidopsis, is in the order of 0.5-2.0 μmol g^{-1} FW min^{-1} with 50 mM external Na^+ (Tester and Davenport, 2003). The high rates of unidirectional influx of Na^+ which do not result in rapid accumulation of Na^+ imply a substantial efflux of Na^+ across the plasma membrane (Tester and

Davenport, 2003). Three pathways are suggested for Na^+ influx: Ca^{2+} sensitive pathway (e.g. HKT1 transporter from wheat), Ca^{2+} insensitive pathway, and bypass flow, i.e. Na^+ entry into the plant by 'leaking' into the root via apoplast (Yeo and Flowers, 1985; Tester and Davenport, 2003). It has been reported elsewhere that the most likely pathways for the Ca^{2+}-sensitive Na^+ influx are non-selective cation channels (Demidchik and Tester, 2002; Tester and Davenport, 2003).

Turgor pressure

Plant growth is ultimately the results of rapid expansion of the young cells produced by meristematic divisions. The cell expansion process depends on an inwardly water potential gradient which generates turgor pressure and cell water uptakes, the hydraulic conductivity of the water uptake pathway, uptake of solutes to maintain osmotic potential and the yielding of the surrounding cell walls (Cosgrove, 1997). Salinity could inhibit growth by affecting any of those activities (Neumann, 1997). Plant cell growth is turgor-dependent because the rate of wall yielding is a function of wall stress (generated by turgor) (Cosgrove, 1997). Many different studies showed that turgor pressure varied with the osmotic pressure of the growth medium. Examples of such studies include: with *Chlorella emersonii*, turgor pressure was in a range of 0.6 MPa for cells in 1.0 mM NaCl, and 0.3 MPa for cells in 335 mM NaCl (Munns et al., 1983); 0.2 - 0.7 MPa for tobacco callus grown at 0 and 125 mM NaCl (Gibbs et al., 1989); whereas no change in turgor pressure was detectable when exposed to 50 mM NaCl in the growing zone of rice leaves (Yeo et al., 1991) and at 100 mM NaCl in maize root tips (Neumann and Azeizeh, 1994; Neumann, 1997). Cell turgor of 0·4 MPa is likely for expanded cells in roots (Munns et al., 2002), compared to ~0.6 MPa in shoots. In transpiring plants, turgor pressure is commonly measured using pressure probe (Tomos, 2000) or by using micromanipulation, i.e. a compression testing for studying cell mechanical properties (Wang et al., 2006), whereas in non-transpiring plant, estimates of turgor pressure can be derived from the osmotic pressure of the cell sap as compared with the bathing medium, such as with culture of maize (Looney and Fry, 2005) and with *Chlorella emersonii* (Munns et al., 1983).

Ion toxicity (dilution, exclusion and compartmentalization)

In transpiring plants, high internal concentrations of Na^+ and Cl^- can inhibit the metabolism of dividing and expanding cells (Neumann, 1997). During the first hour of

salinity (100 mM NaCl) exposure, Na^+ is accumulated mainly in epidermal cells of the elongation zone. After 20-72 h, the accumulation of Na^+ was about 2-fold higher in the mature tissue (~105 mM) of the growing leaf than in growing tissues (~48 mM) (Fricke, 2004). As with roots, the growing leaf cells may be able to sense turgor and use this information to control solute transport (Fricke et al., 2004). However, no information is available on the relationships between Na^+ concentrations and growth in non-transpiring plants.

High Na^+ is translocated from root to shoot via transpiration stream leading to a high accumulation in shoots. Therefore Na^+ 'exclusion' from the transpiring leaf blade is an important mechanism for plant survival under salinity (Munns and Tester, 2008). Within a crop species, it is a common case that varieties that are more tolerant to salinity would have less amounts of Na^+ and/or Cl^- accumulating in the shoots and the sensitivity of rice shoots to salinity could be associated with the inability of shoots to exclude Na^+ and Cl^- and also a poor capacity to accommodate these ions in leaf cells (Tester and Davenport, 2003; Munns and Tester, 2008). Different from shoots which accumulate high Na^+ concentrations in leaves, less variability of Na^+ concentrations in roots than in shoots implies that roots control their internal Na^+ levels (Matsushita and Matoh, 1991; Tester and Davenport, 2003). The evidence indicates Na^+ concentrations in roots is not linearly related to external NaCl concentrations and shows saturation in some conditions (Tester and Davenport, 2003) suggesting a mechanism in roots that regulates root Na^+ concentrations and uptake by controlling their Na^+ transporters appropriately according to the external concentration (Munns et al., 2002). This makes sense, since unlike shoots, roots could regulate their Na^+ concentration by controlling the rates of efflux out of the root to external solution, and into the xylem for translocation to shoots (Tester and Davenport, 2003). In liquid culture of non-transpiring plants, the exposure of shoots to direct contact with nutrient solution may place the shoots in the same position to roots in transpiring plant (i.e. enable direct Na^+ exchange with the medium).

The other possible salt adaptation strategies in glycophytes are salt sequestration and prevention of oxidative damage. Rice plants overexpressing the Na^+/H^+ antiporter (Chen *et al*., 2007) and rice transgenics over-expressing SOD (Tanaka *et al.*, 1999) exhibited better salinity tolerance. However, the mechanisms of salt tolerance, i.e. extrusion, organic solute synthesis and compartmentalization of Na^+ are metabolically energetic and compete with plant growth for resources (Qiu et al., 2007; Munns and

Tester, 2008; Zhang et al., 2010). Hence, in germinating seeds under salinity, the conflicting demands for carbon reserves required for osmotic balance and growth will possibly determine the success of germination and post-germination growth (Zhang et al., 2010).

Different transporters involved in Na^+ and Cl^- fluxes and the importance of cell type-specific processes

Mechanisms for tolerating salinity in plants includes: tolerance to the osmotic effects of exposure to salt; tolerance to the negative effects of sodium ions (Na^+) on cellular function through compartmentalization of Na^+ in specific tissues, cells, or cellular organelles and 'exclusion' of Na^+ from the sensitive shoot tissue (Munns and Tester, 2008). Of these mechanisms, Na^+ 'exclusion' from the shoot is best understood (Plett et al., 2010) and understanding Na^+ transporters is necessary for a targeted genetic modification approach to improve salinity tolerance.

Different responses among different tissues within a plant to salinity have been reported elsewhere (Munns and Tester, 2008). The different responses may include the inhibition level on organ growth rate (i.e. shoot and root, Munns and Tester, 2008) up to the different functions of transporters at cellular level (Shabala et al., 2006). Some studies reported different expressions of HKT family of proteins between species, or even within a plant under salinity. This Na^+/K^+ symporter increased root Na^+ accumulation in wheat, TaHKT2;1 (Rubio et al., 1995); in rice, OsHKT2;1 (Garciadeblas et al., 2003; Horie, et al., 2007; Horie et al., 2012) and in arabidopsis, AtHKT1;1 (Rus et al., 2001). Apart from the function in Na^+ influx in roots, HKT1;1 is also suspected to be involved in Na^+ retrieval from the xylem (Davenport et al., 2007) and support Na^+ recirculation from shoots to roots (Essah et al., 2003). Increasing the expression of HKT genes has been unsuccessful as a strategy to improve salinity tolerance in plants since overexpression of this plasma membrane Na^+ transporter could increase Na^+ fluxes into the cells and hence be counter-productive to the reduction of Na^+ to the shoots (Plett et al., 2010). In rice, OsHKT2;1 gene which is responsible for a major portion of Na^+ uptake, in fact does not cause Na^+ toxicity, owing to a rapid down-regulation of the OsHKT2;1 transporter upon high Na^+ concentration to prevent Na^+ toxicity (Horie et al., 2007). Apart from the cell type-specific expression profiles, the function of at least nine HKT-like genes in rice still remain unknown (Munns and Tester, 2008).

Table 2.1. Some ion transporters involved with Na^+, K^+ and Cl^- transport, presumably associated with salt tolerance. Transporters likely to be adopted during rice germination in salinity and anoxia in respect to the energy available will be discussed in Chapter 5.

Monovalent ion transporters	
Channels	
NSCC (non-selective cation channel)	Schachtman and Schroeder, 1994; Kader and Lindberg, 2005; Wang et al., 2007
CLC (voltage gated Cl^- channel)	Nakamura, 2006.
Carriers	
Symporter	
HKT (high affinity K^+ transporter)	Garciadeblas et al., 2003; Golldack et al., 2003; Horie et al., 2007; Plett et al., 2010.
HAK (high affinity K^+ transporter)	Walia et al., 2007.
CCC (cation chloride co-transporter)	Colmenero-Flores et al., 2007.
NKCC (sodium potassium chloride cotransporter)	Teakle and Tyerman, 2010.
Antiporter	
NHX (Na^+/H^+ exchanger in tonoplast)	Fukuda et al., 2004; Yamaguchi et al., 2005.
	Chen et al., 1999.
CHX (cation/H^+ exchanger)	Senadheera et al., 2009.
SOS1 (salt overly sensitive, Na^+/H^+ antiport in plasma membrane)	Martinez-Atienza et al., 2007.

Other example of the cell-type specific transporters is indicated by the different NaCl induced K^+ efflux responses in arabidopsis shoot and root cells which could be associated with different activity of NSCCs (i.e. at least in their binding site and molecular origin) in both organs (Shabala et al., 2006). These cell-type specific transporters and processes add to the complexity of functions of some already known transporters, e.g. OsHKT family of proteins in rice, which are suspected to act differently in different tissues.

Energy requirement and possible increased demands due to exposure to NaCl

Cell growth, both division and expansion require ion uptake and solute synthesis to generate osmotic potential, nitrogen and carbohydrate supply for protein and cell walls synthesis, as well as ATP synthesis (Munns and Termaat, 1986). It has been estimated that in the presence of 500 mM NaCl, ATP production would have to be increased by 50-70% of the production in non-saline solution (Greenway and Munns, 1983). For halophyte plants *Salicornia fructose* and *Suaeda maritima* growing rapidly at high salinity, ATP production for ion regulation would have been about 10-20% of their total ATP formation (Yeo, 1983). In halophyte plants, e.g. *Aegiceras corniculatum*

and *A. marina*, leaf respiration was raised ~ 2-fold when exposed to 100% sea water (compared to 0-20% sea water), with a higher increase in the more salt-sensitive species, which indicated a high metabolic cost in the shoots of those species at high salinity (Burchett et al., 1989). For non-halophyte, it was reported that respiration of 13 d pea seedling increased by 10-38% when grown in 77 mM NaCl than in non-saline condition (Livne and Levin, 1967). Apart from species differences, growing cells will also require many times more ions for generating osmotic potential than non-growing cells (Greenway and Munns, 1983; Setter and Ella, 1994). However, in air, ATP production could not be fully estimated from respiration rate, because some proportions of respiration in most species are through non-cytochrome electron transfer pathway and yielding less ATP production (Lambers et al., 2008).

Net accumulation of Na^+ into plant cells is due to a balance between influx through ion channels and efflux through a possible Na^+/H^+ antiporter (Blumwald et al., 2000; Tester and Davenport, 2003). The stoichiometry of this exchange will determine the metabolic cost of Na^+ extrusion. A fixed stoichiometry of 1:1 exchange of Na^+ for H^+ would make the cost at 1 ATP hydrolysed per Na^+ extruded (provided a 1:1 ratio of ATP hydrolysed to H^+ extrusion by plasma membrane ATPase; Briskin et al., 1991). However the energy required to move one Na^+ from the cytosol to the external solution against the electrochemical potential gradient will vary depending on external salinity, influx rate and intercellular transport (Tester and Davenport, 2003). The expensive mechanism of Na^+ extrusion has been suggested in some studies, i.e. the increase in respiration far exceeds the theoretical cost of Na^+ extrusion in rice (Kronzucker et al., 2001; Malagoli et al., 2008).

Ca^{2+} can alleviate growth inhibition by high salinity

It has been reported in many studies that Ca^{2+} has a beneficial effect in alleviating growth inhibition by high salinity, such as in maize (Azaizeh, et al., 1992); in pepper plant (Ballesta et al., 2008); in sorghum (Colmer et al., 1996); in cotton (Kent and Lauchli, 1985; Kurth, et al., 1986); in barley (Epstein, 1961) and in rice (Yeo and Flowers, 1985; Lin and Kao, 1995; Alam et al., 2003). Ca^{2+} is an essential divalent cation and plant nutrient, which plays structural roles in the cell wall and membranes. Because of its role in maintaining membrane integrity, it contributes to the ability of different plants to resist salt stress (Ballesta et al., 2008). Ion imbalances in plants may occur when Na^+ displaces membrane-bound Ca^{2+}. This leads to an increase of

membrane permeability and intracellular Na^+ concentrations (Kurth et al., 1986; LaHaye and Epstein, 1969). Na^+ may compete with Ca^{2+} for membrane-binding sites. Studies of cotton (Cramer et al., 1986) and maize root growth (Lynch, et al., 1987) showed that Ca^{2+} activity at the plasma membrane was related to the Ca^{2+}/Na^+ ratio in the external solution.

Ca^{2+} was shown to enhance K^+ uptake, but decrease Na^+ uptake, leading to increased K^+/Na^+ ratio in plants (Cramer, 1987). The decrease in accumulation of Na^+ is associated with the inhibition of the unidirectional influx of Na^+ into the roots by Ca^{2+}, which could also reduce the Na^+ stimulated K^+ efflux (Cramer et al., 1985; Shabala et al., 2006). As with the maintenance of low Na^+ in shoots, the maintenance of high K^+ is also important. The high K^+/Na^+ ratio is more important for many species than simply maintaining a low Na^+ concentration, since K^+/Na^+ ratios in the cell are necessary for normal cellular function under saline conditions (Greenway and Munns, 1980; Cuin et al., 2003; Tester and Davenport, 2003).

Recent studies have started to evaluate the signaling mechanisms underlying the interaction of these three cations. Either low-K^+ or high-Na^+ can trigger cellular Ca^{2+} changes that lead to activation of complex signaling networks, and change in expression and activity of Na^+ and K^+ transporters. The studies revealed that plants cope with limited soil K^+ by coupling K^+ channel activation to a cellular Ca^{2+} signaling network (Hedrich and Kudla, 2006). One such network consists of Ca^{2+} sensor proteins (e.g., CBLs) interacting with their target kinases (CIPKs) to regulate the activities of a number of transporting proteins involved in the uptake and translocation of K^+ and Na^+, maintaining the ‘balance’ of these cations in plants under unfavorable conditions (Luan et al., 2009).

Rice germination in NaCl exposure

Amongst other cereals, rice is classified as sensitive to salinity (Maas and Hoffman, 1977). Transpiring rice seedlings will not survive when grown at 100 mM NaCl (Munns and Tester, 2008). Wheat is a moderately salt-tolerant crop (Maas and Hoffman, 1977), and even barley, the most-tolerant cereal, dies after prolonged periods at higher than 250 mM NaCl (Munns and Tester, 2008). However, during germination stage, crops are likely to be more tolerant to salinity, i.e. barley is able to germinate at about sea level salt concentrations (Zhang et al., 2010). There is lack of information on the limit of NaCl concentration that can be tolerated by germinating rice. Past research

which studied salinity on rice applied various concentrations of NaCl to the nutrient solution (Table 2.2).

Different range of NaCl applied in previous studies of rice seedling tolerance to salinity resulted in broad responses of rice germination and early seedling growth, which could be due to: different varieties, seedling ages and methods (Munns and Tester, 2008; Tavakkoli et al., 2012). These various responses of rice germination and growth to different levels of NaCl concentrations will be overviewed in the Introduction of Chapter 3. Except for callus and germination of seeds, all the studies in Table 2.2 were conducted in transpiring rice seedlings. No information is available on rice seedling tolerance in non-transpiring system. The salinity tolerance mechanism in transpiring plant is likely to be different from non-transpiring plants (Munns and Tester, 2008). When rice will be submerged and exposed to anoxic solution, understanding mechanism of salt tolerance in aerated non-transpiring rice seedling would be necessary to study further responses of rice seedling under combined NaCl and anoxia.

Table 2.2. Different concentrations of NaCl used in previous studies on rice germination and early plant growth

Plant age	NaCl concentration	Authors
Germination	0 – 50 mM	Lutts et al., 1995
	226 mM[1)]	Heenan et al., 1988; Khan et al., 1997
	120 mM[1)]	Punyawardena and Dharmasri, 1989; Gregorio et al., 1997
	20 mM[1)]	Hakim et al., 2010
	Soaking in 472 mM[1)]	Abeysiriwardena, 2004; Subasinghe et al., 2007
Leaf, embryo and anther callus	0 – 200 mM	Kishor, 1988; Ahmad et al., 2007
	0 -130 mM	Sathis et al., 1997; Shankdhar et al., 2000
4-25 d old	43 mM-150 mM[1)]	Rahman et al., 2001
	0 – 50 mM	Ferdose et al., 2009
	50 – 100 mM	Flowers and Yeo, 1981; Yeo and Flowers, 1982; 1983; 1986.
	2 – 80 mM [2)]	John et al., 1977;
	50 – 200 mM	Aslam et al., 1993; Bhowmik et al., 2009
	0 – 100 mM	Yeo, et al., 1991; Djanaguriman et al., 2006; Ammar et al., 2007; Senadheera et al., 2009

[1)] The values are deduced from dS m^{-1}

[2)] A combination of NaCl and waterlogged soil

Interaction between anoxia and salinity

Amongst information on the interaction between anoxia and salinity currently available, no studies were conducted in non-transpiring saline conditions. The term of anoxia in those studies refers to the submerged root condition, while shoots are in air (John et al., 1977; Kriedemann and Sands, 1984; Buwalda et al., 1988; Akhtar et al., 1998; Barrett-Lennard, 2003; Gorai et al., 2010). Since transpiring plants likely respond differently to salinity from non-transpiring plant as described previously, available information is of limited relevance to the current study focused on the submerged coleoptile of rice.

Tolerance of salinity is associated in part with an ability to 'exclude' Na^+ from the leaves (Drew et al., 1988), or tolerance to accumulated Na^+ by compartmentalization of Na^+ and Cl^- at the cellular and intracellular levels to avoid toxic concentrations within the cytoplasm (Yeo et al., 1999; Munns and Tester, 2008). In plants exposed to anoxia, ATP production is much lower than in air (Greenway and Gibbs, 2003). With the combined effect of anoxia and salinity, any energy deficit may be aggravated, since plants have to cope with cell maintenance, such as mitigating ion excess as well as osmotic stress, in addition to energy required for growth. The failure of the Na^+ 'exclusion' mechanism due to energy deficiency in anoxia may be a contributory factor in salinity damage of salt-sensitive glycophytes (Drew et al., 1988; Barrett-Lennard, 2003; Munns and Tester, 2008).

Conclusion

This review summarises the main mechanisms of plant tolerance to salinity as well as rice seedling tolerance to anoxia, and it highlights the gaps in our understanding of the rice salt tolerance mechanism during germination stage and when O_2 supply is not available (Fig. 2.1). The ability of plants to regulate ion uptake and extrusion as well as maintain the turgor and osmotic adjustment potentially plays a role in survival in high salinity. During anoxia, the ability of plants to restrict and prioritize cell metabolic activity with respect to energy production and consumption is likely essential to survival and growth.

Previous studies on salinity were conducted on transpiring plants. Therefore all the information on growth, ion regulation, osmotic relations as well as energy budget was discussed based on this transpiring condition. The current literature does not reflect

the prevalence of salinity in a wide array of conditions, such as in complete submergence when direct seeded germination of rice would have to survive in a very low O_2 supply, in a non-transpiring condition.

With few data available, it is difficult to investigate the potential importance of rice tolerance mechanisms to a combination of salinity and anoxia. This thesis expands the data available on rice salt tolerance mechanisms during germination stage in an anoxic non-transpiring condition. The outcome expected from this study could be different from the existing information as discussed in the previous section of this literature review. When fully submerged in anoxic-saline solution, it is hypothesized that rice seedlings would have to rely on the very low ATP production via anaerobic fermentation to regulate ion uptake, solute transports and metabolic activities. Under submergence, ion uptake into shoots is not driven by transpiration, but the energy crisis due to low O_2 might also impede ion uptake and extrusion. On the other hand, with shoot surfaces in direct contact with external solutions, ions in shoots and roots will be easily exchanged with those in external solutions and together with the non-transpiring condition could probably reduce Na^+ and Cl^- accumulation in shoots, as compared with seedlings with shoots in air. During energy starvation, only important organic solutes will be synthesized or transported. Finally, the inevitable elevation of energy requirement for cell maintenance when challenged by salinity would probably be met by increasing cell catabolism, even in anoxia (i.e. elevated ethanol fermentation).

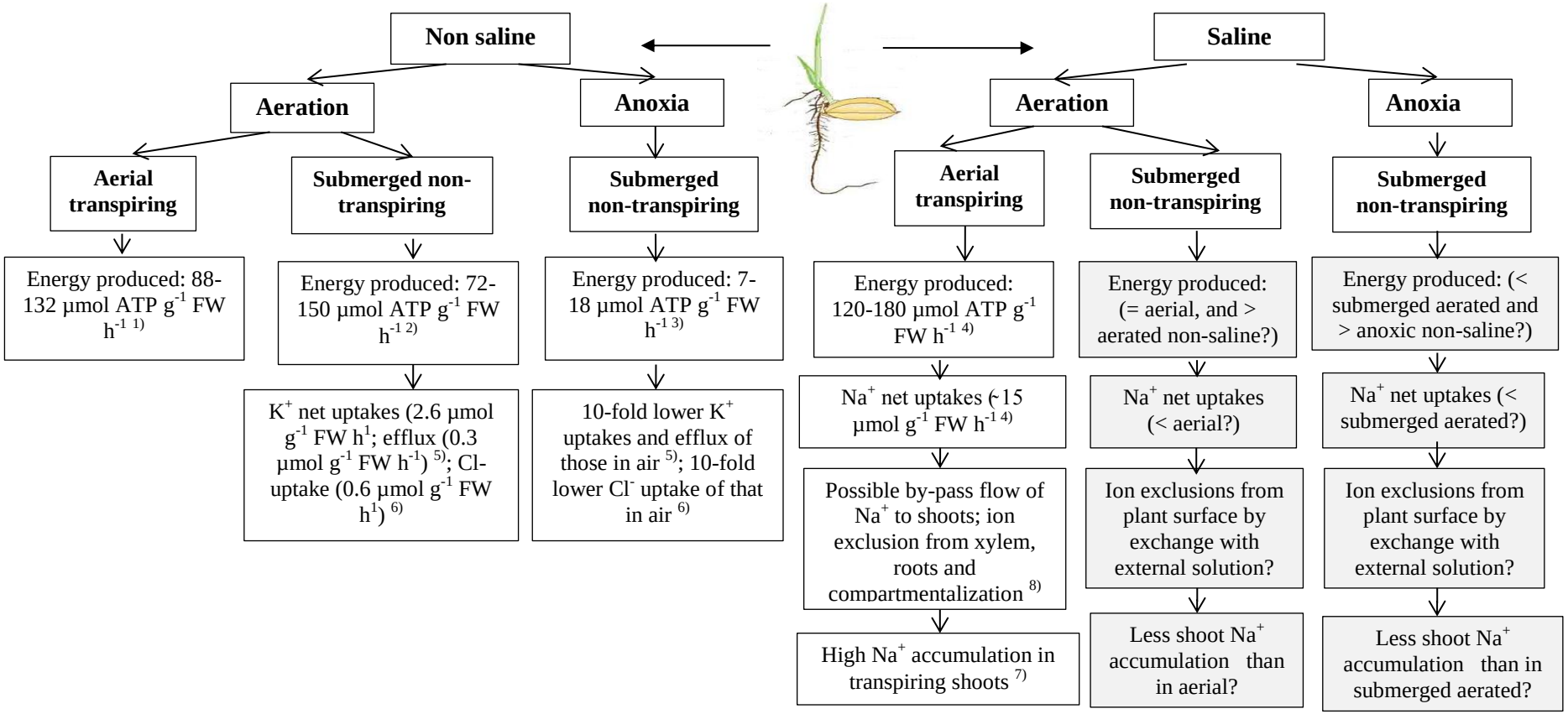

Figure 2.1. Conceptual model of factors contributing to rice seedling tolerance to NaCl exposure in an aerial transpiring and in submerged non-transpiring systems, both aerated and anoxic submerged. Shaded components indicate factors which will be investigated in this thesis.

1) Assessed in rice roots at 1 mM NaCl (Malagoli et al., 2008).
2) Assessed in aerated excised coleoptile tips: 72-108 (Huang et al., 2005); whole excised coleoptiles 150 (Edward et al., 2012).
3) Assessed in anoxic excised coleoptile tips: 7-10 (Huang et al., 2005); whole excised coleoptiles18 (Edward et al., 2012).
4) Assessed in rice roots at 25 mM NaCl (Malagoli et al., 2008).
5) Assessed in whole excised coleoptiles (Colmer et al., 2001).
6) Assessed in anoxic rice coleoptiles (Zhang and Greenway, 1995).
7) (Munns, 2002).
8) (Munns and Tester, 2008)

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CHAPTER 3

Rice Germination and Seedling Establishment under Aeration at 50-200 mM NaCl and Growth Improvement by Supplemental Ca^{2+} *)

*) This chapter has, since my thesis submission, been further revised and published:

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The version in this thesis is the original chapter submitted for examination, with revisions made as suggested by the examiners. The chapter was more extensively re-ordered and revised for the manuscript submitted to, and now published in, *Physiologia Plantarum*.

Abstract

Tolerance to NaCl of rice germination and seedling growth, changes in tissue ions and the contribution to osmotic adjustment were studied in a NaCl dose response experiment and when different exogenous Ca^{2+} concentrations were provided in separate experiments. Seedlings were submerged in aerated nutrient solution.

Rice was able to germinate and the seedlings tolerated and recovered from the adverse effects of high NaCl without substantial injuries. This was despite the large inhibition of by the highest concentration used of 200 mM NaCl. Osmotic adjustment was achieved by using Na^+ and Cl^- as the major osmotica. The internal Na^+ and Cl^- concentrations reached levels similar to those in the external medium.

During early seedling growth, Na^+ and Cl^- concentrations were higher in the coleoptiles than in leaves. By contrast, K^+ concentrations were less in coleoptile than in leaves and this was presumably due to coleoptile senescence. Slow decreases of Na^+ and Cl^- concentrations in shoots and roots in the later sampling time were possibly due to very low ion net uptakes and dilution by growth.

In roots the π_{sap} was 15-30% less negative than the π_{sap} in shoots. The contributions from ions to π_{sap} were 81-92% in roots and 62-74% in shoots which indicated that more organic solutes were required to maintain the low π_{sap} in shoots in saline condition. With less organic solutes available and decrease of Na^+ and Cl^- concentrations with time, osmotic pressure in roots was probably maintained by restricted growth (i.e. less ion diution).

Supplemental Ca^{2+} at 5 and 10 mM alleviated ~24% of the growth inhibition in roots at 200 mM NaCl, by improving root fresh weight, dry weight, length, volume and surface area. Supplemental Ca^{2+} had no effects in shoots. The different responses of shoots and roots to the different addition of Ca^{2+} might be due to the increase of K^+/Na^+ ratio in roots but not in shoots.

Thus, it is suggested that in non-transpiring conditions, the ability of rice seedlings to tolerate at least 200 mM NaCl was associated with an efficient use of Na^+ and Cl^- as osmoticum, at approximately similar concentrations with those in external medium. Growth inhibition which was larger in roots than in shoots might be associated with the lower turgor pressure in roots during prolonged salinity. Adding Ca^{2+} in this high NaCl concentration improved root growth by maintaining a more favourable K^+/Na^+ ratio.

Introduction

Rice tolerance to salinity can greatly differ between germination and older stages of seedling. Previous studies have reported variation in rice seedling growth under a range of salt concentrations. The different responses (Yeo and Flowers, 1985; Flowers et al., 1991; Lutts et al., 1995; Khan et al., 1997; Punyawardena and Dharmasri, 1989; Abeysiriwardena, 2004; Cha-um et al., 2009) might be attributed to various varieties, seedling ages as well as NaCl-treatment methods, i.e. using petri dishes, soils, hydroponics with and without supplemental Ca^{2+} (Chapter 2 Literature Review). Many studies showed that rice, a species which has been considered to be salt-sensitive, suffered decreased rates of germination, reduced water uptake, inhibited embryo axis growth and increased Na^+ and Cl^- accumulation in tissues, as the level of NaCl-salinity was increased (Lutts et al., 1995; Khan et al., 1997; Alam et al., 2003).

The levels of salinity applied in germination tests varied largely among those earlier research papers. The germination percentage in many cultivars including Pokkali, a reputably tolerant genotype was not affected at 50 to 100 mM NaCl, but decreased by about 50% at 200 mM NaCl (Khan et al., 1997). In contrast, Lutts et al. (1995) showed that 50 mM NaCl already inhibited the germination rate by more than 50% of the control in all varieties tested, including Pokkali. This result was similar to that of Punyawardena and Dharmasri (1989) who stated that rice germination under salinity occurred below 8 dS m^{-1} (~ 77 mM). Similarly, IRRI's salinity screening technique for rice in which germination is in salinized nutrient solution by adding NaCl to the desired EC of 6 and 12 dS m^{-1} (~ 56-120 mM) (Gregorio et al., 1997). In experiment by Heenan et al. (1988), NaCl concentrations up to 16.3 dS m^{-1} (~165 mM) only delayed the germination of all rice varieties tested up to 9 days after sowing, but increasing the NaCl concentration to 22 dS m^{-1} (~226 mM) inhibited the germination of all the varieties tested (Heenan et al., 1988; Hakim et al., 2010). Apart from the variation of results on rice tolerance to salinity during germination and early seedling growth, the mechanisms of salt tolerance in seeds is relatively poorly understood, especially when compared with the information on responses to NaCl in older stages of rice seedlings (Ren et al., 2005).

In many crop species, supplemental Ca^{2+} alleviates the inhibition of growth in saline conditions. Ion imbalances in plants can, for example, occur when high concentrations of Na^+ in the soil reduce the amounts of available K^+, Mg^{2+}, and Ca^{2+} or when Na^+ displaces membrane-bound Ca^{2+} (Kurth et al., 1986). Na^+ may compete with

Ca^{2+} for membrane-binding sites and so it has been hypothesized that high exogenous Ca^{2+} levels can protect cell plasma membranes from the adverse effects of salinity (Cramer et al., 1986). Numerous studies have shown that the growth of crops is seriously inhibited by high ratios of Na^{+}/Ca^{2+} characteristic of sodic conditions (LaHaye and Epstein, 1971; Kent and Lauchli, 1985). In maize, the increase of Na^{+} concentrations imposed by NaCl caused a displacement of Ca^{2+} from root cell membranes (Lynch et al., 1987). Increasing the external concentrations of Ca^{2+} counteracted this displacement, which may account for the protective effects of supplemental exogenous Ca^{2+} in plants exposed to salinity (Azaizeh et al., 1992).

LaHaye and Epstein (1969) suggested that Na^{+} acted by displacing Ca^{2+} from membranes, leading to increased membrane permeability and intracellular Na^{+} concentrations. Early physiological studies by Epstein and colleagues (Epstein, 1961) also reported that K^{+} and Na^{+} compete for entry through similar carriers/channels, leading to the hypothesis that one toxicity factor of Na^{+} is inhibition of K^{+} transport causing K^{+} deficiency. Here, Ca^{2+} has a role in the balance of this K^{+}- Na^{+} interaction. Ca^{2+} was shown to enhance K^{+} uptake, but decrease Na^{+} uptake, leading to increased K^{+}/ Na^{+} ratio in plants (Cramer et al., 1987). Ca^{2+} supplement was also mitigated the NaCl-induced growth inhibition of *S. bicolor* by maintaining net K^{+} to Na^{+} selectivity and increasing proline accumulation in the root tip (Colmer et al., 1996).

The enhancement of cotton seedlings root elongation in a saline solution provided with supplemental Ca^{2+} resulted from two distinct processes; improved cell elongation, and maintenance of high rates of cell production (Kurth et al., 1986). Supplemental Ca^{2+} enhanced the rate of cell production by approximately 20 to 30% at salinities ranging from 25 to at least 100 mM NaCl, while NaCl diminished the rate of cell production considerably at 0.4 mM Ca^{2+} level (Kurth et al., 1986).

The ameliorating effect of Ca^{2+} on growth in saline conditions was not, however, observed in rice (Yeo and Flowers, 1985). No effects on growth or ion concentrations in the shoot were found over a range of Na^{+}/Ca^{2+} ratio (5 to 25), and even in extreme Na^{+}/ Ca^{2+} ratios (100 to 500) there were only marginal increases in Na^{+} and Cl^{-} entry to the shoot and did not influence growth (e.g. 0.5 mM Ca^{2+} with 50 mM NaCl). In contrast, Lin and Kao (1995) demonstrated that Ca^{2+} at 10 mM was able to counteract the NaCl-induced inhibition of root growth (measured as length of main root axis) in 3-days old rice seedlings. Since rice seedlings develop many lateral roots and of different thickness, it may be better to measure root fresh weight or dry weight, or total root

length which considers the length of all the roots of the seedling. The contradictory results in these two studies of the response of rice to supplemental Ca^{2+} indicated that shoots may respond differently from roots to supplemental Ca^{2+}. In addition, Alam, et al. (2003) reported that adding Ca^{2+} in combination with NaCl increased the fresh weight in rice seedlings. Supplemental Ca^{2+} (3 to 9 mM) significantly increased final germination percentage compared to seeds without Ca^{2+} in the saline medium (NaCl from 0 to 250 mM). 3 mM Ca^{2+} offset the deleterious effects of 150 mM NaCl in seed final germination, whereas 6 mM Ca^{2+} offset those of 250 mM NaCl. In this experiment, it would have been nice to have that supplemented by dry weight, since the different rate of starch breakdown at early stages might cause different seedling dry weight, which might leave different water content in similar fresh weight of seedlings.

The study reported here had three main objectives: first, to conduct a NaCl dose-response experiment on rice to then select appropriate NaCl treatments to study effects on growth reduction and investigate ion uptakes during the phases of germination up to 138 h after sowing, with the broader aim to determine rice seedling tolerance to salinity during those stages. Second, the role of Ca^{2+} in germination and seedling growth of rice at a low and high concentration of NaCl was evaluated. Since two processes of great importance in the establishment of seedlings in a saline environment: cell elongation and maintenance of a balanced nutrient ion uptake both require Ca^{2+} (Kent and Lauchli, 1985), therefore the influence of Ca^{2+} on NaCl responses of rice was evaluated. Third, this study was done with the principal purpose to establish a reasonable picture of the salinity response of rice seedling, in order to explore the research basic aim, i.e. the interaction between salinity and anoxia (Chapters 4 and 5).

Materials and Methods

Preparation of rice seedlings

Dehulled seeds or rice (*Oryza sativa* L. cv. Amaroo) were surface sterilized with a dilute sodium hypochlorite 0.1% for 20 min and then washed thoroughly with deionized water. Batches of seeds were transferred to 100 ml conical flasks. The composition of the solution is given below; the pH was adjusted to 6.5 using $Ca(OH)_2$. The conical flasks as well as solutions were autoclaved (before adding MES). The seedlings were grown in the dark at 30^{o}C in a controlled-temperature room. This preparation was done for all experiments.

Aerated-NaCl treatments

The seeds germinated and grew in aerated solution (0.25 mM O_2) with a different range of NaCl concentrations. The nutrient composition of the solution was macro nutrients (mM): Ca^{2+} 1, NH_4^+ 0.2, NO_3^- 0.2, SO_4^{2-} 0.95, K^+ 0.3, Mg^{2+} 0.4, H_2PO_4 0.1, and micro nutrients (μM): Fe-EDTA 12.5, H_3BO_3 6.25, Mn^{2+} 0.5, Zn^{2+} 0.5, Cu^{2+} 0.125, Mo^{2+} 0.125, Ni^{2+} 0.25. A syringe was used to withdraw, or add, solution through the outlet of each conical flask and the nutrient solution were refreshed daily.

The rinse procedure used 1 mM $CaSO_4$ and iso-osmotic mannitol (in experiment 1 and 2 and REL measurement in experiment 3) and was carried out in an approximately 3 minutes, to remove surface water and ions in the free spaces. Tissues for sugar measurement (in experiment 2) were rinsed with the nutrient solution (NaCl still present) without sugar in 3 x 3 minutes. In experiment 3, $SrSO_4$ was used instead of $CaSO_4$ because Ca^{2+} in tissues was also measured, therefore rinsing with Ca^{2+} was avoided.

Recovery stage

The recovery treatment was a check whether the non-germinated seeds were still able to germinate and whether the injuries of seedlings during NaCl exposure (if any) were recoverable upon returned to non-saline condition. In this non-saline recovery, tissue samplings were taken at 1 h, 4 h, 24 h, 72 h after recovery (i.e. removal of NaCl) and at the same time the nutrient solutions were also sampled and refreshed to measure the K^+ net loss or uptake and tissue ions. The solutions were stored at -20^{o}C prior to measurement of K^+. The recovery treatment was ended at 72 h. This recovery test was conducted for experiments 1 and 3.

Experiment 1. The NaCl dose responses in aerated intact rice seedlingss

In this experiment, the seedlings were prepared as described above and treated with four levels of NaCl: 0.3; 50; 100 and 200 mM at the time of sowing, with 1 mM Ca^{2+} in the nutrient solution. Na^+ and Cl^- are typically present in all soils, and moreover Cl^- is an essential micronutrient essential for plants (Na^+ is only essential for C4 species, whereas rice is C3). Therefore, as it is not be reasonable to eliminate Na^+ and Cl^- in control treatments, a well-define low level of NaCl (0.3 mM) was used. The NaCl was added to the nutrient solution and the solution was continuously bubbled with air.

This range of NaCl concentration was based on preliminary results which showed that in 200 mM NaCl, the seeds were still able to germinate, but with severe inhibition (about 50% of the seedlings only grew roots and shoots up to <10 mm length). Therefore, 200 mM NaCl was chosen as the highest concentration to be applied.

There were three replicate flasks for each treatment. Each conical flask had 24 seedlings, and at each sampling, four seedlings (approximately 140 mg) were taken for tissue ion concentration and growth rate measurement. The samplings were taken at: 36, 66 and 138 h of salinity. During non-saline recovery period, 2 seedlings were taken at each sampling for ion measurements. The rest of the seedlings were used for growth measurement at the end of the recovery period.

Experiment 2. Dynamics in tissue ion concentrations and contributions to cell π_{sap}

As with experiment 1, the treatments in this experiment were: 0.3; 50; 100 and 200 mM NaCl with 1 mM Ca^{2+} in the nutrient solution. The difference from experiment 1 was the sampling time and duration of NaCl exposure. In this experiment, eight time samplings were conducted, i.e. 18, 42, 66, 90, 114, 138, 162 and 186 h after sowing (NaCl exposure). NaCl exposure was prolonged to 186 h to study the dynamics of Na^+, Cl^- and K^+ concentrations in tissues and in relation to tissue sugars and π_{sap}.

There were three replicate flasks for each treatment. Each conical flask had 24 seedlings, and at each sampling, two seedlings (approximately 70 mg) were taken for tissue ion concentration. The samplings for π_{sap} were conducted at: 114, 138, 162 and 186 h after sowing (NaCl exposure). Sugar analysis was carried out using tissues from samplings taken after 138-182 h after start of NaCl exposure (i.e. at 138 and 182 h after sowing).

Experiment 3. Rice seed germination and seedling growth in different Ca^{2+} *concentration under salinity*

The preparation of the rice material was conducted as described previously. The conical flasks were given nutrient composition as stated previously except Ca^{2+} was given at 1, 5 or 10 mM as $CaCl_2$. The treatments in this experiment included two concentrations of NaCl, i.e. 0.3 and 200 mM, so that there were 5 treatment combinations of ($NaCl+CaCl_2$): (200+1); (200+5); (200+10); (0.3+1) and (0.3+10). The 200 mM NaCl was chosen since previous experiment showed that despite ~70% growth

inhibition at 200 mM NaCl, the seedlings were able to regain growth during non-saline recovery. The range of Ca^{2+} concentrations was determined from previous studies on salinity in rice seedlings (Yeo and Flowers, 1985; Alam et al., 1993; Lin and Kao, 1995), and considered within a physiological range (Shabala et al., 2006).

NaCl exposure was given for 138 h, i.e. the same time as in experiment 1. Samplings were conducted three times at 114, 138 h (the end of salinity) and 72 h after return to non-saline solution (i.e. 210 h). There were three replicate flasks for each treatment. Each conical flask had 20 seedlings, and at each sampling, 6-7 seedlings (approximately 200 mg) were taken out for tissue ion concentration, growth measurement and π_{sap}. Samplings during non-saline recovery were conducted as in experiment 1.

Analytical procedures

Assessment included variables observed in the external medium and in rice tissues. In all experiments, medium pH was measured regularly each time the solutions were refreshed. Relative Electrolyte Leakage (REL) of electrolyte was measured using Electrical Conductivity meter (Cyberscan Con100 Auto-ranging 5-PT Cal, EUTECH Cybernetics, Singapore) at the end of NaCl exposure and at the end of non-saline recovery in experiment 3 by incubating rice tissues in test tubes in nutrient medium without NaCl at 30°C for 2 h to measure the initial conductivity (E1), then E2 was measured after the tubes were boiled for 30 min to release all the electrolytes. The REL was calculated as (E1/E2) × 100 (modified from Dionisio-Sese and Tobita, 1998). Coleoptile samples were rinsed as described previously, prior to incubation in the nutrient solution for electrolyte leakage measurements. Therefore, ion loss from the apoplast was unlikely to be a significant contribution in these measurements. Both medium π (π_{sol}) and tissue π (π_{sap}) were measured using a freezing point depression osmometer (Fiske associate, Norwood, MA, USA). Estimation of turgor pressure (P) was derived from the osmotic pressure of the cell sap (π_{sap}) (Munns et al., 1983).

Na^+, Cl^-, and K^+ concentrations in shoots, endosperms and roots were measured by extracting the tissues in 0.5 M HNO_3, shaked for 48 h at 30^{o}C and then making appropriate dilutions for analyses using Buchler-Cotlove Chloridometer (Buchler Instruments, Model 4-2008, Fort Lee, New Jersey, USA) for Cl^-, and using Flame Photometer (Corning Medical and Scientific, Model 410, Cambridge, UK) for Na^+ and K^+. The Na^+, Cl^- and K^+ concentrations in reference tissue were within 87-94% of

expected; the data were not adjusted. During non-saline recovery period, net uptake or loss of K^+ was also estimated from the changes in K^+ in the external medium during time interval between samplings.

The shoot and root fresh and dry weight as well as their length increments were recorded at each sampling in experiment 1. The seeds were considered as germinated when either the radicle or coleoptile had protruded by more than 2 mm. Sugar concentrations (as hexose equivalents) of the shoots and endosperms from experiment 2 were extracted in 80% ethanol and boiled under reflux for 20 minutes, twice. Sugars were measured colorimetrically using anthrone (Yemm and Willis, 1954) with absorbance measured at 620 nm, using a glass cuvette in a UV-visible spectrophotometer (Shimadzu recording spectrophotometer model UV-1601, Tokyo, Japan).

Root dimensions (total root length, diameter, surface area and volume) at the end of salinity and recovery in experiment 3 were measured using WinRHIZO computer software, connected with a desktop optical scanner as the image acquisition device (Regent Instruments Inc., Quebec City, QC, Canada).

Ca^{2+} concentration in shoot, root and endosperm at the end of salinity period in experiment 3 was extracted using 0.5 M HCl (Hunt, 1982). The diluted extract samples were analysed using an Inductively Coupled Plasma-Mass Spectrometry (ICPMS instrument, Perkin-Elmer, Norwalk, CT, USA). This ICP-MS provided not only Ca^{2+}, but also S, P and Mg^{2+}, Na^+ and K^+ concentrations in the shoots, endosperms and roots at the end of NaCl exposure. The Na^+ and K^+ concentrations obtained from ICP-MS were comparable with the results from the Flame Photometer.

Statistical analyses of data

Data sets were analysed using Genstat 14 ed. All pairwise comparisons were tested using Duncan's multiple range test.

Results

Growth at different levels of NaCl

Since there were large growth inhibitions at 200 mM NaCl (Table 3.1), it was important to establish there was no irrepairable injury, before giving the detail of changes in metabolism during NaCl exposure. No such serious injuries occurred as indicated by the rapid recovery of growth after return to non-saline condition, i.e. after

72 h in non-saline condition, there was no significant differences of shoot fresh weight as well as shoot and root K^+ concentrations between any of the NaCl treatments.

Table 3.1 presents the effect of different levels of NaCl on the fresh and dry weight of shoot, endosperm and roots of rice seedlings at the end of NaCl exposure. Final germination rate was similar in all NaCl treatments (> 98%). Shoot and root fresh weights were not affected by 50 mM NaCl. However, compared to growth at 0.3 mM NaCl, the growth at 200 mM NaCl was only ~40% for shoots and ~8% for roots. This different growth inhibition of shoots and roots lead to a decrease in root-shoot ratio from~0.7 at 0.3 mM NaCl to~0.15 at 200 mM NaCl.

Table 3.1. Length, fresh weight and dry weight of shoot, endosperm and root of intact seedlings at 138 h after sowing (experiment 1). Percentage of water contents in shoots and roots in all NaCl treatments were not significantly different. Water contents in endosperms at 0.3 mM NaCl was 71% and significantly higher than those at other NaCl treatment. Data given are means ± SE, n=3. Superscripts indicate significant differences at P<0.05 (comparisons down columns, within parameters).

Tissue	NaCl (mM)	Length (cm)	Fresh weight (mg) per seedling	Dry weight (mg) per seedling
shoot	0.3	6.6 ± 0.2^{a}	32.3 ± 4.8^{a}	2.5 ± 0.4^{a}
	50	7.1 ± 0.7^{a}	29.8 ± 4.3^{ab}	2.3 ± 0.3^{a}
	100	5.9 ± 0.2^{b}	24.8 ± 2.5^{b}	1.7 ± 0.1^{b}
	200	1.1 ± 0.1^{c}	12.7 ± 2.1^{c}	0.7 ± 0.1^{c}
endosperm	0.3		34.1 ± 2.8^{a}	10.2 ± 1.6^{c}
	50		29.0 ± 4.7^{b}	11.2 ± 1.6^{bc}
	100		28.6 ± 4.7^{b}	13.1 ± 1.6^{b}
	200		27.8 ± 3.2^{b}	15.3 ± 1.8^{a}
root	0.3	6.8 ± 0.5^{a}	24.7 ± 3.9^{a}	1.5 ± 0.1^{a}
	50	5.8 ± 0.4^{b}	21.2 ± 2.6^{a}	1.5 ± 0.1^{a}
	100	4.6 ± 0.6^{c}	13.5 ± 3.9^{b}	1.3 ± 0.1^{a}
	200	1.0 ± 0.3^{d}	1.9 ± 0.5^{b}	0.1 ± 0.03^{b}

K^+, Na^+ and Cl^- in shoots (leaves, coleoptiles) and endosperm

K^+, Na^+ and Cl^- concentrations in shoots were distributed differently in coleoptiles and leaves (Fig. 3.1). In all NaCl treatments, Na^+ and Cl^- concentrations were 20-50% higher in coleoptiles than in leaves. Further, K^+ concentration was~70% lower in coleoptiles than in leaves (at 100 h after sowing), and this value in coleoptile decreased with time (Fig. 3.1).

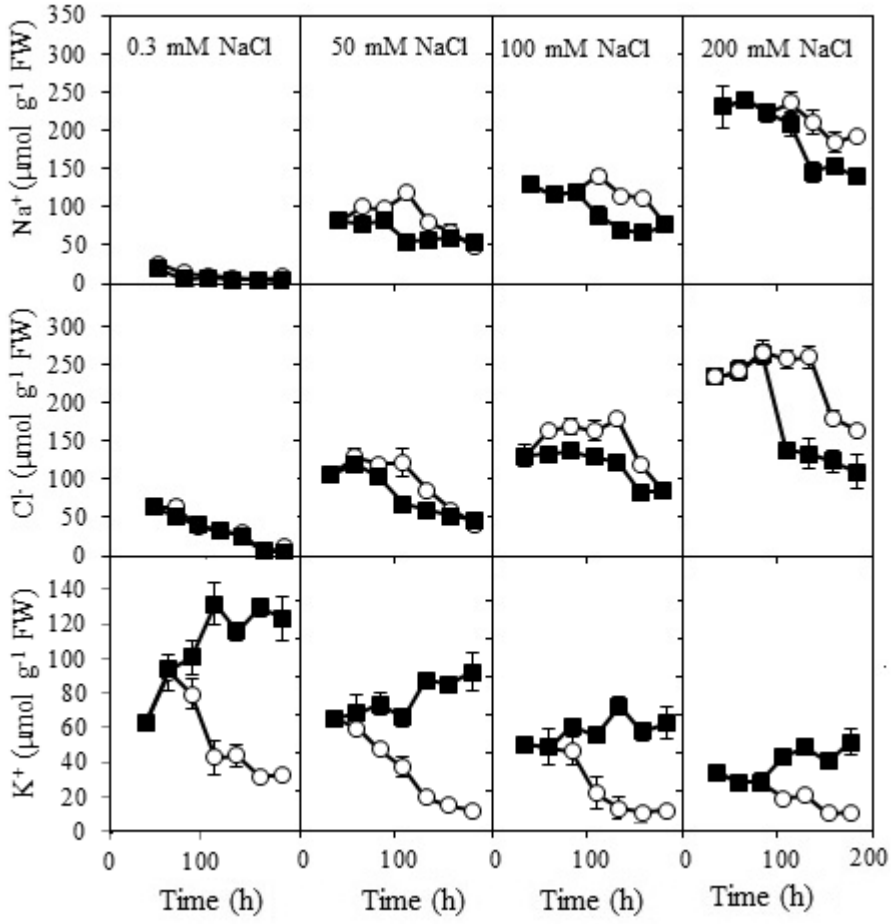

Figure 3.1. Na^+, Cl^- and K^+ concentrations in coleoptiles and leaves of intact rice seedlingss during 42 – 186 h NaCl exposure in experiment 2. NaCl was given at the time of sowing. Na^+, Cl^- or K^+ concentrations in coleoptile, opened circle; in leaf, filled square. Data given are means ± SE, n=3.

The Na^+ and Cl^- concentrations in shoots and roots of seedlings during NaCl exposure increased rapidly after emergence (Fig. 3.2). After reaching approximately similar concentrations as in the medium, these values in the tissues remained fairly steady and then slightly decrease toward the end of NaCl exposure at 186 h after sowing. In endosperm, Na^+ and Cl^- increased only gradually and reached a peak at ~114 h after NaCl was imposed, then slightly decreased to reach concentrations which were close to their initial concentrations.

Despite the large increase in Na^+ concentrations in shoots (i.e. coleoptiles + leaves) (Fig. 3.2), K^+ concentrations in shoots at 100 and 200 mM NaCl were relatively unchanged throughout the NaCl exposure at about 40 and 30 µmol g^{-1} FW at 100 and 200 mM NaCl, respectively. In roots, K^+ concentrations at 50, 100 and 200 mM NaCl decreased at~ 100 h after NaCl was imposed then remained steady toward the end of

NaCl exposure (186 h). In endosperm, K^+ concentrations were similar for all NaCl treatments and decreased gradually with time (Fig. 3.2).

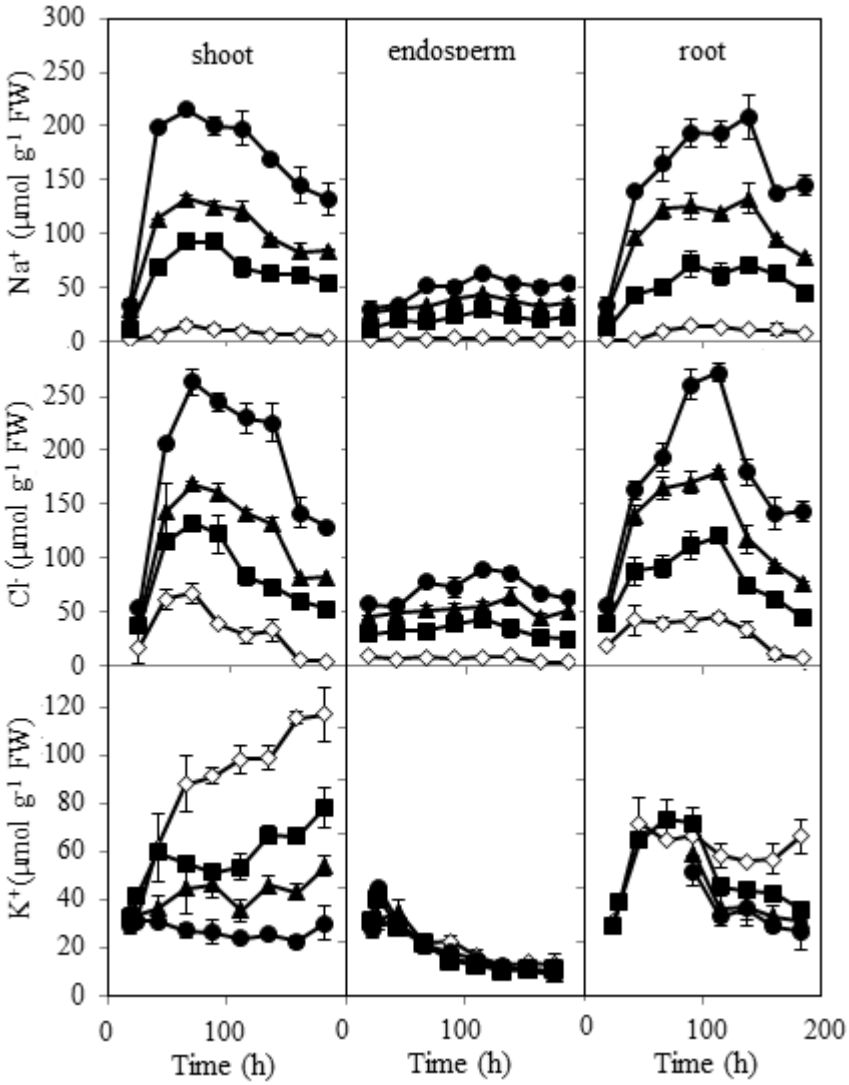

Figure 3.2. Na^+, Cl^- and K^+ concentrations in the shoots (coleoptiles and leaves) (left), endosperms (middle) and roots (right) of rice seedlings in experiment 2. NaCl was given at the time of sowing. Na^+, Cl^- or K^+ concentration in seedlings in NaCl treatments at 0.3 mM (open diamond); 50 mM (filled square); 100 mM (filled triangle); 200 mM (filled circle). Data given are means ± SE, n=3.

Reduction of turgor pressure and different contribution of ions and sugar to π_{sap}

The relationship between turgor pressure and coleoptile elongation has been a matter of debate and it was originally postulated that the rate of cell extension should be directly proportional to turgor pressure (Kutschera, 2004). In this current study, the estimated turgor pressure of rice shoots at 200 mM NaCl was 0.33 MPa. The value of minimum turgor has been estimated in coleoptile segments of *Avena sativa* and *Zea*

mays and found to be in the range of 0.22-0.25 MPa (Hohl and Schopfer, 1992) and 0.33 MPa in rye (Edelmann and Kutschera, 2002). This low turgor pressure in shoots of rice seedlings at 200 mM NaCl therefore could have limited growth.

Table 3.2. Contribution from ions and other solutes in shoots and roots of intact rice seedlings to π_{sap} at 138 h after NaCl was imposed in experiment 2. The π of the medium (π_{sol}) was measured as -0.002, -0.22, -0.44 and -0.88 for the 0.3, 50, 100 and 200 mM NaCl respectively. Turgor pressure (P) was calculated by π_{sap} - π_{sol}. $\Delta \pi_{sap}$ = the difference between π_{sap} measured and π_{sap} at 0.3 mM NaCl. Δ ion = the difference of π_{sap} contributed from ions from that at 0.3 mM NaCl. Values in italic showed the percentage of contribution of the various solutes π to total π_{sap} measured.

NaCl (mM)	Parameter	Shoots		Root	
		π (MPa)	**%**	**π (MPa)**	**%**
0.3	π_{sap}	-0.62		-0.43	
	Turgor P	0.62		0.43	
	Total ions	-0.39	*(62)*	-0.25	*(58)*
	Sugar	-0.13	*(22)*	-0.03	*(7)*
	Others	-0.10	*(16)*	-0.15	*(35)*
50	π_{sap}	-0.81		-0.63	
	Turgor P	0.59		0.41	
	Total ions	-0.50	*(62)*	-0.52	*(83)*
	Sugar	-0.09	*(11)*	-0.05	*(8)*
	Others	-0.22	*(27)*	-0.06	*(9)*
	$\Delta \pi_{sap}$	-0.19		-0.20	
	Δ ion	-0.11		-0.27	
100	π_{sap}	-0.96		-0.81	
	Turgor P	0.52		0.37	
	Total ions	-0.63	*(66)*	-0.75	*(92)*
	Sugar	-0.11	*(11)*	-0.06	*(7)*
	Others	-0.23	*(25)*	-0.01	*(1)*
	$\Delta \pi_{sap}$	-0.34		-0.46	
	Δ ion	-0.24		-0.50	
200	π_{sap}	-1.21		-1.03	
	Turgor P	0.33		0.15	
	Total ions	-0.90	*(74)*	-0.83	*(81)*
	Sugar	-0.13	*(11)*	-0.12	*(12)*
	Others	-0.18	*(15)*	-0.08	*(7)*
	$\Delta \pi_{sap}$	-0.59		-0.60	
	Δ ion	-0.51		-0.59	

At 0.3 mM NaCl, Na^+, Cl^- and K^+ concentrations both in shoots and roots contributed ~ 60 % to π_{sap}. These values in shoots increased with the increase of NaCl concentrations. In roots, the contribution of these ions to π_{sap} increased to 92% at 100 mM NaCl, while decreased to 81% at 200 mM NaCl. This decrease in percentage of ion contributions was presumably compensated by sugar or other organic solutes (Table 3.2). Total contribution from total sugar and other organic solutes to π_{sap} was similar in shoots and roots at non saline condition, however at saline conditions these contributions were 30-75% lower in roots than in shoots (Table 3.2).

Sugar concentrations in shoots during 138-162 h after sowing in experiment 2 were assessed to evaluate total sugar accumulation after Na^+ and Cl^- net uptakes have reached quasi-steady state, since inadequate ion net uptakes may require organic solutes to compensate the decrease in ion contribution to π_{sap} (i.e. to achieve osmotic adjustment). Sugar concentrations in shoots were 20-30% higher at 0.3 and 200 mM NaCl than at 50 and 100 mM NaCl. In endosperm, the lowest sugar concentrations was found at 200 mM NaCl, while in roots, sugar concentrations decreased 60-70% from 138 to 162 h after NaCl was imposed (Table 3.3).

Table 3.3. Sugar concentrations in shoots, endosperms and roots of intact seedlings at 138 and 162 h after NaCl was imposed at sowing in experiment 2. Data given are means ± SE, n=3. Superscripts indicate significant differences at P<0.05 (comparisons down columns, within sampling time).

Tissue	NaCl (mM)	Sugar (µmol g^{-1} FW)			
		138 h		162 h	
Shoot	0.3	53± 11.2	b	57 ± 1.5	b
	50	36 ± 1.3	a	49 ± 1.9	a
	100	42 ± 0.6	a	45 ± 4.8	a
	200	53 ± 6.9	b	56 ± 4.5	b
Endosperm	0.3	248 ± 25	bc	240 ± 22	ab
	50	309 ± 12	c	274 ± 24	b
	100	185 ± 28	b	211 ± 13	ab
	200	94 ± 12	a	176 ± 15	a
Root	0.3	15 ± 2.6	a	18 ± 1.6	ab
	50	19 ± 3.2	a	24 ± 4.1	b
	100	24 ± 4.6	ab	10 ± 1.2	a
	200	47± 15	b	15 ± 3.6	ab

During NaCl exposure, the increase of Na^+ and Cl^- concentrations in shoots of rice seedlings resulted in a decrease (i.e. more negative) of π_{sap} (Fig. 3.3). This correlation indicated that Na^+ and Cl^- were used as osmoticum to maintain a low $\pi_{sap.}$

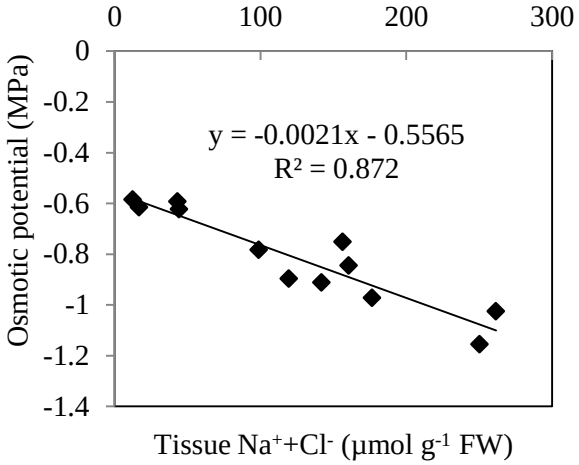

Figure 3.3. The relationship between tissue osmotic potential in shoot (π_{sap}) and Na^+ + Cl^- concentrations in the shoot of rice seedlings exposed to a range of NaCl treatments. Tissues were sampled 138 h after exposure to NaCl treatments. Data from experiment 2.

Ca^{2+} alleviates rice growth inhibition at high salinity

At 200 mM NaCl with 5 and 10 mM Ca^{2+}, rice seedlings showed better growth than those with 1 mM Ca^{2+}, i.e. 2-days earlier germination as well as the emergence of leaves from enclosed coleoptiles. However, final germination rates were not affected by this supplemental Ca^{2+}.

The alleviation of growth inhibition by supplemental Ca^{2+} at 200 mM NaCl was more evident in roots than in shoots. At 200 mM NaCl with 1 mM Ca^{2+}, shoot fresh weight (at 138 h after NaCl was imposed) was only~30% of that at 0.3 mM NaCl (Fig. 3.4). This shoot growth inhibition was not improved by additional Ca^{2+} up to 10 mM. On the contrary, root fresh weight increased by more than 2-fold when 5 mM Ca^{2+} was added to the external solution at 200 mM NaCl (Fig. 3.4). Similar trend were found in shoot and root dry weight (data not shown).

In contrast to saline solutions, in non-saline solutions (0.3 mM NaCl), addition of 10 mM Ca^{2+} did not affect shoot growth but significantly decreased root growth at 138 h after sowing (Fig. 3.4).

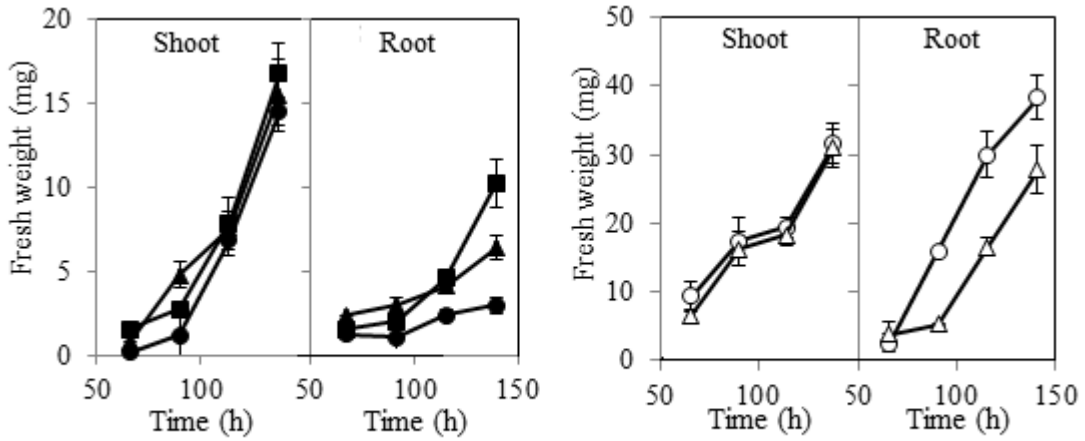

Figure 3.4. At 200 mM NaCl (left), exogenous Ca^{2+} alleviated the root growth inhibition of rice seedlings, but did not significantly affect shoots. At 0.3 mM NaCl (right), despite the inhibition on root fresh weight, no significant effect was found on shoots. NaCl was given at the time of sowing. Fresh weight at 200 mM NaCl + 1 mM Ca^{2+}, filled circle; 200 mM NaCl + 5 mM Ca^{2+}, filled square; 200 mM NaCl + 10 mM Ca^{2+}, filled triangle; 0.3 mM NaCl + 1 mM Ca^{2+}, open circle; 0.3 mM NaCl + 10 mM Ca^{2+}, open triangle. Data from experiment 3, means ± SE, n=3.

Root dimensions were measured to examine the detail of the alleviation of root growth inhibition by supplemental Ca^{2+} added to the NaCl treatment. Measured were root length, volume, surface area and diameter, since merely the length of the longest root may not give a good picture. The addition of 5 and 10 mM Ca^{2+} at 200 mM NaCl significantly improved by~20%, 10% and 11% the total root length, volume and surface area respectively (Fig. 3.5). However, the average diameter of the roots was significantly reduced by this addition of Ca^{2+} (Fig. 3.5). In contrast with the seedlings in saline treatments, the decrease of root growth caused by supplemental Ca^{2+} in non-saline seedlings at 138 h after sowing was likely due to this reduction in root volume, surface area and average diameter.

The beneficial effect on roots from the addition of supplemental Ca^{2+} in high NaCl was probably associated with the higher K^+/Na^+ concentration ratio in the tissues (Table 3.4). Addition of supplemental Ca^{2+} increased the K^+/Na^+ concentration ratio by various levels in endosperm and root, but it was not significantly different in shoots. Close to the end of NaCl exposure (at 114 – 138 h) the increase of ~35% of tissue K^+ with time in the shoot was accompanied by an ~30% decrease of K^+ concentration in the endosperm (data not shown).

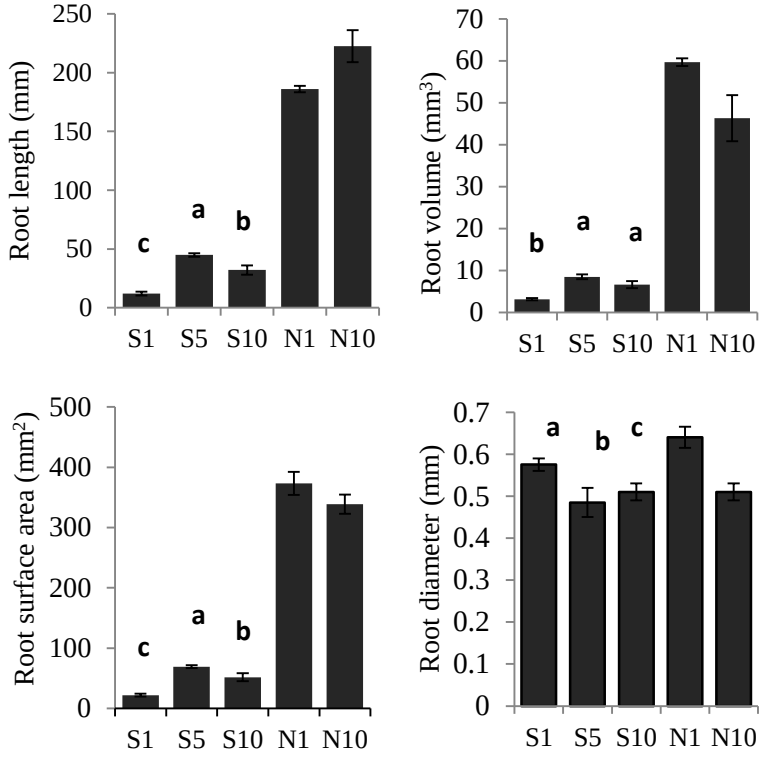

Figure 3.5. Supplemental Ca^{2+} alleviated the inhibition of root length, surface area and volume of rice seedlings at high salinity in experiment 3. NaCl was given at the time of sowing. Despite stimulating longer roots in low salinity, addition of supplemental Ca^{2+} reduced the root's surface area and volume. 200 mM NaCl + 1 mM Ca^{2+}, S1; 200 mM NaCl + 5 mM Ca^{2+}, S2; 200 mM NaCl + 10 mM Ca^{2+}, S10; 0.3 mM NaCl + 1 mM Ca^{2+}, N1; 0.3 mM NaCl + 10 mM Ca^{2+}, N10.

Table 3.4. K^+, Na^+ concentrations and K^+/Na^+ ratio in shoots, endosperm and roots of rice seedlings at 138 h NaCl exposure (experiment 3). NaCl was given at the time of sowing. K^+/Na^+ ratio in shoots at 0.3 mM NaCl was 10 and 17 for 1 and 10 mM Ca^{2+}, respectively. Data given are means ± SE, n=3. Superscripts indicate significant differences at $P<0.05$ (comparisons across row, within ion variable).

Tissue and ions	**200 mM NaCl**		
	1 mM Ca^{2+}	**5 mM Ca^{2+}**	**10 mM Ca^{2+}**
shoot	Tissue ion concentrations (µmol g^{-1} FW)		
K^+	26 ± 7 ^{a}	26 ± 2 ^{a}	30 ± 4 ^{a}
Na^+	237 ± 10 ^{a}	220 ± 26 ^{a}	222 ± 19 ^{a}
K^+/Na^+	*(0.11 ± 0.03)* ^{a}	*(0.12 ± 0.01)* ^{a}	*(0.14 ± 0.03)* ^{a}
Endosperm			
K^+	17 ± 1 ^{a}	18 ± 3 ^{a}	19 ± 2 ^{a}
Na^+	109 ± 12 ^{b}	93 ± 8 ^{b}	94 ± 11 ^{b}
K^+/Na^+	*(0.16 ± 0.02)* ^{b}	*(0.19 ± 0.01)* ^{a}	*(0.20 ± 0.02)* ^{a}
Root			
K^+	29 ± 2 ^{b}	41 ± 5 ^{a}	42 ± 6 ^{a}
Na^+	229 ± 11 ^{a}	212 ± 19 ^{a}	216 ± 14 ^{a}
K^+/Na^+	*(0.13 ± 0.01)* ^{b}	*(0.19 ± 0.03)* ^{a}	*(0.20 ± 0.03)* ^{a}

During high NaCl exposure, K^+ uptake by tissues would have to compete with Na^+ to enter the cell. This K^+ net uptake and loss during NaCl exposure are presented in Fig. 3.6. The greatest K^+ net lost (~ 0.2 μmol g^{-1} FW) occurred at the lowest (1 mM) Ca^{2+} treatment. The high K^+ loss from rice seedling seedlings at 200 mM NaCl with 1 mM Ca^{2+}, however, was not detected in Relative Electrolite Leakage measurement which showed no significantly difference among different Ca^{2+} treatments both at the end of salinity and at the end of recovery in non-saline solutions (data not shown). The portion of K^+ loss was probably only a small part of the total electrolytes. At non-saline condition, K^+ net uptake was similar between rice seedlings with 1 and 10 mM Ca^{2+} (Fig. 3.6).

Addition of supplemental Ca^{2+} also changed the concentrations of Ca^{2+} in tissues. Discussion on the changes of tissue Ca^{2+}, and of other elements in tissues, is given in Appendix 3.2.

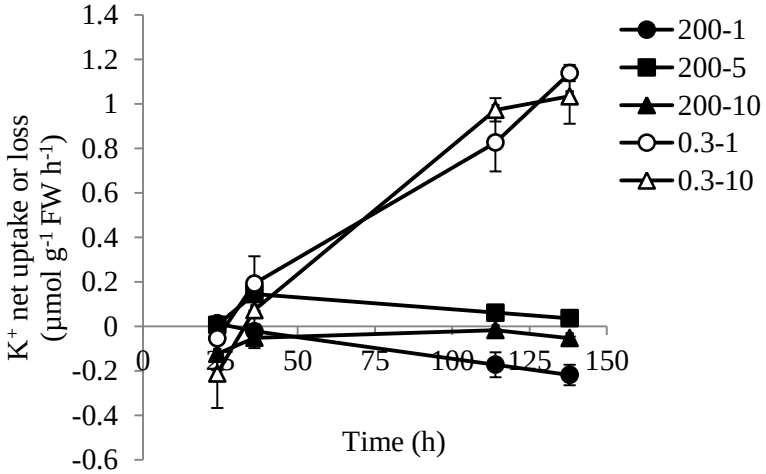

Figure 3.6. K^+ net uptake or loss in 0.3 and 200 mM NaCl with different additional Ca^{2+}. Net K^+ uptakewas calculated based on the K^+ depletion in nutrient solution between time samplings in experiment 3. NaCl was given at the time of sowing. K^+ concentrations in shoots, endosperms and roots at the end of NaCl exposure (138 h) are given in Table 3.4. Data given are means ± SE, n=3.

Discussion

Rice seedling growth at different levels of NaCl

Rice germinated over the full range of NaCl concentrations used, with relatively little effect on growth at 50-100 mM NaCl, but after start of germination, growth was severely reduced at 200 mM NaCl. The severe effects at 200 mM NaCl made it important to establish whether the seedlings had suffered irreparable injury.

Vigorous growth and rapid K^+ uptake after return to non-saline solutions demonstrated that rice seedlings were able to tolerate and recover from exposure up to 200 mM NaCl, without suffering irrepairable injury. At 50 – 200 mM NaCl, the low external osmotic potential caused substantial inhibition in water uptake and lead to a higher dry weight but lower fresh weight in rice endosperms compared to those at 0.3 mM NaCl. The slower starch degradation was also reported in rice seedlings during germination in 75 mM NaCl which was associated with a 36% decrease in activity of α amylase (Shereen et al., 2011).

The growth reductions in shoots were ~60% at 200 mM NaCl, ~30% at 100 mM NaCl, but no growth reductions at 50 mM NaCl, and there were no effects on total final germination percentage in salinity up to 200 mM NaCl, although germination was delayed at the highest salinity. These results were interesting, since other studies reported that inhibition in rice germination rate and growth at similar NaCl concentrations are much larger in magnitude, as described previously (Heenan et al., 1988; Punyawerdana and Dharmasri, 1989; Lutts et al., 1995; Khan et al., 1997; Hakim et al., 2010). One likely possibility is because the germination methods were different, i.e. in those other studies the emerged shoots from the germinating seeds grew in air and transpired, so that ions may have accumulated in shoot tissues. In the present study, complete submergence in the aerated solution system enabled rice seedlings to exchange ions with external solution and therefore reach an equilibrium with the external solution.

K^+, Na^+ and Cl^- in shoot (leaves, coleoptile) and endosperm

A coleoptile is a protective sheath that surrounds the embryonic leaves. This organ has more vacuoles than the leaves (Jones and Rost, 1989; Taylor and Vasil, 1995; Maeda et al., 2002). High concentrations of Na^+ and Cl^- could be present in cytoplasm and vacuoles, but it is technically not easy to measure these compartments. However, in glycophytes, salt compartmentalization is an adaptation strategy to mitigate further injuries by ion toxicity (Greenway and Munns, 1980). The relatively little effect on growth at 50-100 mM NaCl (p. 44) despite the tissues containing these potentially toxic ions could be an implication that Na^+ and Cl^- were stored in vacuoles which occupy the bulk of intracellular volume in coleoptile cells. Meanwhile K^+ in cytosol is essential for plant metabolic processes (Marschner, 1995) and the gradual decrease of K^+ with time might indicate a slow loss of cytosolic K^+ due to cytosol deterioration during Program Cell Death (PCD)

throughout coleoptile senescence (Inada, 1998). These coleoptiles cease to elongate and mature after the emergence of the primary leaves (Kutschera, 2004). The different characteristic between coleoptiles and leaves in respect to Na^+ and Cl^- concentrations found in this non-transpiring experiment may not be as important as that in transpiring condition, i.e. having more Na^+ and Cl^- compartmentalization in the vacuoles of coleoptile may be an adaptive response of rice seedlings in transpiring condition. Furthermore, the exposed coleoptiles would also transpire much more than any enclosed leaves, which may also cause accumulation of Na^+ and Cl^- further higher concentrations in coleoptiles than in enclosed leaves.

There were maximum peaks in Na^+ and Cl^- tissue concentrations during 66-90 h after NaCl exposure in shoots and 66-114 h in roots, followed by a gradual decrease of ion concentrations with time both in shoots and roots. The slow decreases in Na^+ and Cl^- tissue concentrations after internal ion concentrations reached a peak at ~90 h in shoots and ~114 h in roots after NaCl was imposed is interesting, since the relative growth rate of the tissues decreased followed the decreases in Na^+ and Cl^- net uptakes. Study on rice roots in 30 mM NaCl demonstrated that OsHKT2;1-induced Na^+ influx is rapidly down-regulated by post-translational and transcriptional mechanisms, to restrict the amount of Na^+ influx via OsHKT2;1 to prevent Na^+ over-accumulation and Na^+ toxicity (Horie et al., 2007). However, a complete elucidation of the molecular mechanisms that mediate rapid inactivation of OsHKT2;1 under high Na^+ concentrations remains unknown (Horie et al., 2007). Further discussion on the relation between ion net uptake and growth rate is given in Appendix 3.1.

In shoots, the Na^+ and Cl^- net uptakes between 0-66 h after germination in NaCl were 1-5 µmol g FW^{-1} h^{-1}. Then at 66-182 h these rates decreased to < 1 µmol g FW^{-1} h^{-1}. In roots, these rates were 1-3 µmol g FW^{-1} h^{-1} from 0-114 h after NaCl was imposed, followed by < 1 µmol g FW^{-1} h^{-1} up to the end of NaCl exposure at 186 h. The relative different time needed to reach peaks in shoots and roots was presumably determined by either different ion permeability or better ion extrusion in shoots and root plasma membrane, or different biological structure between those organs. The few hours earlier emergence of roots than shoots may add to the length of time needed by roots to reach maximum Na^+ concentration. Different NSCCs channels associated with Na^+ influx which has been reported in shoots and roots plasma membrane (Shabala et al., 2006) may result in different Na^+ accumulation with time. While related with biological structure, vacuolation of rice root tip cells is reported as an adaptive response

to accumulate excess ions under salinity (Miyake, 2006). Having more vacuole volume in roots than in leaves is likely to cause longer time for roots to accumulate ions to reach an equilibrium state with external medium.

Contribution of ions and sugars to π_{sap}

At 200 mM NaCl, rice seedlings were still able to grow with tissue concentrations that reach ~200 mM Na^+ and Cl^-. It is reported that under NaCl exposure cytosolic Na^+ concentrations can reach up to 200 mM level, with most authors favoring values in the 50 to 100 mM range (Maathuis and Amtmann, 1999; Shabala et al., 2006). At 200 mM NaCl, shoots of rice seedlings had to attain more ions and organic solutes for osmotic adjustment. The contribution from Na^+ and Cl^- accounted for about three-quarter of the $\pi_{sap,}$ and the rest of the contribution presumably came from organic solutes. Since the cost to synthesis of 1 mol compatible solutes has been estimated as more than ten times the cost to accumulate 1 mol Na^+ (Raven, 1985; Munns and Tester, 2008), an accumulation of Na^+ and Cl^- in shoots to reach a slightly higher concentrations than that of external solution would be an efficient mechanism to reduce energy cost for osmotic adjustment during high salinity exposure.

At 50 and 100 mM NaCl, the contribution of sugars to π_{sap} was less than those at 0.3 and 200 mM NaCl (Table 3.2). The π_{sap} contribution derived from the sum of ions and sugars was only ~75% which implied that other organic solutes were required to compensate for the remaining 25% contribution. The increase by 10-25% in sugar concentrations in shoots during 138 – 162 h after NaCl exposure (Table 3.3) was likely to provide the larger amount of organic solutes to regain π_{sap} caused by the gradual decrease in Na^+ and Cl^- tissue concentrations. However, this slight increase in sugar concentration (~0.04 MPa) would have not been sufficient to compensate the decrease in shoots Na^+ and Cl^- concentrations during the period of 138-162 h after sowing which was about -0.12 MPa (estimated from Fig. 3.2). The measured π_{sap} of the shoots at 100 mM NaCl during the subsequent period (138-162 h after sowing) which decreased by~-0.08 MPa (data not shown) also indicates that osmotic adjustment was hard to be maintained during the long period at this high NaCl concentration, particularly when internal Na^+ and Cl^- concentrations kept decreasing with time. In another scenario, the sugar accumulation at 200 mM NaCl could be due to very little consumption and the inevitable consequence is that the sugars contributes only a bit to $\pi_{sap.}$

At 138 h after sowing, the calculated turgor (P) in seedlings at 200 mM NaCl compared to P at 0.3 mM NaCl which was 35% in roots and 55% in shoots, indicated that the shoot was closer to osmotic adjustment than the roots. In saline conditions, the contributions from ions to π_{sap} which were 81-92% in roots and 62-74% in shoots indicated that more organic solutes contributed to maintain the low π_{sap} in shoots. In shoots, contribution from sugars and other organic solutes in both saline and non-saline conditions were estimated to be 30-40% of the measured $\pi_{sap.}$ Whereas in roots, the contribution from sugar and other organic solutes were estimated about 42% in non-saline, but only 8-19% in 50-200 mM NaCl. The decrease in soluble sugar concentrations is also reported in rice roots subjected to 342 mM NaCl for 4 days grown in vermiculite, despite a considerable increase of sugar concentrations in rice shoots (Siringam et al., 2012). The ion contributions to π_{sap} which was less in shoots than in roots demonstrated that with less organic solutes available, roots have to rely on ions to generate low π_{sap}. When NaCl was increased to 200 mM, contribution from ions was not enough to decrease the $\pi_{sap.}$ At this stage, π_{sap} in roots was probably maintained by restricted growth (~8% of the root growth at 0.3 mM NaCl). It is also reported in other study with maize, roots growing at low Ψ_w were thinner and with this decrease in volume expansion, substantial decrease in π_{sap} was possible without osmotic accumulation (Sharp et al., 1988).

Rice seedling responses to supplemental Ca^{2+} at 200 mM NaCl

The alleviation of growth inhibition at 200 mM NaCl by addition of supplemental Ca^{2+} was more evident in root (~24%) than in shoots. Growth inhibition in high NaCl was associated with a high accumulation of Na^+ and low K^+ concentrations (Munns and Tester, 2008). Although down-regulation of Na^+ influx through NSCCs could be an important mechanism in Ca^{2+} amelioration of salt toxicity (Demidchik and Tester, 2002; Essah et al., 2003), yet in the present experiment there was no significant difference in Na^+ internal concentrations among Ca^{2+} treatments both in shoots and roots. Instead, there was~ 30% higher K^+ concentration during treatment with exogenous supplemental Ca^{2+} in roots but not in shoots. The better selection for K^+ over other cations in root than leaf cells associated with addition 10 mM Ca^{2+} was reported in *Arabidopsis thaliana* (Shabala et al., 2006), and could be associated with the function of roots in selective uptake of ions from soils. This higher K^+ concentrations in roots with 5 and 10 mM Ca^{2+} lead to an increase of root K^+/Na^+ ratio

from 0.1 to 0.2. This difference in K^+/Na^+ ratio may determine the different responses of shoots and roots to supplemental Ca^{2+}. Since Na^+ and K^+ are known to compete for enzyme activation and protein biosynthesis, it is the cytosolic K^+/Na^+ ratio rather than the absolute quantity of Na^+ or K^+ in the cell, which determines cell metabolic competence, and therefore this ratio is often used as a key determinant of plant salt tolerance (Greenway and Munns, 1980; Munns and Tester, 2008).

At 200 mM NaCl with different Ca^{2+} concentrations, similar π_{sap} was found among shoots of intact seedlings at 1, 5 and 10 mM Ca^{2+} (~ 1.3 MPa) at 114 h after NaCl was imposed (data not shown). This is in agreement with results on Na^+, K^+ and Cl^- concentrations which showed no significant different among shoots at 200 mM NaCl with different Ca^{2+} levels. At 138 h, the π_{sap} in shoots remained the same as at 114 h, with a lower π_{sap} contribution from ions. The decrease in the percentage of π_s contribution from ions between 114 to 138 h after NaCl was imposed was due to a decrease in Na^+ and Cl^- concentrations during the period (i.e. after peaks had been reached at 50-70 h after sowing), and therefore the contribution of π_{sap} from other solutes must be elevated.

In contrast to seedlings in saline conditions, in 0.3 mM NaCl, the adding of 10 mM Ca^{2+} had no effect on shoot growth, but root fresh weight decreased by 26%. This decrease in root fresh weight which was shown by some reduction in root volume, surface area and diameter may be due to root sensitivity to the increase of Ca^{2+} internal concentrations. It is reported that root and shoot plasma membranes have different non-specific cation channels (NSCCs) which respond differently to an alteration of exogenous Ca^{2+} (Shabala et al., 2006).

Conclusions

Rice seedlings were able to grow and tolerate at least 200 mM NaCl in aerated nutrient solution and recovered without substantial injuries by allowing Na^+ and Cl^- to enter the cells and using these ions as osmoticum to reach maximum tissue concentrations which were about similar to those in the external medium. In contrast for transpiring seedlings, ion concentrations in the shoots often rise well above external concentrations (Barrett-Lennard, 2003; Munns and Tester, 2008).

Supplemental Ca^{2+} at 5 and 10 mM alleviated ~24% of the growth inhibition in roots at 200 mM NaCl, by improving root fresh weight, dry weight, length, volume and

surface area. Supplemental Ca^{2+} had no effects in shoots. The different responses of shoots and roots to the supplemental Ca^{2+} might be due to the increase of K^+/Na^+ ratio in roots but not in shoots.

The discussed possibilities on the various processes in air were not explored further, since the experiments in this Chapter were intended to give a good background for the main aim of this study, which focused on the interaction between NaCl and anoxia of submerged seedlings (see Chapters 4 and 5).

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Appendix 3.1.

High Na^+ and Cl^- uptake stimulated growth of shoots of submerged rice seedlings at moderate salinity in aerated solution

It was likely that rapid growth during early growth stage of rice seedlings was related to the Na^+ and Cl^- net uptakes in the tissues. The increase of Na^+ concentrations with time in shoot has a strong correlation with the fresh weight increment ($R^2 = 0.88$). The period when Na^+ and Cl^- concentrations in shoots reached peaks (at~66 - 90 h after sowing) coincided with the period of most rapid growth increment (Fig. 1 of this

Appendix; 3A1.1). The growth rate was calculated on fresh weight basis because (i) there was no significant different in water content among shoots or roots at different NaCl levels. (ii) Analysing dry weight would involve other errors due to very small dry weight per sample. (iii) Large samples would be needed to measure dry weight in all sampling. (iv) Ion concentrations were measured in fresh weight basis, the most appropriate basis for their expression.

The results from Chapter 5 of this thesis with aerated and anoxic excised coleoptile tips (both had much lower growth rate than aerated intact shoots) which showed a similar quasi-steady state Na^+ and Cl^- concentrations at about 70-90 h after NaCl was imposed, suggests that the period of quasi-steady state in ion uptake was not affected by growth. Therefore, in this current experiment, shoot growth rate curve which coincides with Na^+ and Cl^- net uptake curve may indicate that Na^+ and Cl^- net uptakes increased turgor pressure by providing a cheap osmolyte, so leading to shoot growth. This occurred only in a short period when Na^+ and Cl^- were high at initial hours and the shoot fresh weight increments may be due to more cell vacuolation. The decrease in Na^+ and Cl^- net uptakes during later stages in NaCl exposure however remained unknown since the calculated dilution by fresh weight increment underestimated the decrease in shoots Na^+ and Cl^- concentrations. One possibility on the down-regulation of some Na^+ transporters at high salinity as described in the discussion, however, this would need to be confirmed by molecular studies.

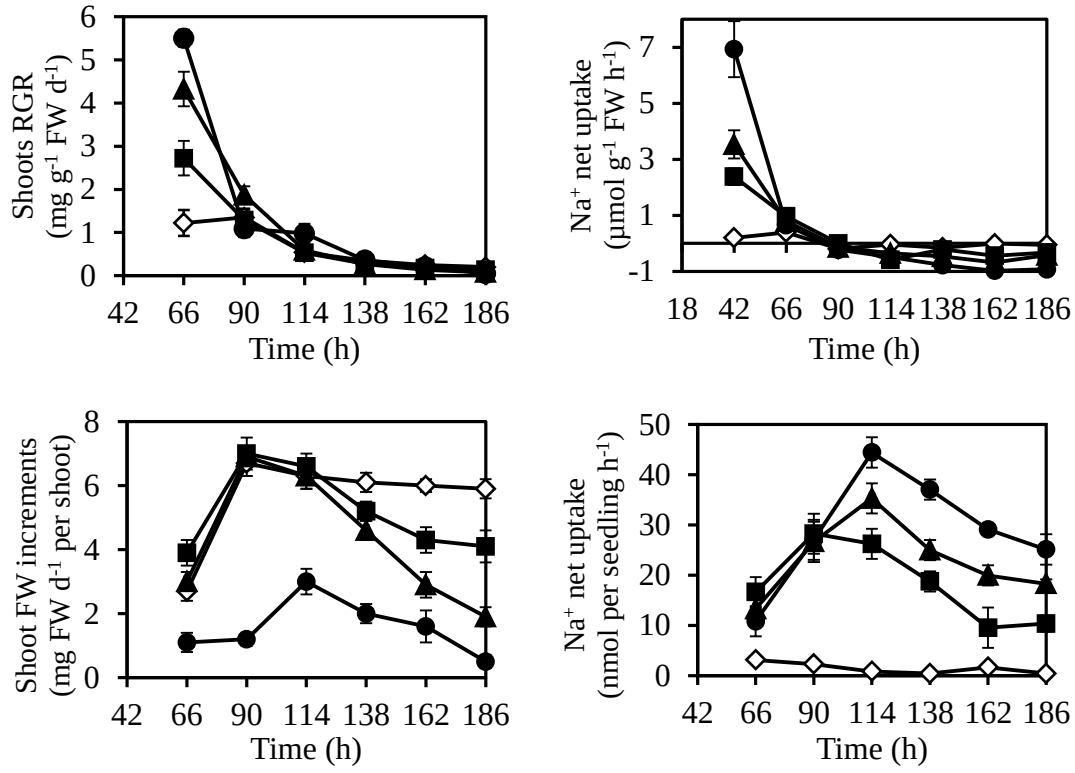

Figure 3A1.1. Shoot relative growth rate, shoot fresh weight increments and Na^+ net uptake (per fresh weight and per shoot) of rice seedlings. Na^+ concentrations or growth at 0.3 mM NaCl, open diamond; 50 mM NaCl, filled square; 100 mM NaCl, filled triangle; 200 mM NaCl, filled circle. Data given are means ± SE, n=3.

Appendix 3.2

Ca^{2+}, Mg^{2+}, P and S concentrations in tissues of rice seedlings in salinity with different exogenous Ca^{2+}

Increasing NaCl concentration of the external medium slightly reduced the concentrations of Mg^{2+} in rice shoot and root, but increased their concentrations in the endosperm. In rice grains, the aleuron layer is an accumulation site of P, K and Mg^{2+} (Ogawa et al., 1979). As the seedling further developed, Mg^{2+} and P were translocated to shoot and root. Therefore, the higher P concentrations in the endosperm might be associated with the slow starch breakdown at higher NaCl treatments. Alternatively, with the slow growth of the shoot, P required and transported could be less. Besides, there is relationship between Mg^{2+} and other cation such as Ca^{2+} and Na^+. The high concentration of Na^+ at 200 mM NaCl and high supplemental Ca^{2+} gave less opportunity to other cations to be taken in the cells. The decrease of Mg^{2+}

concentrations in shoot with addition of 5 and 10 mM Ca^{2+}, but not in roots, may have contributed to the less-beneficial effect of supplemental Ca^{2+} on shoots than on roots.

Table 3A2.1. Ca, Mg, P and S concentrations, in shoots, endosperms and roots of rice seedlings at 138 h NaCl exposure with different levels of external Ca^{2+}. Data given are means ± SE, n=3. Superscripts indicate significant differences at P<0.05 (comparisons across row, within ion variable).

	200 mM NaCl			0.3 mM NaCl	
	1 mM Ca^{2+}	5 mM Ca^{2+}	10 mM Ca^{2+}	1 mM Ca^{2+}	10 mM Ca^{2+}
Element			(µmol g^{-1} FW)		
			shoot		
Ca	0.84 ± 0.1 ^{d}	2.85 ± 0.2 ^{c}	5.14 ± 0.7 ^{b}	3.00 ± 0.3 ^{c}	9.30 ± 1.3 ^{a}
Mg	3.60 ± 0.2 ^{c}	3.13 ± 0.2 ^{d}	2.86 ± 0.2 ^{d}	7.48 ± 0.7 ^{a}	5.72 ± 0.3 ^{b}
P	17.8 ± 2.1 ^{b}	17.7 ± 1.9 ^{b}	14.5 ± 0.9 ^{c}	28.1 ± 3.1 ^{a}	27.5 ± 2.9 ^{a}
S	0.31 ± 0.02 ^{c}	0.55 ± 0.06 ^{b}	0.63 ± 0.05 ^{b}	7.42 ± 0.6 ^{a}	7.90 ± 0.9 ^{a}
			endosperm		
Ca	1.85 ± 0.2 ^{e}	3.33 ± 0.3 ^{d}	5.41 ± 0.7 ^{b}	4.20 ± 0.4 ^{c}	9.76 ± 0.3 ^{a}
Mg	33.2 ± 1.4 ^{a}	33.9 ± 3.2 ^{a}	32.1 ± 2.6 ^{a}	30.3 ± 2.0 ^{a}	31.4 ± 3.3 ^{a}
P	61.5 ± 5.2 ^{a}	60.5 ± 5.8 ^{a}	57.4 ± 3.0 ^{b}	48.1 ± 3.2 ^{c}	51.4 ± 2.5 ^{c}
S	3.20 ± 0.5 ^{b}	3.08 ± 0.5 ^{b}	2.43 ± 0.3 ^{c}	3.23 ± 0.4 ^{b}	4.11 ± 0.4 ^{a}
			root		
Ca	1.19 ± 0.2 ^{d}	2.57 ± 0.2 ^{c}	2.32 ± 0.5 ^{c}	3.03 ± 0.1 ^{b}	3.76 ± 0.1 ^{a}
Mg	4.25 ± 0.2 ^{b}	4.10 ± 0.1 ^{b}	4.13 ± 0.5 ^{b}	6.64 ± 0.6 ^{a}	7.03 ± 0.7 ^{a}
P	35.5 ± 4.1 ^{a}	25.9 ± 1.8 ^{b}	26.3 ± 2.8 ^{b}	21.4 ± 1.6 ^{c}	22.9 ± 0.9 ^{c}
S	14.4 ± 2.9 ^{a}	6.35 ± 0.8 ^{b}	2.58 ± 0.7 ^{c}	2.78 ± 0.5 ^{c}	6.05 ± 0.4 ^{b}

CHAPTER 4

Responses of Rice Seedlings and Excised Coleoptile Tips in a Combination of NaCl and Anoxia

Abstract

The combined effect of NaCl and anoxia on rice seedlings was studied to elucidate responses of survival, K^+, Na^+, Cl^- net uptakes, catabolism rate and growth. The use of excised coleoptile tips as research material was also examined in comparison with responses to those of intact seedlings, and to study ion net uptake and ethanol production.

In a combination of NaCl and anoxia, intact rice seedlings tolerate at least 100 mM and excised coleoptile tips tolerated 50 mM. The rapid resumption of growth and K^+ uptake after return to non-saline aerated solutions confirmed that there was no serious injury in any of the treatments with the possible exception excised coleoptile tips at 100 mM NaCl. In anoxia, the maximum concentrations of Na^+ and Cl^- in shoots of intact seedlings were approximately similar to external Na^+ concentrations. In this energy starvation, these Na^+ and Cl^- concentrations in shoots of intact seedlings were ~ 30% less than those concentrations previously studied in shoots of aerated intact seedlings.

In rice seedlings, an increase of ~ 0.54 µmol ethanol production g^{-1} FW h^{-1} at 50 mM NaCl without significant increase in shoot fresh or dry weight indicated that more energy was required to produce energy for cell maintenance. By contrast, at 75 and 100 mM NaCl in anoxia, the shoot growth decreased and this response implied an alteration of energy allocation from growth to maintenance. In excised coleoptile tips, the extra energy (0.7 - 1 µmol ATP g^{-1} FW h^{-1}) deducted from the difference of ethanol production at 50 and 0.3 mM NaCl, plus the energy reallocated from ~ 74% growth inhibition in these excised tips would meet the additional requirement for cell maintenance in salinity. Assesment of the ethanol formation in the coleoptile of intact seedlings gave a similar value as the measured value for the excised coleoptiles tips.

Overall, with only ~ 25% shoot growth decreased, anoxic rice seedlings were able to survive up to 100 mM NaCl by maintaining K^+/Na^+ ratio and estimated turgor pressure and recovered upon return to non-saline re-aeration. In contrast to intact seedlings, excised coleoptile tips failed to survive at 100 mM NaCl in anoxia. However, at 50 mM NaCl excised coleoptile tips had K^+/Na^+ ratio and ethanol production relatively similar to those of the shoot of intact seedlings. Furthermore, with 90% lower growth rate of excised coleoptile tips at 50 mM NaCl than intact shoots, excised coleoptile tips would be suited to conduct experiments on ion uptake and ethanol

production rate because substantial growth (and presence of other organs) may complicate interpretation of the results on the functioning of coleoptiles.

Introduction

The objectives of these experiments were to examine the interaction between NaCl and anoxia on coleoptile growth, K^+, Na^+, Cl^- net uptakes and catabolism rate by intact seedlings. To further study the dynamics of ion uptake as well as ethanol formation, the use of excised coleoptile tips as research material was also examined for responses to the combined anoxia and NaCl treatments, in comparison to those of intact seedlings.

Irrigated land is often subject to salinization (Flowers, 2004). During salinity stress, the reduction in shoot growth occurs in two phases: a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na^+ in leaves (Munns and Tester, 2008). In maize, as in many other Gramineae, tolerance of salinity is associated in part with an ability to 'exclude' Na^+ from the leaves (Drew et al., 1988), or tolerance of accumulated Na^+ by compartmentalization of Na^+ and Cl^- into the vacuole to avoid toxic concentrations within the cytoplasm (Yeo et al., 1999; Munns and Tester, 2008). Therefore, failure of the Na^+ exclusion mechanism due to energy deficiency may be a contributory factor to salinity damage of salt-sensitive glycophytes, especially when O_2 deficiency occurs in saline waterlogged soils (Drew et al., 1988; Barrett-Lennard, 2003; Munns and Tester, 2008).

Poor drainage (soil O_2 deficiency) can often accompany high salinity in irrigation agriculture (Drew and Lauchli, 1985; Ram et al., 2002). Flooding slows seed germination, imposes fatalities and delays seedling establishment of direct-seeded rice (Ismail et al., 2009). The coleoptile of rice seedlings is one of the few plant organs that can elongate during anoxia (Atwell et al., 1982; Alpi and Beevers, 1983; Menegus et al., 1984; Setter et al., 1994). This elongation is of adaptive significance since contact of the seedling with the atmosphere can be established, hence providing an O_2 source to submerged seedlings (Kordan, 1975). Energy production is ten-fold lower in anoxia than in air, therefore the maintenance of glycolysis and induction of fermentative metabolism are considered to be essential for plants to produce the metabolic energy to survive under these conditions (Kennedy et al., 1992; Ricard et al., 1994; Drew, 1997; Huang et al., 2005). Understanding the mechanism of rice tolerance to flooding during

germination and early seedling growth could therefore help to give new insight on improving crop establishment under this unfavorable environment.

So far, most of the experiments on crop seedlings under salinity, with or without anoxia, focused on roots in treatment solutions, with the shoots in air (John et al., 1977; Brauer et al., 1987; Drew and Lauchli, 1985, 1987; Volkov and Amtmann, 2006). Also, amongst 'exclusion' strategies, root selectivity has received the most attention (Yeo and Flowers, 1982; Ferdose et al., 2009) with saline treatments generally imposed at a range of concentrations a few weeks after sowing (Yeo and Flowers, 1982; Drew and Lauchli, 1985; Drew, et al., 1988; Alamghir et al., 2007; Cha-um, et al., 2009). No experiments have been conducted to determine the responses of rice or other plants if their whole intact seedlings or coleoptiles are submerged and then exposed to both anoxia and salinity. This situation occurs during seed germination and initial growth in direct seeded rice. When submerged, the transpiration stream cannot be involved in ion delivery to shoot tissues. Moreover, since in rice exposed to O_2 deprivation, almost no roots develop, it is interesting to observe how intact coleoptiles regulate ion uptake from the external medium. Apart from this, most of the previous studies on salinity and anoxia in rice have been based on later growth stages by which plants which have a complicated structure, so that some data interpretations become difficult. This research will investigate how rice, as an important crop worldwide and a flood tolerant species can survive under anoxia and the detrimental effects of excess NaCl, during its early stage of seedling growth when under water (i.e. without transpiration). The experiments also explored whether excised coleoptile tips could be used to study the interaction between NaCl and anoxia, since these excised coleoptiles tips can at times be very useful, e.g. to evaluate ion net fluxes and measure ethanol production for this defined, anoxia tolerant tissue.

Materials and Methods

The methods applied in each of the experiments in this chapter are described in Appendix 4.1.

Seedling preparation (for experiments 1 and 2)

Dehulled seeds of rice (*Oryza sativa* L. cv. Amaroo) were surface sterilized with 0.1% sodium hypochlorite for 20 min and then washed thoroughly with deionized water. Batches of 14 seeds were transferred to each 50 ml conical flask containing an

air flushed solution for 36 h for germination stage, prior to being exposed to a 30 h hypoxic pretreatment (0.06 mM O_2) and then anoxia for 72 h (experiment 1) and 78 h (experiment 2). The composition of the nutrient solution is given below. The conical flasks and solutions were autoclaved (before adding MES). The seedlings were grown in the dark at 30^oC. The nutrient solution contained macro-nutrients (mM): Ca^{2+} 2.0, NH_4^+ 0.3, NO_3^- 0.3, SO_4^{2-} 0.9, K^+ 0.3, Mg^{2+} 0.3, H_2PO_4 0.1, and micro-nutrients in (µM): Fe-EDTA 12.5, H_3BO_3 6.25, Mn^{2+} 0.5, Zn^{2+} 0.5, Cu^{2+} 0.125, Mn^{2+} 0.125, Ni^{2+} 0.25. The solution contained 0.5 mM MES and the pH was adjusted to 6.5 using $Ca(OH)_2$.

Excised coleoptile tips preparation (for experiment 3)

Dehulled seeds were germinated and grown in aerated solution (0.25 mM O_2) for 36 h, prior to being exposed to a 30 h hypoxic pretreatment (0.06 mM O_2). During hypoxia and anoxia, the nutrient solution as described above also contained 50 mg l^{-1} ampicillin. Coleoptile tips were excised (15-20 mm) in such a way that tissues inside the base of the coleoptile were avoided. Coleoptile tips were healed for 5 h in hypoxic solution before being given anoxic treatment for 96 h. Glucose 20 mM was given during the healing and at 50 mM during anoxia. These concentrations were determined based on other studies, i.e. applying different levels of glucose on anoxic excised coleoptile tips (Huang et al., 2005). These excised coleoptile tips were used for a preliminary experiment (Appendix 4.1) and in experiment 3.

Method used to impose anoxia in combination with NaCl

The combination of NaCl and anoxia was applied for all experiments, except for the preliminary experiment (Appendix 4.1). The design of the conical flasks ensured anoxia in solution in those tubes continuously flushed with humidified high purity N_2. A gas-tight syringe was used to withdraw, or add, solution through the outlet of each conical flask, while maintaining gas flow. The anoxic treatment solutions were pre-flushed for several hours with high purity N_2 before being injected into the tubes (Colmer, et al., 2001). When the experiments included transfer to aeration and de-salinization, the nutrient solutions were sampled and refreshed in the same way as for the anoxic flasks, but the solutions were aerated and non-saline. The solutions sampled from the flasks were stored at -20^oC prior to measurement of K^+ and ethanol. The non-saline re-aeration (recovery) was applied in experiment 1 for 48 h. During the recovery,

K^+ net uptake or loss was measured at 1, 4, 12 and 24 h after starting re-aeration in non-saline solution. At the end of the recovery stage at 48 h, K^+, Na^+ and Cl^- in tissues were measured. The recovery stage was intended as a validation of system to establish whether there was any tissue injury and if so whether it was repairable.

Experiment 1. Responses of intact seedlings to combined salinity and anoxia

The NaCl treatments were started after seedlings in the conical flasks were in anoxia for 18 h. Four levels of NaCl (0.3, 50, 75 and 100 mM) were imposed. To avoid osmotic shock, 75 and 100 mM NaCl were given in 2 steps; first, 50 mM NaCl was applied at 18 h after starting anoxia. Second, 25 or 50 mM NaCl were added at 24 h to reach the final concentrations. Salinity was imposed for 72 h, prior to return to non-saline aerated solution (recovery) for 48 h. Samplings were conducted at 0, 24, 72 h after the first NaCl was imposed and at the end of recovery. There were three replicate flasks for each treatment. Each conical flask contained 14 seedlings, and samplings of seedlings were: at start salinity (0 h), 6 seedlings were taken out for measurement of tissue organic solutes (sugars, amino acids and organis acids), ion and growth rate analysis; at the end of salinity (72 h after NaCl was imposed), 5 seedlings were taken out for protein, ion measurements and growth analysis, and at the end of recovery, 3 seedlings were taken for measurement of protein, ion and growth analysis. Meanwhile for the samplings during anoxia (24 h after NaCl imposed), 6 separate flasks (three replicate flasks each of 0.3 and 50 mM NaCl) had also been prepared, to leave the remaining flasks undisturbed (since opening would have allowed some O_2 to enter the anoxic solution). From each flask at the 24 h sampling, 3 seedlings were analyzed for shoot and endosperm fresh and dry weight, 3 for measurements of tissue ions, 8 seedlings were used for organic solute analysis. Due to limited tissue samples at later stages of anoxia-NaCl treatment, sugars, amino acids and organic acids were measured in shoot and endosperm only at 0 h and 24 h after NaCl was imposed. At 24 h after commencement of NaCl treatments, the second step had not yet been added, so only tissues at 0.3 and 50 mM NaCl were available to be analysed.

The rinse procedure for ion measurements used 2 mM $CaSO_4$ and iso-osmotic mannitol and was carried out in approximately 3 minutes for seedlings from all the NaCl treatments, to remove ions in the free spaces and surface water that would have contained ions. Tissues for sugar measurement were rinsed with the continued anoxic solution (NaCl still present) without sugar for 3 periods each of 3 min.

Experiment 2. Changes in tissue ion and ethanol production of intact seedlings during salinity and anoxia

This experiment used intact seedlings and was conducted in similar way to experiment 1, to examine the changes in tissue ions which were expected to reach a quasi-steady state after a certain time during NaCl exposure, and the relation of ethanol production to the changes in ion concentrations. The main differences were time of samplings and the NaCl concentration used. Ion measurements from experiment 1 were used to determine the appropriate times for samplings in the subsequent experiments. In experiment 2, samplings of ion concentration in the tissues (shoot and endosperm) at 50 and 100 mM NaCl were conducted more frequently: i.e. at 0, 24, 30, 48, 54, 72 and 78 h after starting salinity, to obtain the trend of ion accumulation during theses time intervals, while seedlings at 0.3 mM NaCl were only sampled at 0, 24 and 72 h. The 75 mM NaCl treatment (used in experiment 1) was not included in this experiment, as only certain number of samples could be accomodated, since for each sampling separate flasks had to be prepared so that other flasks were left undisturbed in anoxia. Ethanol production was measured within 2 h periods at: (1) starting salinity 0-2 h; (2) stepping to NaCl final concentration (24-26 h); (3) in the middle of the salinity treatment (48-50 h) and (4) near to the end of salinity (70-72 h). Shoot and endosperm π_{sap} were measured at the end of NaCl exposure. The large numbers of flasks involved meant that the experiment was conducted at two different batches (i.e. two times), with each batch containing a full set of two replicates, so that overall, there were 4 replicates per treatment and sampling time.

Experiment 3. Responses of excised rice coleoptile tips in combined NaCl and anoxia

Approximately 0.1 g fresh weight of excised coleoptile tips (prepared as described above and in Appendix 4.1) were transferred into each conical flask for 5 h healing in hypoxic nutrient solution containing 20 mM glucose, prior to anoxia treatment. Glucose at 50 mM was given since starting anoxia, whereas NaCl was given 18 h after starting anoxia. There were three treatments, 0.3, 50 and 100 mM NaCl, each with 3 replicate flasks. The 100 mM NaCl treatments was stepped up as described in experiment 1. Ethanol measurements were conducted at the same time intervals as in experiment 2. This experiment was repeated also with 3 replicates, to have more accuracy on ethanol data. At the end of NaCl exposure (72 h), coleoptile tips were harvested for ion measurements and osmotic potential. The rinse procedure for ion

measurements used 2 mM $CaSO_4$ and iso-osmotic mannitol in anoxic solution for approximately 3 min, to remove ions in the free spaces and surface water that would have contained ions.

Analytical procedures

In experiment 1, shoot and endosperm fresh and dry weights were recorded at each sampling. The growth rate of the shoot of intact seedlings was calculated based on fresh weight increment per coleoptile (mg d^{-1} per coleoptile). Whereas in experiment 3 for excised coleoptile tips, fresh weight increment was calculated per gram fresh weight (mg g^{-1} FW d^{-1}). The seeds were considered as germinated when either the radicle or coleoptile had protruded by more than 2 mm.

In experiment 1, the net uptake or loss of K^+ was measured from the depletion of K^+ in the medium between sampling times, during recovery in non-saline aerated solution. In all experiments, Na^+, Cl^-, and K^+ concentrations both in intact seedlings and excised coleoptile tips were measured by extracting the tissues in 0.5 M HNO_3, placed on a shaker for 48 h in a dark room at 30^{o}C. Cl^- in the diluted extracts was measured using a Buchler-Cotlove Chloridometer (Buchler Instruments, Model 4-2008, Fort Lee, New Jersey, USA), Na^+ and K^+ were measured using a Flame Photometer (Corning Medical and Scientific, Model 410, Cambridge, UK). The Na^+, Cl^- and K^+ concentrations in reference tissue were within 87-95% of expected; the data were not adjusted.

The medium and tissue sap were assessed for osmotic potential (π_{sol} and π_{sap}). Sap was expressed from freeze/thawed coleoptile or endosperm samples. Coleoptiles or endosperm were blotted to removes surface solution, sealed in cryo-vials and frozen in liquid N_2. Samples were thawed while still in their vials and then crushed in a stainless steel press to extrude sap and this was immediately analysed. Sap and solutions were measured using a freezing point depression osmometer (Fiske 210 micro-sample/20 µl sample size) calibrated with standards.

In experiment 2 and 3, ethanol in the gas stream exiting flasks was trapped in vials containing deionized water in an ice bath connected to the outlet of each flask. All samples from medium and trap were stored at -20 ^{o}C prior to the assay. Ethanol was assayed in a 1 ml cuvette containing 100 mol m^{-3} glycylglycine buffer and containing 300 mol m^{-3} KCl, 1.7 mol m^{-3} NAD^+, aldehyde dehydrogenase and alcohol

dehydrogenase (adapted from Beutler, 1983). The reaction was monitored at 340 nm using a UV-visible spectrophotometer (Shimadzu, model UV-1601, Tokyo, Japan). The recovery of ethanol from checks containing no tissues but spiked with ethanol into the conical flask was more than 95%. Blank vials (contained nutrient solutions without rice tissues) were also included as a check.

Protein in shoot and endosperm was measured based on the Lowry et al., (1951) method, using folin phenol reagent and bovine serum albumin as standard. The assay was measured at 750 nm using the spectrophotometer described above.

Amino acids and organic acids in the coleoptiles were measured from tissues collected and frozen in liquid N_2. Tissues were freeze-dried, extracted twice with ice-cold 5% (w/v) perchloric acid, centrifuged at 4^{o}C, and following collection of the supernatants, K_2CO_3 was added to reach pH 3-5 (for amino acids) and pH <3 (for organic acids) using pH meter (Cyberscan pH 510, Serial no 140234, Eutech Instrument, Singapore). The precipitated potassium perchlorate was removed by centrifugation and collection of the supernatant. The extracts were analyzed using a HPLC [600E pump, 717 plus autoinjector, 996 photodiode array detector (PDA), Waters, Milford, MA, USA] method described by Cawthray (2003). The recoveries of the internal standards ranged between 92 – 104% of the expected values.

Soluble sugars in shoots and endosperms were extracted twice in 80% ethanol and by boiling under reflux for 20 minutes. Total sugars (hexose equivalent) in each sample were measured colorimetrically using anthrone (Yemm and Willis, 1954) by measuring the absorbance at 620 nm, using a glass cuvette in a UV-visible spectrophotometer (described above). Soluble sugar recoveries using glucose spiked into additional tissue samples prior to extraction were within 86-92%.

Statistical analyses of data

Data sets were analysed using Genstat 14 ed. All pairwise comparisons were tested using Duncan's multiple range test.

Results

Growth and ethanol production of intact rice seedlings submerged in different NaCl concentrations under anoxia

The following results from the recovery in aerated non-saline solution are evidence that rice seedlings were able to recover from NaCl exposure without having substantial injury. After transfering intact seedlings from saline anoxia to aerated-non saline solution, rates of fresh weight increments and K^+ uptake were 3.5-5 times higher in shoots which had been during anoxia at 100 mM than in shoots which had been at 0.3 mM NaCl. For fresh weight increments of shoots, the rates were 0.03 and 0.07 mg per shoot h^{-1} for previous anoxia at 0.3 and at 100 mM NaCl, respectively. Values for K^+ net uptakes were 0.1 and 0.5 μmol g^{-1} FW h^{-1}. By the end of anoxia and NaCl exposure, intact seedlings from all NaCl treatments had similar fresh weight and tissue K^+ concentrations. Thus, even the seedlings exposed to 100 mM NaCl were able to recover, so that the changes during anoxia as described below were not due to deterioration of the tissues.

During anoxia, shoot fresh and dry weight of rice seedling at 50 mM NaCl were similar to those at 0.3 mM NaCl, but about 20% higher than those of shoots at 75 and 100 mM NaCl (Table 4.1), and there was no difference in endosperm dry weight among NaCl treatments at the end of NaCl exposure. The water content of the shoot (± 95%) and endosperm (± 45-50%) during NaCl exposure in anoxia was not significantly different among seedlings in the various treatments (data not shown).

Table 4.1. Data of shoot and endosperm fresh weight and dry weight of rice seedling during 72 h NaCl exposure in anoxia (experiment 1). NaCl was given 18 h after starting anoxia and increased by steps of at most 50 mM NaCl. At 0 h NaCl exposure, the shoot fresh weight was 3.9 mg (the enclosed leaf fresh weight was 0.7 mg), whereas endosperm fresh and dry weights were 24.3 and 15.2 mg respectively. Data given are means ± SE, n=3. Superscripts indicate significant differences at P<0.05 (comparisons down columns, within parameter and organ).

	Fresh weight (mg)		Dry weight (mg)	
NaCl (mM)	**shoot**	**endosperm**	**shoot**	**endosperm**
0.3	6.1 ± 1.2 [a]	26.8 ± 0.9 [b]	0.23 ± 0.02 [a]	14.6 ± 1.6 [a]
50	5.5 ± 0.6 [a]	27.6± 0.5 [b]	0.27 ± 0.06 [a]	13.1 ± 1.1 [a]
75	4.4 ± 0.5 [b]	29.3 ± 0.5 [a]	0.18 ± 0.02 [b]	14.1 ± 0.8 [a]
100	4.4 ± 0.3 [b]	29.2 ± 1.3 [a]	0.17 ± 0.03 [b]	14.0 ± 0.3 [a]

During NaCl exposure, ethanol production per unit fresh weight was about 13-25% higher per intact seedling at 50 mM NaCl than in the non-saline controls. The period of this salt-induced catabolism started from 24 h after NaCl was imposed, and ended during 50-70 h (Table 4.2). After 70 h ethanol production became similar to that of tissues at 0.3 mM NaCl. This extra energy produced indicated that there was an increase of energy requirement for cell maintenance in intact seedlings during NaCl

exposure in anoxia. Ethanol production for intact seedlings was measured on a seedling basis (Table 4.2). To interpret these data, assessment has to be made of the ethanol production by the shoots and in the endosperms. This will be done in the Discussion.

Table 4.2. Ethanol production in rice seedlings per seedling basis, i.e. shoots + endosperms, with data from experiment 2. At the end of salinity, as with the fresh weight basis, the ethanol production calculated based on protein basis by adding the soluble protein concentration in both shoot and endosperm showed no significant difference among 0.3, 50 and 100 mM NaCl respectively. Data given are means ± SE, n=4.

Time (in anoxia/in salinity)	NaCl		
	0.3 mM	50 mM	100 mM
	Ethanol (μmol per seedling h^{-1})		
Start salinity step 1 (18-20 h/0-2 h)	0.11 ± 0.01 [a]	0.12 ± 0.01 [a]	
Start salinity step 2 (42-44 h/24-26 h)	0.16 ± 0.02 [b]	0.18 ± 0.03 [a]	0.17 ± 0.03 [a]
Middle salinity (66-68 h)/48-50 h)	0.14 ± 0.01 [b]	0.18 ± 0.02 [a]	0.09 ± 0.01 [c]
End salinity anoxia (88-90 h/70-72 h)	0.12 ± 0.01 [a]	0.10 ± 0.01 [a]	0.08 ± 0.01 [a]

K^+, Na^+ and Cl^- in shoot and endosperm

The results from experiment 2 with frequent ion samplings shows that after the second steps of increase in NaCl (given at 24 h), K^+ concentrations in shoots of seedlings at 50 and 100 mM NaCl dropped rapidly, then remained constant (~ 70 μmol g^{-1} FW) toward the end of NaCl-anoxic period (i.e. at 78 h after NaCl was imposed). In endosperm, K^+ concentrations decreased gradually during the exposure to NaCl (Fig. 4.1).

Na^+ concentrations in shoots increased with time at 50 and 100 mM NaCl and reached maximum levels of approximately 60 and 100 μmol g^{-1} FW, i.e. close to the concentrations at external solution for the seedlings at 50 and 100 mM NaCl. In endosperms, by contrast, the internal Na^+ concentrations were about 50% of those in the external medium. The rate of increase of internal Na^+ became less with time in salinity and had plateaued towards the end of the salinity treatment (Fig. 4.1). The Na^+ net uptakes in shoots at 50 and 100 mM NaCl were 1.9 and 5.1 μmol g^{-1} FW h^{-1} (~ 0.01 and 0.02 μmol g^{-1} per shoot h^{-1}) during the initial 24 h and net uptake had decreased to almost zero in the final period just prior to 78 h. The Na^+ net uptake in endosperm at 50 and 100 mM NaCl was ~ 1.3 μmol g^{-1} FW h^{-1} at initial hours and decreased to ~ 0.13 μmol g^{-1} FW h^{-1} in the final period just prior to 78 h.

Similar to Na^+ trends, internal Cl^- concentrations showed rapid increases early during the exposure to salinity, and then reached a near-steady state at about 54 h after starting salinity (approximately 60 and 90 μmol g^{-1} FW for the shoots at 50 and 100

mM NaCl respectively) (Fig. 4.1). The net uptakes of Cl^- in shoot during the initial 24 h of salt exposure were 0.7 and 3.7 µmol g^{-1} FW h^{-1} for 50 and 100 mM NaCl respectively. Cl^- net uptakes then decreased gradually to almost zero in the final period just prior to 78 h, both for seedlings in 50 and 100 mM NaCl. The Cl^- net uptake in both the shoot and endosperm which were 30-60% lower than the Na^+ net uptake at first 24 h could be due to the initial Cl^- concentrations in both shoot and endosperm at starting NaCl treatments which were higher than Na^+ concentrations in both organs.

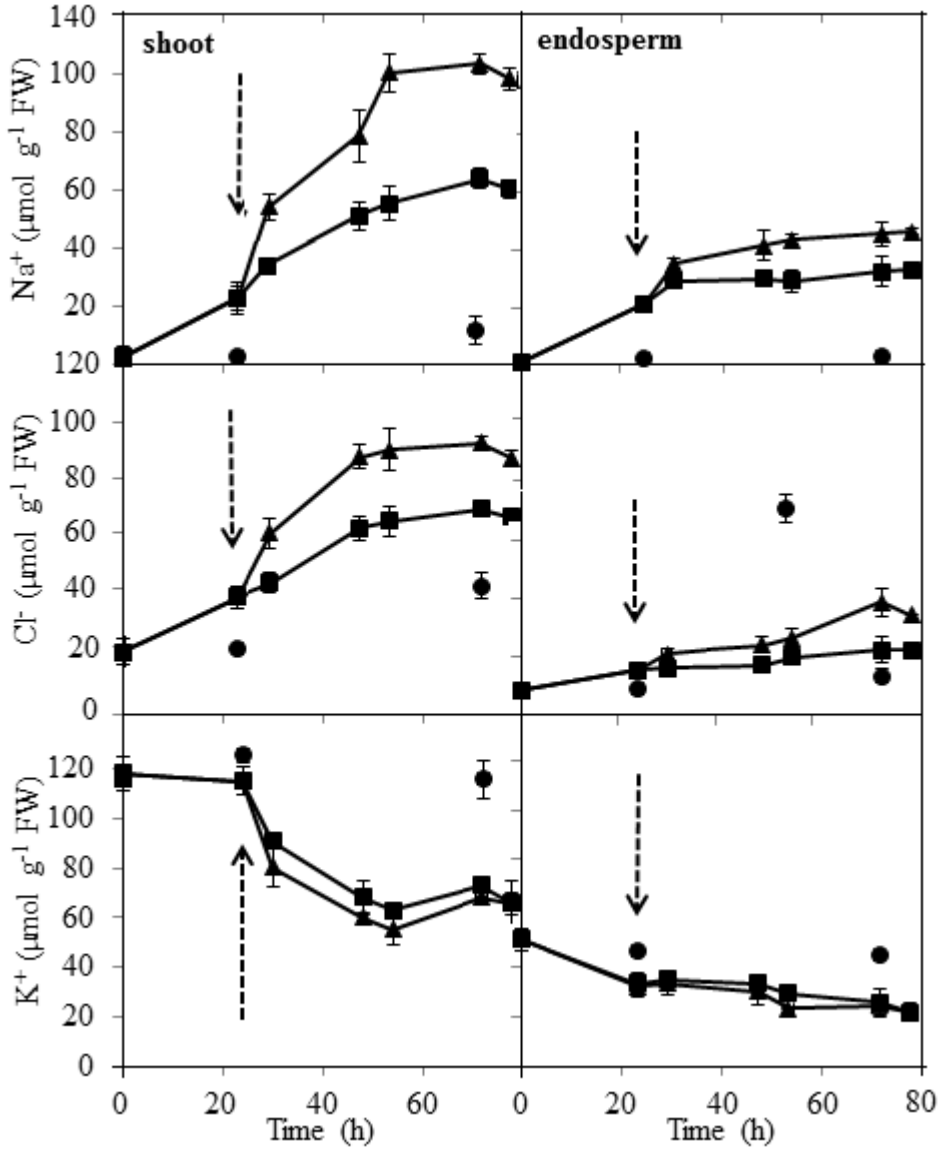

Figure 4.1. Na^+, Cl^- and K^+ concentration in the shoot (left) and endosperm (right) in rice seedlings in combined anoxia and salinity. Three levels of NaCl were given 18 h after starting anoxia. First step of NaCl was at time 0 h. Second step of NaCl was at the time shown by dashed arrow. Ion samplings were taken from intact seedlings in separate flasks (experiment 2). Na^+, Cl^- and K^+ concentrations at 0.3 mM, filled circle; 50 mM filled square; 100 mM filled triangle. Data given are means ± SE, n=4.

Sugars, organic acids and amino acids

Total sugar concentrations on a fresh weight basis both in endosperm and in shoots remained constant within 24 h at both 0.3 and 50 mM NaCl (Table 4.3). There was ~20% increase in amino acids per FW basis; i.e. alanine and GABA in rice shoots at 0.3 mM NaCl during 24 h after starting treatments (i.e. 42 h in anoxia). The ~30% lower amino acids at 50 mM NaCl per FW basis was due to dilution by growth. The values based on content per seedling showed that the increase of organic and amino acids during 24 h in salinity was not significant both at 0.3 and 50 mM NaCl, and indicated that there was hardly any organic solutes synthesised during the first 24 h of NaCl exposure (Table 4.3).

Table 4.3. Sugar, organic acids (OAs) and amino acids (AAs) concentrations and contents (values in brackets shows content per seedling of rice seedlings in 0 and 24 h salinity at 0.3 and 50 mM NaCl (experiment 1). NaCl exposure was started after 18 h starting anoxia. Data given are means ± SE, n=3. Values in sugar concentrations, as well as amino acids and organic acids contents per seedling with time (across row) are not significantly different at P<0.05, both at 0.3 and 50 mM NaCl.

Solutes	**Sugar, AAs and OAs**					
	0 h		**24 h**			
			0.3 mM NaCl		**50 mM NaCl**	
Soluble sugar	μmol g^{-1} FW (μmol per seedling)					
Shoot	22		26		25	
Endosperm	177		192		189	
AAs						
Alanine	26.4	(0.18)	31.4	(0.19)	19.3	(0.19)
Gaba	13.8	(0.07)	16.7	(0.09)	7.0	(0.07)
Asparagine	2.1	(0.02)	2.6	(0.02)	1.6	(0.02)
Others	3.5	(0.07)	6.6	(0.08)	3.1	(0.07)
Total AAs	46	(0.35)	57	(0.37)	31	(0.36)
OAs						
Malic acids	5.0	(0.03)	5.3	(0.03)	2.8	(0.03)
Others	0.3	(0.01)	0.3	(0.01)	0.2	(0.01)
Total OAs	5.3	(0.04)	5.6	(0.04)	3.0	(0.04)

Excised coleoptile tips, a comparison with intact seedlings for growth rate, ethanol production, K^+/Na^+ ratio and ion contribution to π_{sap} in salinity during anoxia

Different growth rate between intact seedling and excised coleoptile tips

The growth rate of shoots of intact seedlings in anoxia and NaCl at 50 and 100 mM decreased by 10 and 31%, respectively, compared to that in 0.3 mM NaCl. Excised coleoptile tips however, showed different growth response from those of shoots of

intact seedlings at the same conditions. The growth of excised coleoptiles tips at 50 mM NaCl in anoxia declined by 74% compared to growth in 0.3 mM NaCl, and at 100 mM NaCl, there was no fresh weight increments of the excised coleoptile tips. Instead, the final fresh weight had decreased by ~ 15% of the initial fresh weight at 0 h, when NaCl was imposed (Table 4.4). The excised coleoptile tips water content in these different saline treatments were 99, 96 and 95% for 0.3, 50 and 100 mM NaCl, respectively.

Table 4.4. The fresh weight increment (mg d^{-1}) of the shoot of rice seedling and excised coleoptile tips during 0 to 72 h in NaCl exposure in anoxia. Data from experiments 1 and 3. Data given are means ± SE, n=3.

		NaCl concentration (mM)		
		0.3	**50**	**100**
Anoxia	Intact shoot (coleoptile + leaf) (mg d^{-1} per shoot)	1.37 ± 0.32	1.23 ± 0.16	0.94 ± 0.13
	Excised coleoptile tips (mg g^{-1} FW d^{-1}) *)	23.04 ± 4.3	5.9 ± 0.9	-42.7 ± 1.6

*) Since the weight of a single excised coleoptile tip is about 5 mg, this means that 1 g FW contains about 200 excised coleoptile tips. The increment of 23 mg g^{-1} d^{-1} FW excised coleoptile tips at 0.3 mM NaCl will be approximately similar to 0.12 mg FW increment in each excised coleoptile tips d^{-1}, or about 10% of the FW increment of the intact shoots of seedlings in anoxia. Using similar calculation, the decrease of excised coleoptile tip fresh weight at 100 mM NaCl by 42.7 mg g^{-1} FW d^{-1} implied that ~ 0.2 mg g^{-1} FW d^{-1} solutes and water had been losts to the external medium from each excised coleoptile tip at 100 mM NaCl.

Ethanol production in excised coleoptile tips and intact seedlings

The comparison of ethanol production by intact seedlings and excised coleoptile tips on a FW basis is presented in Table 4.5. The ethanol production of excised coleoptile tips (µmol g^{-1} FW h^{-1}) was approximately twice of the production in intact seedlings also on a FW basis. At 24 h after NaCl was imposed, the ethanol production per fresh weight basis of both intact seedlings and excised coleoptile tips at 50 mM NaCl were approximately 8-15% higher than the production at 0.3 mM NaCl. At the end of salinity and anoxia (72 h), the production at 50 mM NaCl was still high in excised coleoptile tips, but had decreased in intact seedlings. In both materials, ethanol productions were significantly lower at 100 mM NaCl than at 0.3 and 50 mM NaCl, especially after 48 h in salinity (Table 4.5).

Table 4.5. Ethanol production (μmol g^{-1} FW h^{-1}) of intact rice seedlings and excised coleoptile tips (experiment 2 and 3). Data given are means of replicates ± SE. Superscripts indicate significant differences at $P < 0.05$ (comparisons across row, within time interval).

Trapping time (anoxia/ salinity)	NaCl treatments (mM)					
	0.3		50		100	
	Ethanol production (μmol g^{-1} FW h^{-1})					
Intact seedlings						
Start salinity step 1 (18-20 h/0-2 h)	3.21	± 0.2 ^{a}	3.18	± 0.3 ^{a}	-	
Start salinity step 2 (42-44 h/24-26 h)	4.39	± 0.2 ^{b}	4.75	± 0.1 ^{a}	4.78	± 0.2 ^{a}
Middle salinity (66-68 h)/48-50 h)	3.96	± 0.2 ^{b}	4.68	± 0.4 ^{a}	2.71	± 0.2 ^{c}
End salinity anoxia (88-90 h/70-72 h)	3.48	± 0.7 ^{a}	2.73	± 0.8 ^{a}	2.15	± 0.5 ^{a}
Excised coleoptile tips						
Start salinity step 1 (18-20 h/0-2 h)	7.99	± 1.5 ^{a}	6.58	± 0.9 ^{a}	-	
Start salinity step 2 (42-44 h/24-26 h)	7.71	± 0.2 ^{b}	8.38	± 0.3 ^{a}	5.48	± 1.1 ^{c}
Middle salinity (66-68 h)/48-50 h)	6.2	± 0.3 ^{b}	7.23	± 0.2 ^{a}	5.31	± 0.4 ^{c}
End salinity anoxia (88-90 h/70-72 h)	6.08	± 0.3 ^{b}	6.78	± 0.1 ^{a}	3.56	± 0.4 ^{c}

K^+/Na^+ ratio in shoot and endosperm of intact seedlings, compared to excised coleoptile tips

Similar to K^+ concentrations, K^+/Na^+ ratio in shoot of intact seedlings decreased sharply 24 h after NaCl was imposed, then remained constant during ~ 50 – 78 h. This ratio in endosperm decreased only gradually during 24 -78 h after starting NaCl treatment.

Different from intact seedlings at 100 mM NaCl which had K^+/Na^+ ratio (0.5 – 0.8) at the end of the NaCl-anoxia period, K^+/Na^+ ratio of excised coleoptile tips at 100 mM had dropped to 0.05. At 50 mM NaCl however, K^+ concentration in excised coleoptiles tips was not different from that at 0.3 mM NaCl, therefore K^+/Na^+ ratio in excised coleoptile tips at the end of NaCl exposure was only slightly lower than that in shoots of intact seedlings (0.7 in excised coleoptile tips and 1.1 in shoots of intact seedlings) (Table 4.6).

Table 4.6. K^+/Na^+ ratio in shoot of intact seedlings and in excised coleoptile tips at 0 and 72 h after NaCl was imposed in anoxia (experiment 2).

K^+/Na^+	Time	NaCl treatments (mM)		
	(h)	0.3	50	100
Coleoptile + any leaves	0 h	54		
	72 h	9.5	1.1	0.7
Excised coleoptile tips	0 h	42		
	72 h	8.2	0.7	0.05

Contributions of ions and organic solutes to π_{sap} in shoots of intact seedlings and excised coleoptiles tips

High external NaCl concentrations increased the contribution of Na^+, K^+ and Cl^- to π_{sap}, both in intact seedlings and in excised coleoptile tips. At 0.3 mM NaCl, the contribution of these ions to π_{sap} in the shoot of intact seedling was ~40% higher than that in excised coleoptile tips. In this non-saline condition, ions contributed 54% of the total π_{sap}, of which K^+ contributed 60-70% of the total of the three ions to $\pi_{sap,}$ both in the shoot of intact seedlings and in excised coleoptile tips. At 50 mM NaCl, the contribution of K^+ to total ions π was about 40% in the shoot of intact seedlings and 30% in excised coleoptile tips. At 100 mM NaCl however, K^+ contribution to total ion π decreased to become 28 and 3%. The less contribution of K^+ in excised coleoptile tips, which presumably due to Na^+ induced membrane depolarization required organic solutes to be produced to maintain the low $\pi_{sap.}$ However, the accumulation of amino acids in the excised coleoptile tips in anoxia was not possible because coleoptiles typically acquire these via their translocation from the endosperm. Since net protein hydrolysis did not occur in rice coleoptiles (Mocquot et al., 1981), therefore excised coleoptiles were unlikely to produce sufficient organic acids or amino acids to compensate the different π_{sap} due to lower ability to retain K^+.

Contribution of K^+ to total ions π in endosperm of intact seedling was similar to that in shoot of intact seedlings. However, contribution of ions to total π_{sap} measured in endosperm was less than half of that in shoot. Since the concentrations of organic acids in rice endosperm under anoxia is less than 10% of that in shoot (Avadhani et al., 1978), the major contribution to π_{sap} in endosperm presumably came from sugars which contributed about -0.5 MPa at 24 h after starting salinity and would be even higher at 72 h at the end of salinity.

At 0.3 mM NaCl the π_{sap} of the excised coleoptile tips were less negative than in the intact seedlings, although excised coleoptile tips were in glucose with a π_{sap} of -0.12 MPa (Table 4.7). Consequently, the estimated P was lower in the excised coleoptile tips though it was still within the usual range of anoxic rice coleoptiles (Atwell et al., 1982). Further the estimated P was lower at 50 mM NaCl than at 0.3 mM NaCl and still lower at 100 mM NaCl. However, only the excised coleoptile tips at 100 mM NaCl showed extremely low turgor pressures, presumably since they had lost a lot of K^+. The calculation of $\Delta\pi_{sap}$ (π_{sap} at salinity - π_{sap} at non-saline condition) derived from Table 4.7, i.e. the measure used for shoot tissues in air and taken as a measure of 'osmotic

adjustment' (Munns, 1988), showed partial, not complete osmotic adjustment in both shoots and excised coleoptile tips, since $\Delta\pi_{sap}$ at 50 and 100 mM NaCl were 25-50% less negative than $\Delta\ \pi_{sol}$.

Table 4.7. Osmotic potential (π) contributions from ions in rice shoots, endosperm and excised coleoptile tips in combined salinity and anoxia (experiments 1 and 3). The sampling was taken at the end of salinity and anoxia. NaCl was given 18 h after starting anoxia, for a period of 72 h. Glucose 50 mM (π = 0.12 MPa) was only added in the medium for excised coleoptile tips. Values in brackets show the percentage contribution compared to total π measured.

NaCl (mM)		Intact seedling				Excised Coleoptiles	
		Shoot		Endosperm			
		π (MPa)		π (MPa)		π (MPa)	
0.3	Total π_{sap}	-0.69		-0.77		-0.58	
	Estimated turgor (P)	0.66		-		0.43	
	Total ions [1)]	-0.37	*(54)*	-0.17	*(22)*	-0.18	*(31)*
	Soluble Sugar	-0.07	*(10)*	-0.50	*(65)*	-	
	AAs and OAs	-0.25	*(36)*	-		-	
	π_{sol}	-0.01		-0.01		-0.13	
50	Total π_{sap}	-0.74		-0.90		-0.71	
	Estimated turgor (P)	0.52		-		0.36	
	Total ions [1)]	-0.52	*(70)*	-0.19	*(21)*	-0.41	*(58)*
	Soluble Sugar	-0.06	*(8)*	-0.50	*(56)*	-	
	AAs and OAs	-0.16	*(22)*	-		-	
	π_{sol}	-0.22		-0.22		-0.35	
100	Total π_{sap}	-0.85		-1.07		-0.68	
	Estimated turgor (P)	0.40		-		0.10	
	Total ions [1)]	-0.63	*(74)*	-0.29	*(27)*	-0.47	*(69)*
	π_{sol}	-0.45		-0.45		-0.58	

Discussion

Growth and catabolism rate of intact seedlings exposed to NaCl and anoxia

The rapid resumption of growth and K^+ uptake after return to non-saline aerated solutions showed that there was at most very little irrepairable injury after 72 h exposure of rice seedlings to the taxing combination of anoxia and up to 100 mM NaCl. Establishment of recovery is critical, since otherwise effects during anoxia may be consequences of serious injury, not present acclimative responses (Thomson and Greenway, 1991). This confirmation that there was usually no serious injury reinforces the reputation of the high tolerance to anoxia of rice seedlings, i.e. despite the energy crisis, the anoxic intact seedlings readily survived the additional exposure to 100 mM

NaCl. Reasons why this tolerance broke down in excised coleoptile tips at 100 mM will be discussed later in this section.

An increase in the rate of cell catabolism (~0.54 μmol ethanol production g^{-1} FW h^{-1}) during 24-50 h after exposed to NaCl with no significant increase in shoot fresh or dry weight at 50 mM NaCl was likely to be associated with the energy requirement for cell maintenance. The energy cost of maintenance is presumed to be greater under high concentrations of NaCl, as a result of requirements for ion compartmentation and ion extrusion, as well as possibly for repair of cellular damage (Flowers et al., 1985). After a period of stimulated ethanol production, however, there was no significant difference of ethanol production among NaCl treatments both or on a fresh weight and protein basis at the final few hours at end of salinity and anoxia (70- 72 h). The increase of catabolism rate on a fresh weight basis will be discussed further, in comparison with catabolism by excised coleoptile tips in a later section. In contrast to 50 mM NaCl, seedlings at 75 and 100 mM NaCl only showed significant stimulation of catabolism during the first two hours after the NaCl was increased to 75 or 100 mM. The shoot growth decrease by ~0.45 mg d^{-1} indicates that at these higher NaCl treatments, less energy will be spent on growth and so will become available for maintenance. Alternatively, this slow growth required less energy and therefore no more catabolism stimulation occurred after 2 h NaCl final step being imposed, as indicated by the reduction in the ethanol production at 100 compared to 0.3 mM NaCl. The indication that ethanol formation can be down regulated is consistent with the hypothesis of Huang et al. (2005) that rice coleoptile tips, even under an energy crisis, glycolysis (as gauged from ethanol formation) is regulated depended on the energy requirements of the tissues.

In the present experiment there was no separation of the ethanol produced by the shoot and by the endosperm. However, Setter et al. (1994) showed rice endosperm produced ~40% ethanol less than the shoots (on fresh weight basis). This low ethanol formation in endosperm might be associated with lower activities of PDC and ADH enzymes in rice endosperm than in shoots (~30% less for PDC and ~90% less for ADH in protein basis) (Guglielminetti et al., 2001).

K^+, Na^+ and Cl^- in shoot and endosperm of intact seedlings

Despite the energy crisis, there were no large differences in ion relations under salinity compared to the well-established behavior of most plant tissues under aerated conditions. This conclusion can be best seen from the changes in K^+ and K^+/Na^+ ratios.

The decrease of K^+ after the addition of high NaCl occurred for the endosperm when NaCl was raised to 50 mM, but only for shoots when NaCl was increased further to 75-100 mM NaCl (Fig. 4.1). The similar decrease of K^+ concentrations by shoots at 50 mM NaCl was mostly due to the ~20% higher fresh weight increment at 50 mM NaCl than at 100 mM NaCl during the rapid uptake of Na^+ and Cl^- (24-48 h NaCl exposure) which then diluted the ~22% higher K^+ content in shoots at 50 mM NaCl than at 100 mM NaCl and lead to a similar K^+ concentrations between seedlings at 50 and 100 mM NaCl. The decrease of K^+ concentrations was at the same time that Na^+ in tissues sharply increased (Fig. 4.2). Due to the similarity in physicochemical properties between Na^+ and K^+ (i.e. ionic radius and ion hydration energy), K^+ and Na^+ are known to compete for uptake sites at the plasma membrane (Kurth et al., 1986; Cramer et al., 1987; Marschner 1995; Shabala et al., 2006), including both low-affinity (non-selective cation channels) and high-affinity (e.g. HKT) transporters (Shabala and Cuin, 2007). All the same in most species, high external concentration of Na^+ will cause a displacement of K^+ from the transport systems.

This internal K^+ concentration came to a new quasi-steady state (~60 – 70 µmol g^{-1} FW) at 54 h after start of the NaCl treatments when Na^+ and Cl^- concentrations also reached a quasi-steady state at 60 µmol g^{-1} FW in rice seedlings at 50 mM NaCl. Interestingly, this quasi-steady state condition coincided with ethanol formation dropping to the same rate as for seedlings at 0.3 mM, suggesting less energy was required for cell maintenance. The relationship between this quasi-steady state and the energy requirement will be discussed further in Chapter 6, Concluding discussion.

Responses of excised coleoptile tips to a combination of NaCl and anoxia, in comparison to shoots of intact seedlings

The shoot of intact seedlings is not strictly comparable to the excised coleoptile tips, because apart from the shoot consisting of coleoptile and any enclosed leaves, which is not so for the coleoptile tips, the basal part of whole coleoptile is also not included in the excised coleoptile tip. Studies on ultra-structure of rice embryo

development showed that the coleoptile is more vacuolated than the primary leaf (Jones and Rost, 1989; Taylor and Vasil, 1995; Maeda et al., 2002). Having these biological differences, shoots of intact seedlings and excised coleoptile tips might respond differently to anoxia and or salinity.

Despite these difficulties, some comparisons between intact seedlings and excised coleoptile tips remain possible. This comparison is aided by the measured ion concentrations in coleoptile and leaf from Chapter 3, as well as data from Setter et al. (1994). Setter et al. (1994) established different rates of ethanol formation between coleoptile, seed and leaves, as well as between basal and apical parts of the coleoptile of rice. Thus, the possible different responses between shoot of intact seedlings and coleoptile tips to NaCl exposure could be evaluated.

The inhibition of growth in excised coleoptile tips at 50 mM NaCl was not found in shoots of intact seedlings. No growth rate inhibition in intact seedlings at 50 mM NaCl indicated that extra energy produced during salt-induced catabolism, deducted from comparison with ethanol production by seedlings at 0.3 mM NaCl (0.4-0.7 µmol ATP g^{-1} FW h^{-1}) was required not only for cell maintenance, but also for maintaining growth. In excised coleoptile tips, the extra energy (~ 0.7 - 1 µmol ATP g^{-1} FW h^{-1}) produced at 50 mM NaCl was presumably supplemented by reduced energy requirements for growth, which was inhibited by ~74% compared to 0.3 mM NaCl. The details of this energy requirement in excised coleoptile tips at 50 mM NaCl will be evaluated further in Chapter 5 of this thesis.

Ethanol production and different substrate available for intact seedlings and excised coleoptile tips

The rate of ethanol production (µmol ethanol per seedling) by coleoptiles + embryo was reported at ~40% of the rate in intact seedlings whereas the rate in the coleoptile was ~70% of the total coleoptile + leaf ethanol synthesis rate (Setter et al., 1994). The highest rate of ethanol synthesis within coleoptiles was also associated with the basal segments which had ~3-fold higher ethanol production rate (on fresh weight and protein basis) than the apical segments (Setter and Ella, 1994; Setter et al., 1994). By considering the proportion of FW in coleoptile, leaf and endosperm (caption Table 4.1) and the total ethanol production rate by intact seedlings, the rate of ethanol production by coleoptile, leaf and endosperm could be estimated separately. With an average production rate of 4 µmol ethanol g^{-1} FW h^{-1} in intact seedlings, the rate of

ethanol production (µmol g^{-1} FW h^{-1}) can be estimated at ~7.4 in 'whole coleoptile', at ~ 17 in leaf and at ~3.0 in endosperm. The rate of approximately 7.4 µmol ethanol g^{-1} FW in the 'whole coleoptile' came from basal and apical segments which have different rates of ethanol synthesis. The basal segments of 'whole coleoptiles' which was avoided in excised coleoptile tips (to avoid leaves) have a 3-fold higher ethanol synthesis rate compared to the rate in coleoptile tips (based on FW and protein, Setter et al., 1994). By considering the fresh weight of these basal segments (10-20% of the 'whole coleoptile' Setter et al., 1994), this rate could be estimated at ~18 µmol g^{-1} FW h^{-1} for basal and ~6.2 µmol g^{-1} FW h^{-1} for apical segments, respectively.

Using the assumption that the extra ethanol produced by intact seedlings at 50 mM NaCl came from shoots and endosperm with the same composition as described above (40 and 60% were produced from shoots and endosperm, respectively), the extra energy produced could be estimated as approximately 0.9 µmol g^{-1} FW h^{-1} was produced in shoots and 0.4 µmol g^{-1} FW h^{-1} was obtained from endosperm. Again, this estimated ethanol production from shoot was close to the value of the extra energy produced by excised coleoptile tips at 50 mM NaCl compared to those at 0.3 mM NaCl (0.7 – 1 µmol g^{-1} FW h^{-1}).

There are some possibilities related to the absence of salt induced catabolism in intact seedlings at 100 mM NaCl: (i) slower starch degradation caused substrate limitation, (ii) inhibition on transportation of sugar to the shoot, (iii) sugar in the shoots was needed for osmotic adjustment, (iv) low growth at 100 mM NaCl required less energy associated with growth. Unfortunately, there are no sugar data available for endosperm and shoots at 100 mM NaCl. So, at present it is impossible to choose between the four possibilities.

In anoxia, the metabolism of rice seedlings allows protein synthesis (Mocquot et al., 1981) as well as increases the activity of glycolytic and fermentative enzymes, ADH and PDC (Ricard et al., 1991; Guglielminetti et al., 2001). These rice seedlings are still attached to their endosperms which have starchy reserves, therefore those metabolism under anoxia seem to relate with the role of endosperm as their reserve. Anoxic rice seedlings possess enzymes that allow an efficient metabolism of starch and sucrose to fructose-6-phosphate. The sucrose metabolism in anoxic rice seedlings takes place mainly through sucrose synthase pathway which results in a considerable ATP saving when compared with the invertase pathway (Ricard at al., 1991; Guglielminetti et al., 2001). In contrast to shoots of intact seedlings, in excised coleoptile tips, the only

available substrate was glucose which was added to the external solution. Therefore, more ATP was presumably spent by excised coleoptile tips for sugar catabolism.

Na^+, Cl^- and K^+ concentration and contribution to π_{sap}

Na^+, Cl^- and K^+ concentrations of shoot of intact seedling were slightly different from those in excised coleoptile tips. K^+ concentration in shoot at 0 h salinity was ~2-fold higher than that in excised coleoptile tips, which might be due to K^+ translocation from endosperm to the intact shoot. However, at later stage on anoxia, the K^+ concentration was almost similar in intact shoots and excised coleoptile tips. Meanwhile, Na^+ concentration in shoots of intact seedlings (~ 60 µmol g^{-1} FW) was slightly lower than the concentrations in excised coleoptile tips (~80 µmol g^{-1} FW); in both cases, at later anoxia. This difference in Na^+ concentrations might be associated with the different biological structure of both materials. The anatomy of coleoptile cells which are more vacuolated (Jones and Rost, 1989; Taylor and Vasil, 1995; Maeda et al., 2002) could enable these tissues to sequester more Na^+ and Cl^- than leaves of intact seedlings. Results from Chapter 3 also show that coleoptiles have approximately 20% higher Na^+ and Cl^- concentrations than leaves. Since shoot of rice seedlings consists of leaves and coleoptile, the total concentrations were presumably lower than in excised coleoptile tips (without leaves).

Intact shoots and excised coleoptile tips behaved differently in their responses to low osmotic potential (π_{sap}) in anoxia. In shoots of intact seedlings, the increased net uptakes of Na^+ and Cl^- raised contribution of these ions to π_{sap} by -0.15 to -0.2 MPa and reduced the contribution from organic solutes by ~-0.1 MPa. Ion contribution to π_{sap} in excised coleoptile tips which was only -0.41 MPa (58%) of total π_{sap} measured compared to -0.52 MPa (70%) in shoots, was presumably compensated by almost 2-fold higher soluble sugar concentrations in excised coleoptile tips (refer to data in Chapter 5:~ 57 µmol g^{-1} FW) than in the shoots (~26 µmol g^{-1} FW). Although the π_{sap} measured in both intact seedlings and excised coleoptile tips were similar (-0.7 MPa), addition of exogenous glucose to the medium with excised coleoptile tips decreased the π_{sol} by -0.13 MPa. Thus, the estimated turgor pressure at 50 mM NaCl in excised coleoptile tips was slightly less than that in shoots of intact seedlings (0.4 and 0.5 MPa for excised coleoptile tips and shoots, respectively). This incomplete turgor maintenance, i.e. P declines, was presumably due to the less decrease of π from total

ions than the decrease of π in the external medium. P in excised coleoptile at 100 mM NaCl dropped to only -0.1 MPa.

Transport of endogenous K^+ from caryopses to coleoptiles was inhibited less by anoxia than net K^+ uptake from the solution (Huang et al., 2005), so it was perhaps to be expected that comparing the osmotic adjustment between rice shoots and excised coleoptile tips in anoxia, almost 38% of the π_{sap} of shoots was derived from K^+, whereas in excised coleoptile tips, K^+ contribution to π_{sap} was only ~18%. Excised coleoptile tips could not survive in 100 mM NaCl, since the main contributor for the π_{sap} in anoxia, i.e. K^+ which is mostly transported from endosperm, was less likely to be available by the following mechanisms. First, strong membrane depolarization caused by high Na^+ uptake favors K^+ loss via depolarization-activated outward-rectifying K channels (Shabala et al., 2006) and led to a low π_{sap} and consequently low turgor pressure. Alternatively, the inability of excised coleoptile tips at 100 mM NaCl to adjust their π following the Δ of external π further might have resulted in membrane depolarization which lead to possible leakage of K^+ to the external medium, and caused a low tissue K^+/Na^+ ratio (approximately 0.05). Since Na^+ internal was about similar to Na^+ external, it looks more likely that the decrease in K^+ caused the decrease in turgor than *vice versa*.

Conclusions

Despite an energy crisis with less than 10% in energy production of that in air, rice seedlings could survive under combination of anoxia and 100 mM NaCl with only ~25% reduction in shoot growth as compared with in non-saline anoxia. K^+/Na^+ ratio in the shoot was maintained presumably by translocation of K^+ from endosperm, whereas turgor pressure was developed mostly by the contribution of ions ~70% to π_{sap}. Different from intact seedlings, excised coleoptile tips showed signs of injury at 100 mM NaCl, indicated by a failure to maintain turgor, a loss of K^+ and sharply decreased K^+/Na^+ ratio.

During 24-70 h after 50 mM NaCl was exposed, an increase of ethanol production both in intact seedlings and excised coleoptile tips indicated a higher energy requirement for cell maintenance, which might be associated with a period of high Cl^- uptake and Na^+ exclusion (i.e. extrusion) prior to the quasi-steady state. In excised coleoptile tips, energy consumption would also have been reduced by~90% in the already slow growth at 0.3 mM NaCl. Yet, these excised coleoptiles at 50 mM NaCl

had K^+/Na^+ ratio and ethanol production which were almost similar to those of the shoots of intact seedlings. Thus, excised coleoptile tips will be a useful system to conduct experiments to measure ion fluxes and rate of ethanol production by this specific tissue as long as 50 mM NaCl is the highest salinity treatment imposed. As well, interpretation can be facilitated because the excised coleoptile tips have little or no growth and certainly less than the shoot of intact seedlings.

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Appendix 4.1.

Experimental design

The effect of a combination of NaCl and anoxia was evaluated on intact rice seedlings and excised coleoptile tips. Before conducting experiment on the interaction of salinity and anoxia, some factors have to be considered. First, assuming anoxia aggravates adverse effects of salinity, NaCl concentration for this experiment might need to be lower than those used in studies solely on salinity. A preliminary experiment on the combination of salinity and anoxia showed that 100 and 200 mM NaCl given at the start of experiment resulted in severe inhibition of coleoptile growth. Having reasonable coleoptile growth is important in conducting further experiments using

excised coleoptile tips (i.e. providing enough material to excise). Therefore, three levels of NaCl were tested in the present experiments, i.e. 50, 75 and 100 mM.

Second, the timing of the start of hypoxia would determine the extent of coleoptile elongation. Combination treatment which resulted in shorter coleoptiles was avoided. Hypoxia was started at 30 h after sowing or when the coleoptile length is about 2-3 mm. Hypoxia prior to this stage resulted in growth delay, while after this stage, there would be more root growth. Since salinity delays germination, there would be a time lag in saline treatments. So, to enable starting of hypoxia for coleoptiles at the same stage of development, hypoxia should be started before applying NaCl.

Third, there are some advantages if NaCl were given at sowing: i.e. simpler and probably broader effect on rice coleoptile growth by longer NaCl exposure. However, measuring Na^+ and Cl^- net uptake since the first hour in the combined of NaCl and anoxic treatments is only possible if NaCl is given after starting anoxia. Moreover, responses of rice seedlings would be easier to interpret when the treatments were started on uniform seedlings. Thus, in these experiments, NaCl was given during anoxia.

Fourth, using intact seedlings vs excised coleoptiles includes some advantages and disadvantages for each material. The use of coleoptile tips as a model system was previously highlighted in the published literature (Colmer et al., 2001; Huang et al., 2003; Huang et al., 2005). Excised coleoptiles have some advantages: first, there is no doubt on where the ethanol comes from. Second, sugar availability is easily manipulated by glucose feeding. Third, since growth is less, catabolism rate could be measured directly by ethanol production and used to infer rates of ATP synthesis required for maintenance. On the other hand, when ion mobilization and accumulation are part of the study, intact seedlings would be a better option. Besides, intact seedlings will be more relevant to field situations. In the first and second experiments intact seedlings were selected. The survival mechanism of intact seedlings is much more complex than excised coleoptiles, since there are ion translocations among root, endosperm and shoot which may all be important for cell maintenance. The fast growing of shoots and roots in intact seedlings may also mitigate increases in Na^+ and Cl^- concentrations. However, when the measurements included ethanol production, with intact seedlings one could not distinguish ethanol produced by coleoptile or endosperm. Also in studying ion uptake, ion uptake from medium is also confounded in intact seedlings, since ions can flow from endosperm to shoot. Homologous tissues

were therefore required to assist interpretations, and the third experiment was conducted using excised coleoptiles tips.

Appendix 4.2.

Combination of aeration-hypoxia period to obtain a longer coleoptile with less leaves inside: improvement of the excised coleoptile tip system

An experiment was conducted to obtain good material of rice coleoptiles: i.e. long coleoptiles with less leaves. A combination of aeration-hypoxia period was applied for this purpose. The seeds were germinated and grown in aerated solution (containing 0.25 mol m^{-3} O_2) for 48, 36 or 24 h, prior to being exposed to 18, 30 or 42 h hypoxic pretreatment (0.06 mol m^{-3} O_2). The experiments included anoxia treatments and then re-aeration, Cl^- at 0.25 mM was added to the basal medium, to test for energy dependent anion uptake, as vigorous uptake would indicate no permanent injury. Recovery of K^+ and Cl^- uptake by excised coleoptiles tips following 90 h of anoxic treatment were assessed.

Data from this experiment showed that different lengths of time in aerated and hypoxic conditions also resulted in different rice coleoptile growth characteristics. Applying aeration for 48 h produced longer primary leaves and higher seedling fresh weight, compared to those of other treatments with shorter aeration period; but this was not desirable as the focus of this study was on the coleoptile tissues.

Tissue K^+ concentrations in the coleoptile tips in all treatments at the end of hypoxia period were in the range of 101-122 µmol g^{-1} FW, and there was no significant difference among tissues from the various hypoxia-time treatments. Similar to this, at the end of recovery at 24 h, all coleoptile tips also showed similar K^+ concentration, at approximately 80 µmol g^{-1} FW. In re-aeration recovery when Cl^- was given to the basal medium to test for energy-dependent anion uptake, all coleoptile tips performed vigorous uptakes which indicated no permanent injury has occurred. The Cl^- total uptakes during recovery was 35-51 µmol g^{-1} FW, and resulted in the final internal concentrations of 50–60 µmol g^{-1} FW.

A question which could be raised here is whether the inhibition of growth of primary leaves is due to the hypoxia, or period of aeration prior to hypoxia. According to Atwell et al. (1982), the first 2-5 mm of coleoptile growth was shown to be most intolerant to O_2 deficits, while later stages of coleoptile elongation were unaffected by

low O_2 supply. The processes specific to germination and early stages of coleoptile elongation (possibly cell division) would have greater O_2 requirements than cell extension. Coleoptile elongation is due largely to cell extension. Cell division requires DNA, RNA and protein synthesis, all relatively high-energy processes. In view of the low energy yield from anaerobic metabolism, it is possible that synthesis of these compounds may be inhibited in O_2 deficits. Therefore the earlier low O_2 was applied (0/66 h and 24/42 h), the less opportunity rice seeds have to complete the cell division. Cell division is suggested to be complete in about 10 mm coleoptiles length (Atwell et al., 1982). In this experiment, ~ 10 mm was obtained in about 48 h after sowing in aeration (Fig 4A2.1). Exposing hypoxia after this may less inhibit the growth of the coleoptile, but might also have enabled commencement of growth of the primary leaves. The insensitivity is probably associated with a shift from cell division to cell elongation. Therefore, 48 h aeration prior to hypoxia resulted to the primary leaf to coleoptiles length ratio is about 40-50%. The less inhibition on primary leaves growth stimulated coleoptiles to open their tips to allow leaves to emerge (Fig 4A2.1). This was not found for seedlings in shorter aeration period. Therefore it is suggested that less primary leaf growth is due to the shorter aeration, rather than longer hypoxia. The lengths of time in hypoxia may determine the coleoptiles length (Table 4A2.1). However, the ratio of primary leaf to coleoptiles length will be determined by the length of aeration prior to hypoxia (Fig. 4A2.2).

Table 4A2.1. The fresh weight and length of excised coleoptile tips at the end of hypoxia treatments. Data given are means ± SE, n=20. Superscripts indicate significant differences at $P < 0.05$ (comparisons down columns, within aeration-hypoxia treatments).

Treatment			
Aeration Time (h)	**Hypoxia**	**Fresh weight (mg)**	**Length (mm)**
0	66	2.6 ± 0.33 [a]	10.7 ± 0.8 [a]
24	42	4.4 ± 0.63 [b]	15.6 ± 0.9 [b]
36	30	5.3 ± 0.45 [b]	16.7 ± 0.7 [b]
48	18	3.0 ± 0.48 [a]	9.8 ± 0.4 [a]

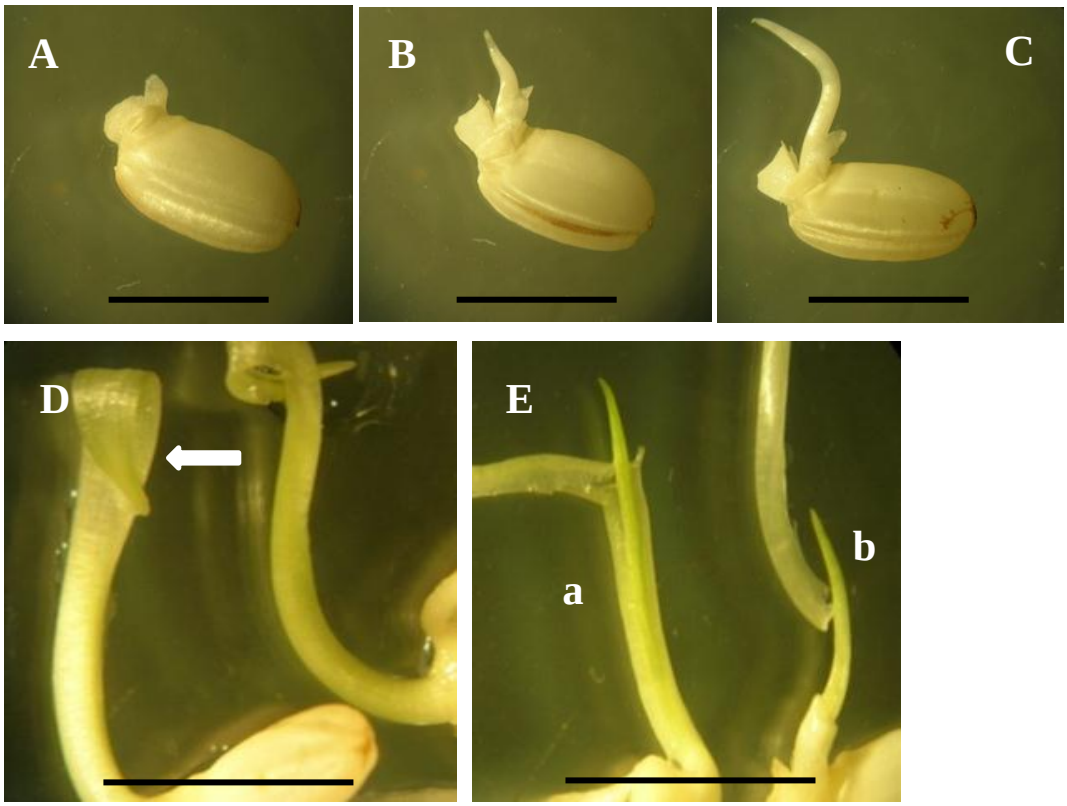

Figure 4A2.1. Different growth stages of rice seedlings (h after sowing) when hypoxia were started: 24 (A), 36 (B) and 48 (C). Rice coleoptiles have been treated with 48 h aeration followed by 18 h hypoxia; the coleoptile tips split (signed by arrow) to allow primary leaves to emerge (D). Primary leaves at 66 h after sowing in two different treatments: 48 h aeration followed by 18 h in hypoxia (a) and 36 h aeration followed by 30 h hypoxia (b) (E). Scale bar = 5 mm.

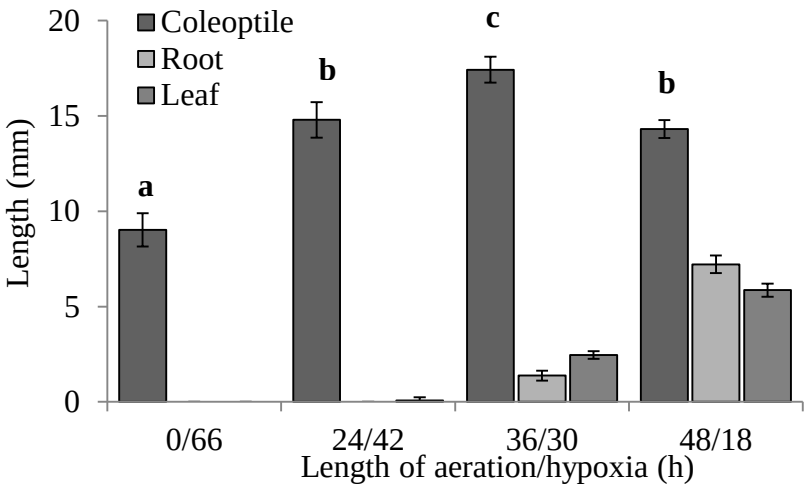

Figure 4A2.2. The length of coleoptile, root and leaf at the end of hypoxia treatment. Sowing seed was conducted at the same time, followed by different duration of aeration. Total time of aeration and hypoxia is equal for each treatment (66 h), so that coleoptiles tip excision and healing could be carried out at the same time, prior to anoxia. Data given are means ± SE, n=20. Letters indicate significant differences at $P < 0.05$ (comparisons within aeration-hypoxia treatments).

CHAPTER 5

Excised Coleoptile Tips Acclimation to a Combination of 50 mM NaCl and Anoxia

Abstract

Rice coleoptiles can survive without O_2 for several days, although anoxia severely curtails energy production. Salinity may, however, impose additional energy costs for cell maintenance, so salinity (50 mM NaCl) in combination with anoxia was studied. Growth, ion uptakes and rates of catabolism by rice coleoptiles were evaluated.

Excised coleoptile tips survived 50 mM NaCl and anoxia in N_2 flushed nutrient solution for 90 h. A very low leakage of inorganic P and vigorous K^+ and Cl^- net uptakes during non-saline re-aeration indicated that the coleoptile tips survived without substantial injuries and recovered from the combination of anoxia and 50 mM NaCl.

Despite 50-75% reduction in growth, ethanol produced by coleoptile tips at 50 mM NaCl was 10 – 16% higher than that at 0.3 mM NaCl. So the energy spent on acclimation to 50 mM NaCl was derived from both reductions in energy consumption during growth and enhanced glycolysis linked to ethanol formation. This energy was presumably consumed during Na^+ 'exclusion' (i.e. Na^+ export from the cytoplasm) and Cl^- uptake, when Na^+ and Cl^- net uptakes increased prior to reach a quasi-steady state. However, calculation of the energy expenditure using the assumption that Na^+ was transported back out to the medium via Na^+-H^+ antiporter could never meet these requirements, indicating other types of transport, involving less energy expenditure must be involved during initial phase of acclimation to 50 mM NaCl.

Thus, the survival of the coleoptile of rice in combined anoxia and 50 mM NaCl was associated with management of energy production and consumption required for solute transport, which is likely to have priority for available energy to enhance survival in these unfavorable conditions.

Introduction

The objectives of the experiments described in this chapter were to test the combined effects of NaCl and anoxia on growth, ion uptakes and rates of catabolism of rice coleoptiles. This question is of both ecological and physiological relevance. Soil O_2 deficiency can often accompany high salinity in irrigation agriculture (Drew and Lauchli, 1985). Secondly, anoxia severely curtails energy production (Greenway and Gibbs, 2003), so it is of interest to establish whether the anoxic tissue still can cope with the additional energy costs likely to be associated with tolerating NaCl in combination with anoxia.

Some plants can survive without O_2 for prolonged periods. Examples of anoxia tolerant plant organs and tissues are rice coleoptiles (Gibbs and Greenway, 2003), the coleoptiles of the paddy weed *Echinochloa* (Fox et al., 1994) and aged storage tissue of beetroot (Zhang and Greenway, 1995). In addition, germinating rice and *Echinochloa* extend coleoptiles in anoxia (Ricard et al., 1991; Alpi and Beevers, 1983; Huang et al., 2003, for rice; Fox et al., 1994 for *Echinochloa*).

Growing in anoxia requires energy for maintenance and for growth. So, it is no wonder these growing tissues show accelerated rates of glycolysis (Pasteur Effect) in the coleoptile of rice (Menegus et al., 1991; Gibbs and Greenway, 2003; Huang et al., 2005) and of *Echinochloa* (Fox et al., 1994). Despite the high rates of glycolysis, the ATP produced under anoxia is much less than the rate when O_2 is present. The high rates of ethanol or ATP production in anoxic rice coleoptiles at 50 mM exogenous glucose were still 7-10 fold lower than the assessed ATP production in aerated coleoptiles (Huang et al., 2005). Moreover, following acclimation to anoxia the coleoptiles of rice appears to be able to down-regulate ethanol production, which presumably would prolong substrate availability for survival of longer-term anoxia (Huang et al., 2005).

The key question addressed in this chapter is whether the likely energy demands at 50 mM NaCl will have repercussions on survival or growth and/or stimulate ethanol formation in response to increased ATP demand, the later has been used as a reasonable indication of ATP synthesis in glycolysis by plants during anoxia (Greenway and Gibbs, 2003).

The present work is the first to address the consequences of a combination of NaCl and anoxia for the coleoptile of rice. Results from Chapter 4 with intact seedlings in combined salinity and anoxia showed Na^+ and Cl^- concentrations approached a quasi-steady state at approximately 72 h after starting salinity. However, intact seedlings are unsuitable to accurately assess the effect of NaCl on ethanol formation by the anoxia-tolerant coleoptile, since the ethanol leaking to the medium is a composite of the production by the endosperm, embryo and coleoptile. Further, without the chance of translocation from the endosperm it becomes possible to evaluate the repercussions of 50 mM NaCl on net K^+ uptake or loss by the coleoptile. This study investigated how excised rice coleoptiles can survive under anoxia and the detrimental effects of NaCl, by evaluating the dynamics of ion net uptakes during exposure to a combination of salinity and anoxia. For comparison with anoxia, a separate experiment in aerated-

salinity was also conducted. A concentration of 50 mM NaCl was applied, because results from Chapter 4 showed that anoxic excised rice coleoptiles could survive in 50 mM NaCl without significant injuries.

Materials and Methods

Raising of rice coleoptiles

Dehulled seeds of rice (*Oryza sativa* L. cv. Amaroo) were surface sterilized in 0.1% sodium hypochlorite for 20 min and then washed thoroughly with deionized water. Batches of 10 g seeds were transferred to each 4 l plastic vessel of aerated nutrient solutions. The solution contained (mM): Ca^{2+} 1, SO_4^{2-} 1.6, K^+ 0.3, Mg^{2+} 0.3; the pH was adjusted to 6.5 using $Ca(OH)_2$. The plastic vessels were surface sterilized, all solutions were autoclaved (before adding 0.5 mM MES), as was any glassware used (see below). The seedlings were grown in darkness at 30^{o}C.

The seeds germinated and grew in non-saline aerated solution for 36 h, prior to hypoxia pretreatment for 30 h (0.06 mM O_2). Mass-flow meters (Bronkhorst HI-TEC, Nederland B.V.) were used to control the composition of O_2 and N_2 coming into each vessel during hypoxia pre-treatment. Coleoptile tips were excised (at 7-10 mm) so that the young leaf tissues were excluded and thus obtaining uniform tissues of coleoptile tips only for the experiments. Excised tips were healed for 5 h in 50 ml conical flasks of hypoxic solution (0.06 mM O_2) containing 20 mM exogenous glucose and 50 mg l^{-1} ampicillin, and then transferred to anoxia or aerated treatments.

Aerated-NaCl treatments

For aerated treatment, nutrient solution (composition as stated above) also contained 50 mg l^{-1} ampicillin and 5 mM glucose. These flasks were continuously air flushed (0.28 mM O_2). There were three replicate flasks for each of 0.3 and 50 mM NaCl. NaCl was given 23 h after transferring to conical flasks (i.e. 18 h after switch from hypoxia back to aeration). Subsequently, samplings were taken at 2, 6, 36, 72, 90 h after starting NaCl treatments, and at the end of recovery (72 h after removal of NaCl and with aeration continued). For controls at 0.3 mM NaCl, three samplings were conducted at 2 h, 90 h (to coincide with the end of salinity) and at the end of the recovery period (to coincide with 72 h after removal of NaCl). To avoid osmotic shock (particularly when high glucose was supplied, see next section), glucose and NaCl were

not given at the same time; glucose was given immediately after excision and then the concentration adjusted after healing when either aeration or anoxia was commenced, whereas NaCl was applied 18 h after glucose was adjusted. NaCl was applied when the tissues would have acclimated to anoxia, to avoid having two perturbations at the same time.

O_2 consumption by excised coleoptile tips in air-saturated incubaton medium during exposure to 50 mM NaCl and non-saline controls was measured at 30 ^oC using a Clark-type electrode to monitor the depletion of O_2 in a sealed, stirred cuvette (Unisense Microrespiration Equipment, see Colmer and Pedersen, 2008).

Anoxic-NaCl treatments

In anoxic treatments, anoxia was imposed by flushing the solutions in sealed flasks with high-purity N_2 gas. Nutrient solution (composition above) also contained 50 mg l^{-1} ampicillin and 50 mM glucose (50 mM glucose was chosen since lower levels resulted in decreases of endogenous sugar levels with time, whereas in aeration 5 mM glucose achieved that objective (Huang et al., 2005); if 50 mM were used in aerated solutions, coleoptile tips would have accumulated high tissue sugar concentrations (Kulichikin et al., 2009). During anoxia, a syringe was used to withdraw, or add, solution through the outlet of each conical flask, while maintaining N_2 gas flow. The solutions were refreshed with anoxic solution. Ethanol was trapped in vials containing ice-cold water and connected to the outlet tube of each flask. All samples were stored in sealed tubes at -20 ^oC. The recovery of ethanol from checks was 96% (flasks containing nutrient solution, spiked with ethanol and flushed with N_2 at 30^oC for the same periods as the experimental units and connected to cold traps). NaCl was given at the same time as in aerated treatments.

Return to aeration and 0.3 mM NaCl

Coleoptiles in non-saline or in 50 mM NaCl anoxic nutrient solution were maintained for 108 and 90 h respectively, prior to return to aerated solution all at 0.3 mM NaCl for another 72 h. The coleoptiles returned to 0.3 mM NaCl might conceivably experience adverse effects from hypo-osmotic shock as well as from the large reduction in Na^+ and Cl^- concentrations. Therefore, a check was included in which the coleoptiles at the time of return to aerated solution were transferred from 50 mM NaCl to nutrient solution containing iso-osmotic mannitol to avoid hypo-osmotic

shock. During re-aeration glucose was reduced from 50 to 5 mM (Kulichikin et al., 2009) and the difference of osmotic potential was taken into account when preparing the iso-osmotic solution for recovery.

For the sampling at the start of return to aerated solutions coleoptiles were rinsed for approximately 3 minutes, to remove ions from the free space and from any surface water. The rinse solution contained 1 mM $CaSO_4$, and for coleoptiles previously in 50 mM NaCl, the rinse solution also contained iso-osmotic mannitol.

After return to aerated solution and 0.3 mM NaCl, the non-saline solutions were sampled and refreshed after 1, 4, 8, 12, 24, 48 h and the test on recovery was ended at 72 h. During these 72 h the volume of external solution was maintained according to tissue/volume ratio and length between samplings, so that the depletion of K^+ would be in the range of 5-25%, to enhance accuracy of the measurements on K^+ net uptakes based on changes in concentrations in the incubation medium. The solutions were stored at -20^{o}C prior to measurement of K^+.

Analytical procedures

Coleoptile tips in anoxia sampled for total sugars, amino acids, organic acids and tissue Pi, were washed for 3x3 minutes with anoxic nutrient solution without glucose (but maintaining the previous osmotic potential with mannitol) and then killed by plunging into liquid N_2 immediately, and were stored at -80^{o}C prior the lyophilization.

In ethanol and sugar assays, the same spectrophotometer (model UV-1601, Shimadzu, Tokyo, Japan) was used. Biochemicals were purchased from Sigma. Ethanol in the treatment solutions and traps was assayed in a 1 ml cuvette containing 100 mM glycylglycine buffer and containing 300 mM KCl, 1.7 mM NAD^+, and aldehyde dehydrogenase (adapted from Beutler, 1983). The reaction was started by addition of alcohol dehydrogenase and absorbance was monitored at 340 nm.

Total sugar were measured using anthrone, after extraction of coleoptiles twice in 80% ethanol boiled with reflux for 20 min. Anthrone reagent is strongly acidic to hydrolize all polymers of di-hexoses (Yemm and Willis, 1954). The results are expressed as equivalent ‘hexose units’. Ethanol-insoluble dry weight after extraction with 80% ethanol was measured for coleoptile tips from the various treatments.

The medium and tissue sap were assessed for osmotic potential (π_{sol} and π_{sap}). Osmotic pressure of sap (π_{sap}) expressed from freeze/thawed coleoptile tips and

endosperm samples was measured using a freezing point depression osmometer (Fiske 210 micro-sample/ 20 µl sample size) at the end of salinity period.

Lyophilized coleoptile tips were extracted using a procedure designed for analysing low molecular weight metabolites (amino acids and organic acids), but these extract were also assayed for Pi (see below). Coleoptiles were extracted twice using ice-cold 5% (w/v) perchloric acid (PCA), centrifuged at 15,000 rpm, 4^{o}C (Beckman Coulter Microfuge 22 R Centrifuge, Fisher Biotec, Australia) and the supernatant collected and then slowly adjusted using K_2CO_3, to reach pH 3-5 (for amino acids) and pH <3 (for organic acids). Organic acids in tissue extracts and in nutrient solution samples were subsequently analysed using HPLC [600E pump, 717 plus autoinjector, 996 photodiode array detector (PDA), Waters, Milford, MA, USA] following the method described by Cawthray (2003). Amino acids were analysed with a Phenomenex EZ: faast analysis kit (Lane Cove, NSW, Australia), adapted from the method of Nozal et al. (2004). Pi was measured in these PCA exctracts from the coleoptile tips and also in nutrient solutions samples, using the molybdatemalachite green method (Motomizu et al., 1983) and absorbance was monitored at 650 nm using the same spectrophotometer described above.

Na^+ and K^+ of the coleoptile tips extracted for 2 d in 0.5 M nitric acid were analysed using a flame photometry (Corning Medical and Scientific, Model 410, Cambridge, UK). Cl^- was measured with a Buchler-Cotlove chloridometer (Buchler Instruments, Model 4–2008, Fort Lee, New Jersey, USA). Measurements on reference tissues showed Na^+, Cl^- and K^+ concentrations within the range 91-97% and no adjustments were made to the data presented.

Statistical analyses of data

Data sets were analysed using the program of general analysis of variance of Genstat 14 edition. Pairwise comparisons used Duncan's multiple range test.

Results

Growth of excised rice coleoptiles under aerated and anoxic-50 mM NaCl

In anoxia at 0.3 mM NaCl for 108 h, fresh and ethanol insoluble dry weights of excised coleoptile tips increased somewhat, in agreement with Huang et al. (2005). In anoxic-50 mM NaCl, growth reduction was approximately 50-75% of that at 0.3 mM

NaCl in anoxia. The growth of excised coleoptile tips in aerated-50 mM NaCl increased by almost two-fold compared to those in aeration at 0.3 mM NaCl (Table 5.1).

Table 5.1. Relative growth rate (on a fresh weight and on an ethanol insoluble dry weight basis) of excised rice coleoptile tips at 0.3 and 50 mM NaCl in aerated and anoxic solutions. Data given are over 108 h and means ± SE, n=6.

NaCl (mM)	Relative growth rates	
	Fresh weight (mg g^{-1} FW d^{-1})	Ethanol-insoluble dry weight (mg EIDW g^{-1} EIDW d^{-1})
Anoxia		
0.3	23.0 ± 4.3	25.8 ± 3.7
50	10.9 ± 2.9	8.5 ± 0.3
Air		
0.3	26.7 ± 1.1	28.1 ± 0.2
50	50.1 ± 2.7	49.5 ± 0.9

Inorganic phosphate (Pi) in tissues and medium

Loss of Pi to the medium has been proposed as a good indicator of injury in anoxic tissues (Menegus et al., 1991). In the present study, no evidence of substantial Pi decrease in coleoptile tips during anoxia indicated that there were no appreciable injuries during anoxia. The concentration of Pi in tissues of anoxic tips of 23-26 and 21 µmol g^{-1} FW at 0.3 and 50 mM NaCl respectively, the total Pi loss during the 72 h NaCl exposure in anoxia which was less than 3 µmol Pi g^{-1} FW did not significantly reduce the initial Pi concentrations in rice coleoptile tips. The rates of Pi loss to the external medium during salinity were approximately 0.03 µmol g^{-1} FW h^{-1} both at 0.3 and 50 mM NaCl. The coleoptile tissue Pi concentrations in the present work compare to results by other workers for anoxic rice coleoptiles of: 7.7 – 8.2 µmol g^{-1} FW (Kato-Noguchi, 2002) and 12-14 µmol g^{-1} FW (Menegus et al., 1991). So the further observations on ion transport are almost certainly for uninjured tissues which had acclimated to a combination of anoxia and 50 mM NaCl.

Effects of NaCl on rates of catabolism in air and anoxia

O_2 uptake by coleoptile tips in aerated solution and ethanol formation in anoxia were both stimulated by 10-17% following exposure to 50 mM NaCl (Table 5.2). In anoxia, the increase of cell catabolism at 50 mM NaCl started at about 6 h after NaCl was imposed and ended approximately after 90 h. The difference in catabolism rates between tissues in 0.3 and 50 mM NaCl were assessed to increase ATP production in

anoxia via glycolysis by ~0.8 µmol ATP g^{-1} FW h^{-1} and in aeration via respiration by a much larger ~12 µmol ATP g^{-1} FW h^{-1} (Table 5.5).

Table 5.2. Effects of 50 mM NaCl on ethanol formation (anoxic) and O_2 consumption (aerated) by excised rice coleoptile tips. Data given are means ± SE, n=3.

Time in salinity (h)		Rate of catabolism		As % of control	
		0.3 mM NaCl	50 mM NaCl		
Anoxia		Ethanol production (µmol g^{-1} FW h^{-1})		(%)	l.s.d.
	2	5.6 ± 0.70	5.2 ± 0.90	ns	
	6	5.7 ± 0.11	6.8 ± 0.39	116	0.68
	36	6.7 ± 0.11	7.4 ± 0.14	110	0.47
	72	6.3 ± 0.18	7.2 ± 0.11	114	0.70
	90	4.2 ± 0.01	4.4 ± 0.7	ns	
		O_2 consumption (µmol g^{-1} FW h^{-1})			
Aerated	2	15.8 ± 1.03	16.9 ± 1.45	ns	
	6	17.5 ± 0.49	20.5 ± 0.89	117	1.64
	36	19.1 ± 0.47	21.1 ± 0.63	111	1.14
	72	17.5 ± 0.68	20.1 ± 0.99	115	1.77
	90	17.0 ± 1.04	19.9 ± 1.06	117	1.89

Na^+, Cl^-, and K^+ concentrations in coleoptiles during salinity in aeration and anoxia

A reduced Cl^- uptake occurs in anoxic-50 mM NaCl compared to aerated-50 mM NaCl (Fig. 5.1), as has been observed earlier for anoxic tissues with low external Cl^- concentrations (Zhang and Greenway, 1995). The high Cl^- uptake in aerated solution, but lower uptake in anoxia demonstrated that the energy available might limit the net Cl^- uptake during the energy crisis in anoxia.

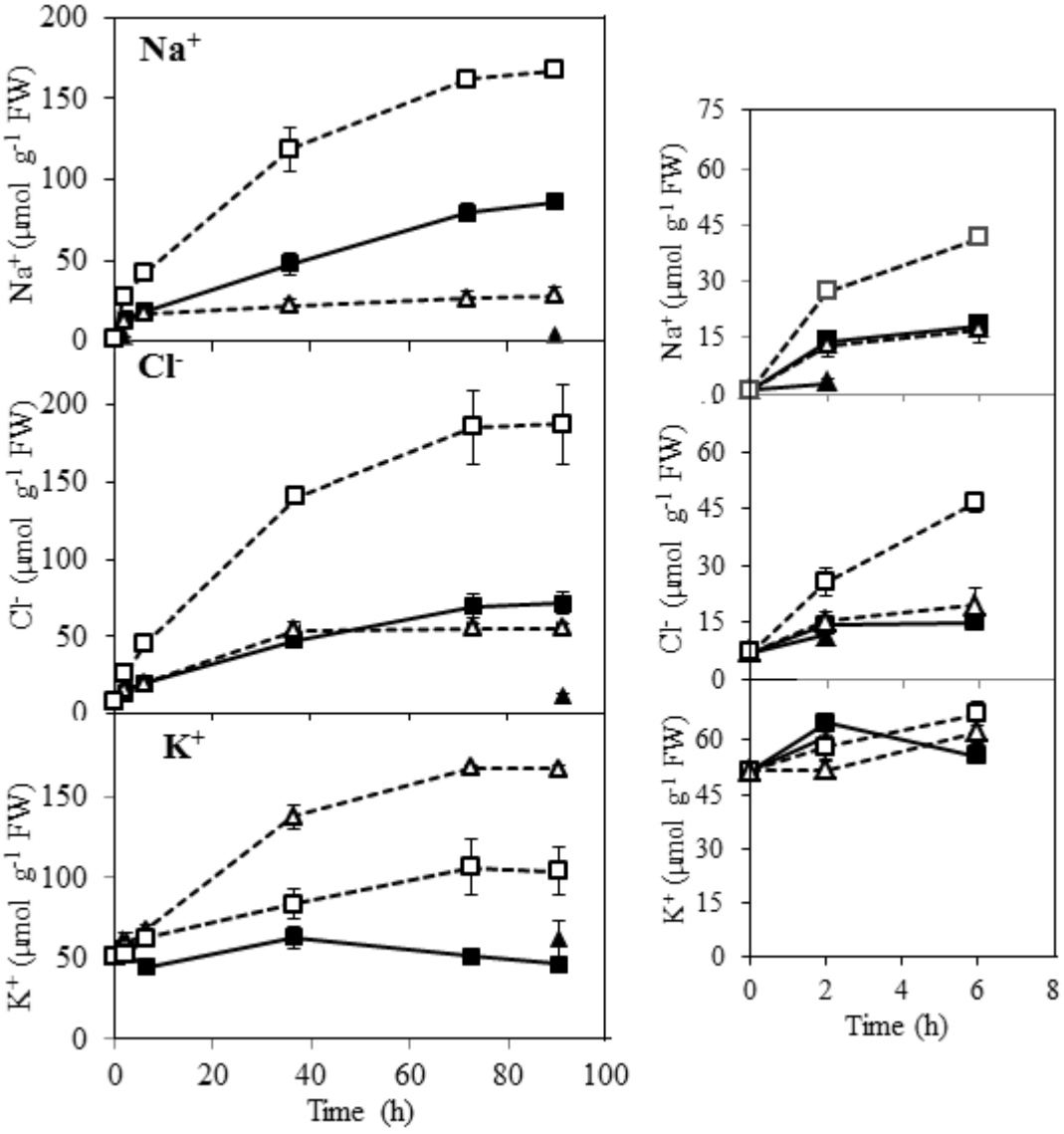

Figure 5.1. Na^+, Cl^- and K^+ concentrations in excised rice coleoptile tips at 0.3 and 50 mM NaCl, when NaCl was given 18 h after starting anoxia. Exogenous K^+ was 0.3 mM and exogenous glucose at 50 mM in anoxia and 5 mM in aerated solution. Anoxia at 0.3 mM NaCl, filled triangle; anoxia at 50 mM NaCl, filled square; aerated at 0.3 mM NaCl, open triangle; aerated at 50 mM NaCl, open square. Data given are means ± SE, n=3. Graphs on the right show concentrations over the first 6 h after transfer to 50 mM NaCl (i.e. first 6 h of main graphs expanded).

For coleoptiles in anoxia, Na^+ sharply increased during the first 6 h after addition of 50 mM NaCl, and then increases were gradually slowed down (Fig. 5.1). Throughout the time course, Na^+ concentrations in anoxic coleoptiles were somewhat less than half those in aerated coleoptiles. Between 0-6 h at 50 mM NaCl, the gradual increase in Na^+ (at about 3 µmol g^{-1} FW h^{-1}), but for Cl^- at only about 1.5 µmol g^{-1} FW h^{-1}, seems to be associated with a 2 µmol g^{-1} FW h^{-1} decrease also in K^+ for this period (Fig. 5.1 and Fig. 5.3). The increase of Na^+ together with the decrease of K^+ between 2-6 h at 50 mM NaCl resulted in a decrease of K^+/Na^+ ratio from 5 to 3. At the end of anoxia, the

K^+/Na^+ ratio had decreased from 21 to 15 in coleoptiles at 0.3 mM NaCl and from 5 to 0.5 in those at 0.3 and 50 mM NaCl.

In anoxia, net Na^+ and Cl^- uptakes, which were calculated from changes with time of the Na^+ and Cl^- concentrations in the tissues, decreased with time of exposure to 50 mM NaCl approaching 70 h in salinity. In aerated solutions, the Na^+ and Cl^- net uptakes were more than 2-fold higher than the rate in anoxia at initial hours to 36 h after NaCl was imposed (Fig. 5.2).

During first hours at NaCl exposure in anoxia, net K^+ uptakes decreased by ~1 and 2.5 μmol g^{-1} FW h^{-1} for coleoptile tips at 0.3 and 50 mM NaCl, respectively (Fig. 5.4). As discussed above, this decrease of net K^+ uptakes at initial hours after NaCl was imposed was associated with the gradual increase in Na^+ both at 0.3 and 50 mM NaCl. After about 50 h, the K^+ slightly loss to the external medium, with 50% less rate in coleoptiles tips at 0.3 mM than at 50 mM NaCl. At the end of NaCl-anoxia at 90 h, net K^+ loss at both NaCl treatments were about 0.5 μmol g^{-1} FW h^{-1}.

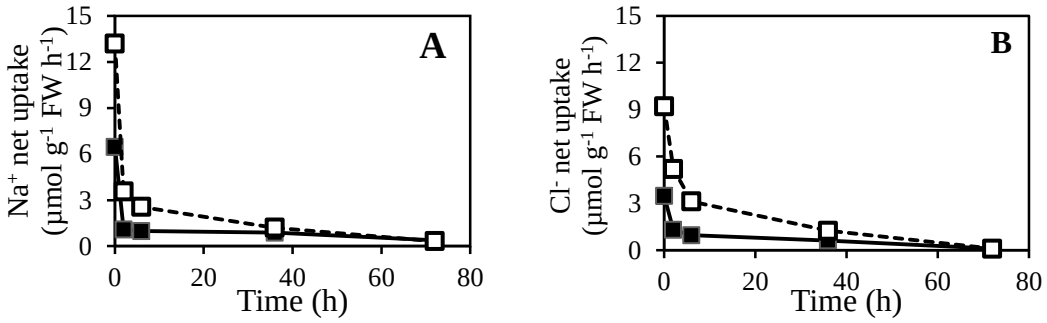

Figure 5.2. The Na^+ (A) and Cl^- (B) net uptake rates by excised rice coleoptile tips during 50 mM NaCl for 90 h in aerated or in anoxic solution, calculated based on increases in Na^+ and Cl^- tissue concentrations between each sequence of sampling times. Ion net uptakes at 50 mM NaCl in anoxia, closed square; 50 mM NaCl in aerated solution, open square.

Na^+, Cl^- and K^+ concentrations after re-aeration in 0.3 mM NaCl, following anoxic treatment for 90 h

The vigorous Cl^- uptakes during non-saline re-aeration by coleoptiles which had been in anoxia either at 0.3 or 50 mM NaCl, so that tissue concentrations reached 120-130 μmol g^{-1} FW (Fig. 5.3), strongly indicated that excised coleoptile tips survived anoxia with 50 mM NaCl without substantial injuriy. This large Cl^- uptake would balance the large K^+ uptake also during the same period (Fig. 5.3). The Na^+ concentrations in the tissues which had been previously been exposed to 50 mM NaCl decreased only gradually during non-saline re-aeration to reach ~51 μmol g^{-1} FW at the

end of recovery period (72 h after end of anoxia) or 58% of the concentration at the end of salinity period (Fig. 5.3). Further these decreases in tissue Na^+ would have been partly due to dilution by volume expansion, which is discussed in the next paragraph.

Both iso-osmotic and hypo-osmotic external solutions resulted in similar rates of decreases in Na^+ concentrations during the period of return to the aerated non-saline condition (Fig. 5.3). The decrease in tissue Na^+ concentrations was associated with decrease in external ionic concentration rather than to a decrease in total external osmotic potential (Willmer, 1978). The increases of fresh weight during non-saline re-aeration between the iso-osmotic and hypo-osmotic solution were 10% and 26% respectively, whereas the water content of the rice coleoptiles in iso-osmotic solution was 5% lower than those in the hypo-osmotic solutions. Thus, the decrease of tissue Na^+ concentration in hypo-osmotic solution was not only due to Na^+ efflux to the external solution (74%), but also to ion dilution (26%) by expanding tissue volume during re-aeration. Meanwhile in iso-osmotic solution, 90% of the decrease in Na^+ tissue concentration was due to loss to external solution, and the remaining 10% was diluted by growth. Overall, the Na^+ loss from the tissues during re-aeration was about 0.5 µmol g^{-1} FW h^{-1} both in iso-osmotic and hypo-osmotic solutions.

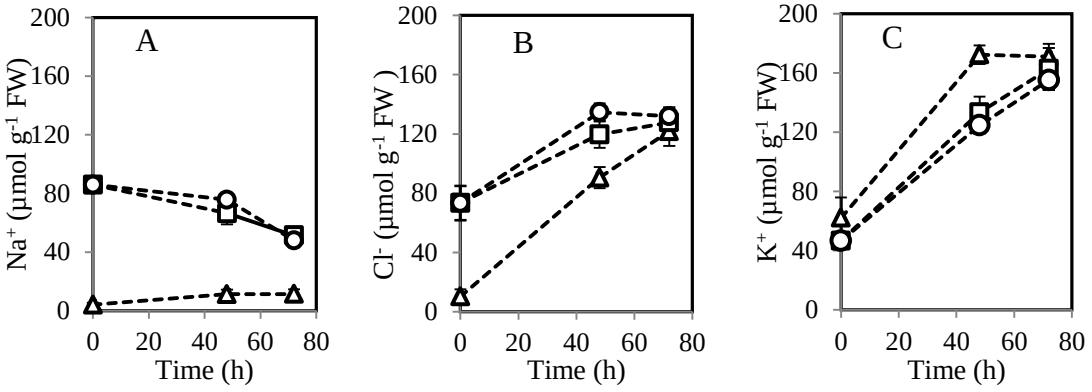

Figure 5.3. Na^+, Cl^- and K^+ concentrations of excised rice coleoptile tips after exposure to salinity treatments (0.3 and 50 mM NaCl) in anoxia for 90 h, followed by non-saline re-aeration for 72h. The recoveries from 50 mM NaCl and anoxia were carried out using re-aeration with and without iso-osmotic mannitol. Recovery from anoxia-0.3 mM NaCl, open triangle; recovery from anoxia-50 mM NaCl without iso-osmotic mannitol, open square; recovery from anoxia-50 mM NaCl with iso-osmotic mannitol, open circle. The time axes refer to time after re-aeration. Data given are means ± SE, n=3.

The high net K^+ uptake during early hours of re-aeration by tissues which had been previously at 0.3 and 50 mM NaCl resulted in high tissue K^+ concentration towards the end of the re-aeration period (Fig. 5.3). The coleoptiles showed large

increases in the rates of net uptake of K^+ within 4 h after the start of re-aeration, and then the rates declined (Fig. 5.4).

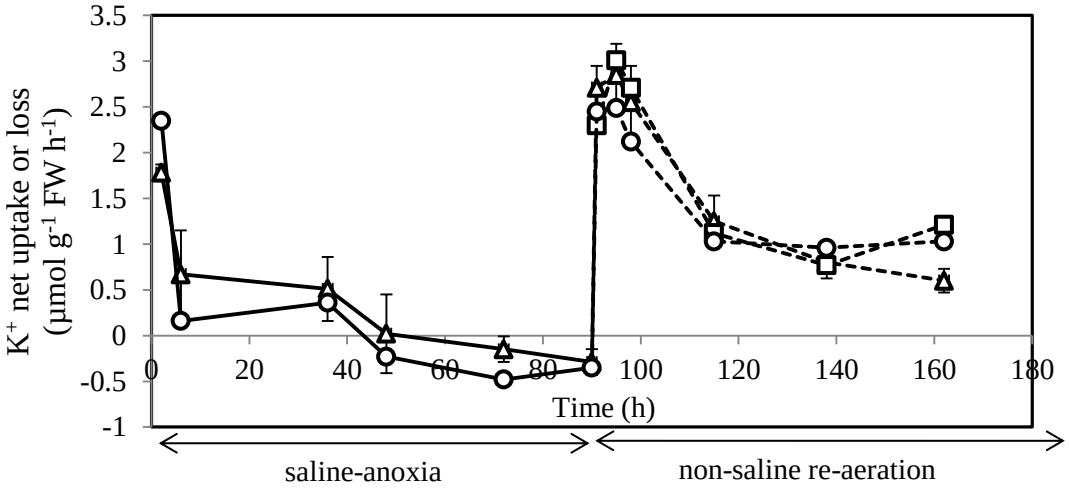

Figure 5.4. K^+ net uptake or loss in excised coleoptiles at 0.3 and 50 mM NaCl, when NaCl was given 18 h after starting anoxia. K^+ was at 0.3 mM in the medium during salinity and exogenous glucose was at 50 mM in anoxia and 5 mM in aerated solution. The recoveries from 50 mM NaCl and anoxia were carried out using non-saline re-aeration with and without iso-osmotic mannitol for 72 h period. Recovery from 0.3 mM NaCl, open triangle; recovery from 50 mM NaCl with iso-osmotic mannitol, open circle; recovery from 50 mM NaCl without iso-osmotic mannitol, open square. Data given are means ± SE, n=3.

Amino acids, organic acids and total sugars in tissues

Organic solutes would contribute to osmotic pressure, while high internal Na^+ and Cl^+ could affect their synthesis. Sugars needed also be determined to check adequacy of substrates for glycolysis.

The alanine concentrations were 23 and 16 µmol g^{-1} FW at 70 h in anoxic-0.3 mM or 50 mM NaCl treatments, so net synthesis in the two treatments were ~0.3 and 0.2 µmol g^{-1} FW h^{-1}. By contrast, rates of ethanol production during anoxia were 6-7 µmol g^{-1} FW h^{-1}. This indicates that ethanol fermentation accounted for 96% of the carbon flux from pyruvate and alanine accounted for only 4%. This is in agreement with the study by Kato-Noguchi and Ohashi (2006) which showed that in rice coleoptiles, about 92, 1 and 7% of pyruvate was metabolized into ethanol, lactate and alanine, respectively.

Alanine is an end product of glycolysis during certain periods. After transfer to 50 mM NaCl under anoxia alanine concentrations decreased (to~ 60% of the concentration at 0.3 mM NaCl) during the first 6 h, followed by a gradual increase towards the end of the exposure to 50 mM NaCl. Similar to alanine, GABA

concentration which decreased during first hours of exposure to 50 mM NaCl, slowly increased and regained to the level at 0.3 mM NaCl (Fig. 5.5).

Among amino acids detected in coleoptile tip samples, only three major amino acids contributed a large percentage of total amino acids, i.e. alanine, GABA and asparagine. Proline which is usually abundant in plants exposed to salinity was not detected in the samples. The absence of proline indicated that pattern of solute accumulated in anoxic rice coleoptile may be different from that in aeration (Singhaki-Wells et al., 2011).

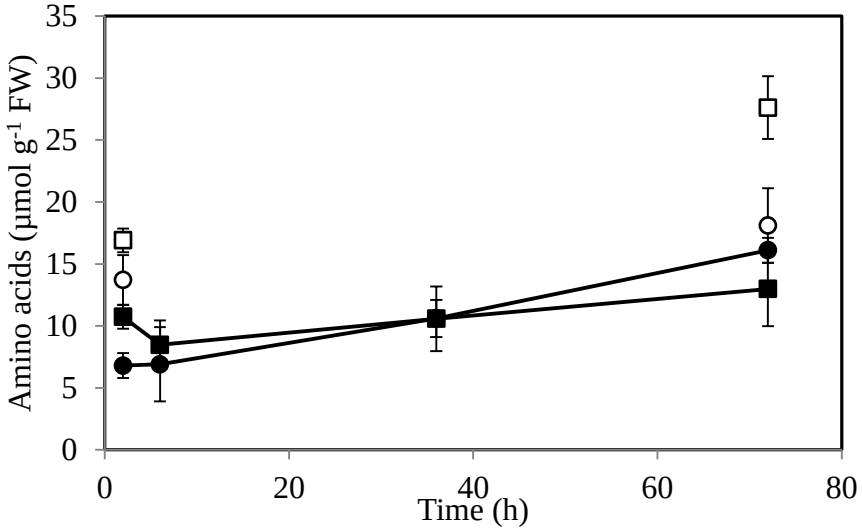

Figure 5.5. Concentrations of alanine and γ-aminobutyric acid (GABA) in excised rice coleoptiles tips during anoxia at 0.3 and 50 mM NaCl. Salinity exposure was started after 18 h starting anoxia. Other detected amino acids were usually less than 1.5 µmol g^{-1} FW, except asparagine (2.7 µmol g^{-1} FW). The total amino acid concentration at 0.3 mM NaCl and 50 mM NaCl was 44 and 25 µmol g^{-1} FW at 2 h; 55 and 38 µmol g^{-1} FW at 72 h in salinity. Alanine at 0.3 mM NaCl, open square; alanine at 50 mM NaCl, filled square; GABA at 0.3 mM NaCl, open circle; GABA at 50 mM NaCl, filled circle. Data given are means ± SE, n=3.

The decrease of malate concentration at early stages of NaCl exposure (Fig. 5.6, only anoxic samples were assayed) is in agreement with the result from Gong et al. (2005); Sanchez et al. (2008; 2011) and NaCl elicited a decrease in organic acids may compensate for an ionic imbalance. The decrease of $[(Na^+ + K^+)/Cl^-]$ during 50 mM NaCl exposure from 7.2 at 0 h to 1.8 at 70 h showed a lower requirements for anions other than Cl^- and the common response of decrease in organic anions (Fig. 5.6) (Greenway et al., 2012). The decrease of malate at 50 mM NaCl by ~3 µmol g^{-1} FW seems to be associated with the decrease of $[(Na^+ + K^+)\text{-}Cl^-]$ from 0 h to 78 h which was about 6 µmol g^{-1} FW, whereas the increase of malate at 0.3 mM NaCl by ~1.5 µmol g^{-1} FW was also connected to the increase of $[(Na^+ + K^+)\text{-}Cl^-]$ by 3 µmol g^{-1} FW during the same period.

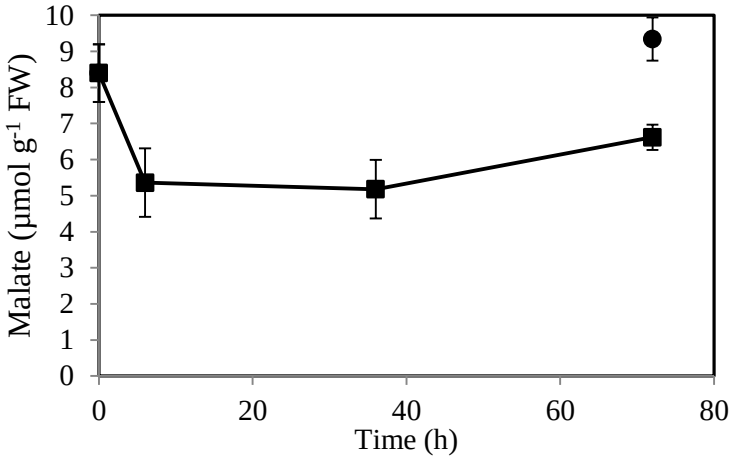

Figure 5.6. Malate in excised tips of rice coleoptiles in anoxia. Other organic acids were either below 1 µmol g^{-1} FW or below LOD. For aerated excised coleoptiles, at 0 h salinity the malate (±SE) was 9.2 ± 1.3. At 72 h the malate concentrations were 17.6 ± 2.0 and 4.9 ± 0.9 (µmol g^{-1} FW) for 0.3 and 50 mM NaCl, respectively. In anoxia at 0.3 mM NaCl, filled circle; at 50 mM NaCl, filled square. Data given are means ± SE, n=3.

Sugar concentrations were assayed because ethanol formation by excised coleoptiles is strongly dependent on carbohydrate supply (Huang et al., 2005). Sugar concentration (expressed as hexose units) in the coleoptiles increased with time. Sugar concentration in rice coleoptiles at 50 mM NaCl, both in aerated solution and in anoxia were however lower than those in non-saline solutions, but at sufficient levels (Huang et al., 2005) (Table 5.3). The increasing sugar concentration with time in all coleoptiles indicated sufficient substrate for catabolism and for growth when that occurred. Endogenous sugar levels are only indicative, but further proof of adequate substrate can only be achieved easily for tissues in aerated solution, where there is no problem increasing exogenous glucose concentrations and hence the osmotic pressure of the solution. In contrast, in anoxia a substantial increase in exogenous glucose level, e.g., to an exogenous level of 100 mM rather than 50 mM would make the external osmotic potential of the glucose ~-0.25 MPa, which might be particularly counterproductive at 50 mM NaCl, which already contributes ~-0.24 MPa to the osmotic potential.

Table 5.3. Sugar concentration in excised rice coleoptile tips at 0.3 and 50 mM NaCl. Exogenous glucose was provided at 50 mM in anoxia and 5 mM in aeration. Data given are means ± SE, n=3.

O_2 treatment	Time after starting salinity (h)	NaCl (mM)	
		0.3	**50**
		Sugar (μ mol g^{-1} FW)	
Anoxia	36	n.a.	56.5 ± 3.7
	72	103.5 ± 7.0	86.5 ± 4.7
Aeration	36	n.a.	20.7 ± 1.3
	72	94 ± 8.9	66.5 ± 3.2

Contributions of ions, sugars, amino acids and organic acids to π_{sap}

The low osmotic potential in the external solution required coleoptile tips to adjust their internal osmotic potential (π_{sap}). The contribution to π_{sap} was obtained from the data on ions and organic solutes in the tissues. In anoxia, the contribution of ions to π_{sap} was 22 and 51% in 0.3 and 50 mM NaCl, respectively, whereas in aerated solution, the contribution of ions at 0.3 mM NaCl was more than 2-fold of coleoptiles in anoxia (Table 5.4).

Table 5.4. Contributions of ions, total sugars (hexose equivalents), amino acids and organic acids to π_{sap} and estimated turgor pressure (P) in the excised rice coleoptile tips at 0.3 and 50 mM NaCl. Glucose 5 and 50 mM were added to the external solution in aeration and anoxia respectively. P was estimated from ($\pi_{sap} - \pi_{sol}$). Values in brackets show the percentage contribution to total π measured. Δ π was estimated from π at 50 mM NaCl - π at 0.3 mM NaCl.

NaCl (mM)		Aeration π (MPa)	Anoxia π (MPa)
0.3	Total ions	-0.46 (60)	-0.15 (22)
	Sugar	-0.22	-0.25
	AAs and OAs	-0.12	-0.29
	π_{sap} measured	-0.79	-0.69
	Estimated P	0.75	0.53
50	Total ions	-0.83 (80)	-0.41 (51)
	Sugar	-0.16	-0.22
	AAs and OAs	-0.06	-0.18
	π_{sap} measured	-1.03	-0.81
	Estimated P	0.79	0.46
	$\Delta \pi_{sol}$	-0.20	-0.57
	$\Delta \pi_{sap}$	-0.25	-0.12

Discussion

Anaerobic catabolism under anoxia and 50 mM NaCl

During O_2 deprivation when energy supply is low, any demands for energy associated with the exposure to NaCl, for example for Na^+ extrusion (Munns and Tester, 2008) need additional economies in other metabolism and or an increase in energy production. In the present experiment these extra demands at 50 mM NaCl were presumably met partly by 50% slower growth at 50 mM NaCl and partly by the observed increase in glycolysis linked to ethanol formation. In some more detail, exposure to 50 mM NaCl will increase energy requirements for ion transport, including Cl^- influx to balance the lower external π and when tissue concentrations reach quasi steady state, particularly for Na^+ extrusion, since this ion at high external concentration would flow inwards along a large inward free energy gradient, which is composed of both concentration and electrical components (Britto and Konzucker, 2009). Mechanisms by which energy requirement associated with the exposure to 50 mM NaCl during anoxia (i.e. an energy crisis) may be mitigated will be discussed in the section after next.

In contrast to the response under anoxia, in aerated solutions 50 mM NaCl stimulated fresh weight increases almost 2-fold relative to 0.3 mM NaCl. This growth stimulation by 50 mM NaCl is unusual for a glycophyte, and since it did not occur for intact seedlings (Chapter 4) it may be an artifact of the excised coleoptile tips. The causes for the growth stimulation may be associated with the high Na^+ and Cl^- uptake via (i) more readily developing turgor pressure and or (ii) substitution of organic solutes in the vacuole, generating osmotic pressure by Na^+ and Cl^- (Table 5.4). Discussion on the growth, respiration rate and ionic balance in aerated solution is given in Appendix 5.1.

Rates of anaerobic catabolism

Gibbs and Greenway (2003) stated that anoxia in plant tissues reduces the rates of energy production by 65 – 97% compared with rate in air. In the present study, the energy requirements for growth and maintenance during anoxia can be estimated from the ethanol production rate by these tips at 0.3 mM NaCl; which was reasonably stable between 30 h and 72 h in salinity or 48 – 90 h in anoxia, at 6.3 – 6.7 μmol g^{-1} FW h^{-1} or 6.3-6.7 μmol ATP g^{-1} FW h^{-1}. In 50 mM NaCl, the energy requirements for growth and

maintenance were 10-16% higher at 7.2 - 7.4 μmol ATP g^{-1} FW h^{-1} based on the increased production of ethanol, although the reduced growth at 50 mM NaCl indicated the actual energy requirements for maintenance were higher than assessed only on the change in ethanol formation. The energy production by excised coleoptiles under anoxia was reduced by 93% compared to the rate in air. The usual reservations that hydrolysis of sucrose via sucrose synthase (Gibbs and Greenway, 2003; Harada et al., 2005; Jackson and Colmer, 2005) would increase ATP production under anoxia does not apply in this case since the exogenous substrate was glucose.

Before assessing the energy demand by coleoptile tips at 50 mM NaCl, there was evidence for down regulation of glycolysis in anoxia under benign conditions. The increase of 10-16% in ethanol formation in response to salinity supports the suggestion by Colmer et al. (2001) and Huang et al. (2005) that under benign conditions, these excised rice coleoptiles down regulate ethanol formation below its possible maximum. The suggestion by Huang was based on the decrease of ethanol formation rate during the first 24-70 h in anoxia, despite the increase of endogenous sugar, which indicated the need of energy for acclimation at early stage in anoxia. The present experiment strengthens this notion, by exposure to 50 mM NaCl, which is likely to increase the energy requirements for maintenance, and indeed resulted in a 10-16% increase in ethanol formation.

For anoxic coleoptiles, the increase of ethanol formation was despite a 50-75% lower gain in fresh weight (i.e. less growth), both these observations support the notion that at 50 mM NaCl there is higher maintenance requirement for energy. Accordingly, the additional energy required for maintenance at 50 mM NaCl would be the sum of the ATP produced via the increase in ethanol formation and the energy saved by the reduction in growth. The increase in energy produced was presumably used for Cl^- uptake, vacuolar compartmentalization of Cl^- and Na^+, and Na^+ extrusion. So, these maintenance processes apparently had a higher priority for energy than growth.

At 50 mM NaCl, an up to ~75% decrease in coleoptiles growth implied that ~ 2.4 μmol ATP g^{-1} FW h^{-1} (extracted from Table 5.5, energy associated for growth at 0.3 mM NaCl was ~3.2 μmol ATP g^{-1} FW h^{-1}) was diverted to the increased demand of energy for cell maintenance associated with exposure to NaCl. To this, the increase of ATP production compared to production at 0.3 mM NaCl (~0.8 μmol ATP g^{-1} FW h^{-1}) can be added, so the extra energy requirement of maintenance associated with exposure to 50 mM NaCl becomes~3.2 μmol ATP g^{-1} FW h^{-1}. Table 5.5 shows the different

energy produced and spent at 0.3 mM and 50 mM NaCl during anoxia and in aerated solution. Despite the large decreases of ATP production during anoxia, the ratio of hexose being used in anaerobic catabolism and in respiration showed that the hexose being fermented in rice coleoptiles during anoxia increased to be~110% of the amount respired in aeration.

The reduced growth of coleoptile tips at 50 mM NaCl under anoxia implied a higher energy requirement for cell maintenance so that less energy was available to support growth. The energy requirement for maintenance by coleoptile tip cells at non-saline condition was 3.3 µmol g^{-1} FW h^{-1} (Huang et al., 2005). Hence, when deducted from total ATP produced (~6.5 µmol g^{-1} FW h^{-1}), about 3.2 µmol ATP g^{-1} FW h^{-1} was likely to be used for other purposes including cell wall and net protein synthesis associated with growth at 0.3 mM NaCl.

Table 5.5. Different energy produced and spent by excised rice coleoptiles tips in anoxia and aeration, in non-saline and saline conditions.

Parameters related to energy	**Anoxia**		**Aeration**	
NaCl	**0.3 mM**	**50 mM**	**0.3 mM**	**50 mM**
O_2 consumption (µmol g^{-1} FW h^{-1})	-	-	17-19	20-21
Ethanol production (µmol g^{-1} FW h^{-1})	6.3-6.7	7.2-7.4	-	-
ATP (µmol g^{-1} FW h^{-1})[1)]	6.3-6.7	7.2-7.4	68-114	80-126
Hexose consumed during anaerobic/ aerobic catabolism (%) [2)]	111	109		
Assesment: Energy requirement for maintenance (µmol g^{-1} FW h^{-1}) [3)]	3.3	?	18-29	?
Total energy – maintenance requirement (µmol g^{-1} FW h^{-1})	3.0-3.4	?	50-85	?

1) Assumption: 6 mol O_2 are needed for each mol hexose consumption in glycolysis linked to the TCA cycle. The ATP produced per mol glucose will be 24-36 mol ATP. The value of 24 is considering a 40% contribution by the alternative oxidase which produced 1 mol ATP instead of 3 mol ATP per mol glucose respired (Huang et al., 2005)
2) The ratio of hexose being used in anaerobic and aerobic catabolism
3) Based on Huang et al., 2005 from excised coleoptile tips with 2.5 mM exogenous glucose; and calculation from Colmer et al., 2001 from other leaf tissues (de Visser et al.,1991 and Penning de Vries, 1975)

The extra energy produced can now be compared with requirements for Na^+ and Cl^- fluxes, which can be deduced from Table 5.6. This consideration allows some speculation to what extent the deduced ATP spent on ion fluxes can give clue on what type of ion transporters are likely to be engaged during exposure to 50 mM NaCl.

Na^+ and Cl^- net uptake during salinity in anoxia

In anoxic-50 mM NaCl, there was less vigorous uptake of Cl^-. The limited ATP produced in anoxia (5-10% of the rate in air) resulted in less energy for active Cl^- uptake into the cells and therefore leading to a lower net uptake of its counter cation, Na^+. This lower uptake is presumably not due to an inadequate energy for the Cl^-/H^+ cotransporter rather there would be down-regulation of Cl^- transporters which resulted in an attenuation of Cl^- internal concentration as suggested in Greenway and Gibbs (2003).

Assessment of energy requirements for Na^+ and Cl^- fluxes

The assessed energy requirements associated with exposure to 50 mM NaCl though far from precise (section above) can still be usefully compared with energy requirements of various transporters, thus allowing speculation on which of these transporters are likely to function under anoxia. The assessment of energy requirement for Na^+ and Cl^- fluxes includes information on: (i) estimation of individual rates of Na^+ and of Cl^- influxes and effluxes, (2) the free energy of Na^+, Cl^- and H^+ movement across the plasma membrane which could determine the stoichiometry of the transporters and (3) the kind of transporters involved in the processes. Additional energy expenditure required for vacuolar compartmentation was not included in the present consideration, so as to avoid more complexity, and as will be seen below does not substantially impact on the main point to be made here in this Discussion.

Estimation of Na^+ and Cl^- influx and efflux in anoxia and air

The best method to estimate Na^+ and Cl^- fluxes would be by using ^{22}Na and ^{36}Cl tracers. However such experiments could not be carried out due to the limited time available. An approach to estimate the Na^+ and Cl^- fluxes during the combination of NaCl and anoxia was by applying the efflux-influx ratio which has been postulated by some other studies, combined with the data on Na^+ and Cl^- net uptakes for the present coleoptiles as deduced from changes in internal concentrations with time.

In aerated tissues, Na^+ fluxes are largely cyclical; the accumulation of Na^+ in the plant is very little compared to the large amount that initially enters and subsequently exits the cells (Cheeseman, 1982; Essah et al., 2003; Britto and Kronzucker, 2009). This system resulted in an efflux/influx ratio of 0.86-0.9 in rice (Malagoli et al., 2008),

0.92-0.95 in *Puccinellia tenuiflora* and wheat (Wang et al., 2009) and 0.95 in barley (Kronzucker et al., 2006). Since Na^+ efflux of the cycle is almost equal to the influx, the influx measurement could be used to estimate the approximate efflux (Britto and Kronzucker, 2009). Meanwhile, data of Cl^- fluxes in aerated barley roots with variable addition of $CaCl_2$ (calculated from Britto et al., 2004) shows an efflux/influx ratio of approximately 0.7 both for 10 mM and 100 mM external Cl^-.

Table 5.6. The estimation of Na^+ and Cl^- fluxes at 50 mM NaCl in anoxia and aerated solution.

O_2 treatments	**Cl^-**		**Na^+**	
Time of exposure to NaCl	**Influx**	**Efflux**	**Influx**	**Efflux**
Anoxia[1)]	(μmol g^{-1} FW h^{-1})			
0-2h	7.9	4.5	21.8	14.8
2-6h	2.9	1.7	3.8	2.6
6-36h	2.2	1.2	3	2.0
36-72h	1.4	0.8	2.7	1.8
72-90h	0.27	0.15	1.2	0.8
Aeration[2)]				
0-2h	30.7	21.5	88.0	74.8
2-6h	17.3	12.1	23.3	19.8
6-36h	10.3	7.2	17.3	14.7
36-72h	4	2.8	8.0	6.8
72-90h	0.33	0.23	2.1	1.8

1) In anoxia, the rate of Na^+ efflux at 0.3 mM NaCl compared to the rate at 50 mM NaCl was about 21% and 4% at early hours and end of salinity respectively. Whereas Cl^- influx for those time ranges was 63% and 33%, respectively.
2) At early hours and at the end of salinity in aeration, the Na^+ efflux rate at 0.3 mM NaCl compared to the rate at 50 mM NaCl was about 45% and 31% at early hours and end of salinity, respectively. Cl^- influx for the same time range was 45% and 30%, respectively.

The ratio of efflux/influx stated above was obtained with aerated conditions. Fortunately, some studies on different ions indicated that the efflux/influx ratio is not affected by O_2 condition (NH_4 in hypoxia by Kronzucker et al., 1998). The efflux/influx ratio of K^+ in aeration and anoxia for rice coleoptile tips was also very similar (estimated from Colmer et al., 2001). For the current study, efflux and influx over each time interval was estimated based on the data on Na^+ and Cl^- net uptake rates calculated from differences in concentration between various intervals and the ratio of Na^+ influx to efflux on rice (Malagoli et al., 2008), and Cl^- influx in aerated barley root (Britto et al., 2004) (Table 5.6).

During the early h of exposure of anoxic tissues to 50 mM NaCl, all the estimated fluxes were substantial (Table 5.6) and how the associated increase in energy demand could be coupled with despite an energy crisis will be considered shortly. Further, all the estimated fluxes of Cl^- and Na^+ greatly decreased with time (Table 5.6).

Free energy gradients for Na^+, Cl^-, K^+ and H^+ movement across plasma membrane

Information on the free energy of individual ions to move across the plasma membrane is needed to evaluate feasible stoichiometries of the transport systems, be it by H^+/ion symporter or by H^+/ion antiporter or by other coupled transporters (Table 5.7). The different free energy gradient among ions will be discussed in a later section on postulated mechanisms of solute transport.

Table 5.7. The free energy required for Na^+ and Cl^- fluxes during anoxia and in aeration

O_2 treatments	Time of exposure to NaCl	Free energy (kJ mol^{-1})[1)]			
		Na^+ Efflux	Cl^- influx	K^+ efflux	H^+ extrusion
Anoxia	0-2h	15.2	8.6	1.2	13.3
	2-6h	14.3	9.3	1.35	
	6-36h	11.9	11.7	1.39	
	36-72h	10.6	12.7	1.69	
	72-90h	10.4	12.7	1.2	
Air	0-2h	14	10.7	0.48	13.3
	2-6h	12.9	12.3	0.85	
	6-36h	10.3	15	0.97	
	36-72h	9.5	15.6	1.71	
	72-90h	9.3	15.8	2.2	

1) Based on assumption that membrane potential of rice coleoptile under aeration = -129 mV and in anoxia = -122 mV (Zhang and Greenway, 1995), at 30°C. The internal Na^+, Cl^- or K^+ concentration refer to the concentration in coleoptile tissues at different sampling time.
$\Delta G = -(zF\,\Delta\pi - RT \ln \frac{(X)_{out}}{(X)_{in}})$ (Nobel, 1974)
Where: R is the universal gas constant; T is the temperature in Kelvin; z is the valence of the ionic species; F is the Faraday's constant; $(X)_{out}$ is the concentration of the ionic species in the extracellular fluid and $(X)_{in}$ is the concentration of the ionic species in the intracellular fluid. It is assumed that the magnitude of this pH gradient is about 2.3 pH unit (apoplast pH~ 5.0; cytosol pH ~7.3).

Speculation on different transporters

Table 5.8 presents the energy available in rice coleoptiles during anoxia and NaCl exposure and 4 different scenarios of the most likely transporters involved in the rice

coleoptiles. Additional energy expenditure spent on vacuolar compartmentation was not included since there is no information on the fluxes across the tonoplast under anoxia.

Table 5.8. Total ATP required for ion transport during anoxia and in air compared to Δ ATP produced by coleoptile tips, using different assumption of transporters involved.

O_2 treatments and times of exposure to NaCl	Energy available	Different schemes on energy required			
		a	b	c	d
Anoxia		(μmol ATP g^{-1} FW h^{-1})			
0-2h	2.3	30.6	14.8	7.4	5.9
2-6h	2.5	8.4	2.6	1.3	0.8
6-36h	4.1	6.4	2	1	0.7
36-72h	3.9	4.6	3.2	1.6	1.4
72-90h	1.1	1.34	1.07	0.5	0.5
Aeration					
0-2h	50-72	136.2	74.8	74.8	60
2-6h	64-94	54.4	19.8	19.8	12
6-36h	66-97	35.3	14.7	14.7	10.5
36-72h	62-92	14.8	14.8	10.8	9.4
72-90h	62-90	2.5	2.46	2.13	1.9

1) ATP produced at 0.3 mM NaCl – ATP requirement for cell maintenance at non-saline condition (~3.3 μmol ATP g^{-1} FW h^{-1} in anoxia (Huang et al., 2005); 18-29 μmol ATP g^{-1} FW h^{-1} in aeration (Colmer et al, 2001)).
2) Different energy expenditure scenarios:
   a. With assumption of 1:1 Na^+/H^+ stoichiometry for Na^+ export; all Cl^- enters the cell via Cl^-/H^+ symport with 1:2 Cl^-/H^+ stoichiometry, and H^+ was extruded with a 1:1 H^+ pumped per ATP.
   b. With assumption that Na^+ exported from the cell via Na^+ /H^+ antiport with 1:1 stoichiometry of Na^+ /H^+, Cl^- influx could be passive at high external Cl- and before the cytoplasmic Cl- concentration has stabilized at higher levels (Teakle and Tyerman, 2010) during 0-36 h. Subsequently between 36-90 h, Cl^- entered cells via Cl^-/H^+ symport with 1:2 Cl^-/H^+.
   c. Similar to assumption b), except the assumption that the H^+-ATPase stoichiometry during energy deficit was 2:1 pumped per ATP (Warncke and Slayman, 1980).
   d. Similar to c) except 30-70% Na^+ was exported from the cell via Na^+ K^+2Cl^- co-transporter (i.e. with 1:1:2 stoichiometry). The rest of Na^+ was transported via H^+/Na^+ antiporter.

Taking the assumption that the Na^+ efflux rate during first hour in anoxic-salinity, the Na^+/H^+ antiport has a stoichiometry 1:1 (Malagoli et al., 2008) (scheme a Table 5.8), then H^+ influx into the cytoplasm would be equal to Na^+ efflux. The maximum energy needed to extrude this incoming H^+ would be either 14.8 μmol g^{-1} FW h^{-1} or 7.4 μmol g^{-1} FW h^{-1} if the H^+/ATPase under energy deficiency has a stoichiometry of 2:1 (Warncke and Slayman, 1980) (scheme c Table 5.8). Even the lower values exceeded

the different energy produced by anoxic coleoptile tips at 50 mM NaCl compared to 0.3 mM NaCl (Table 5.8).

This section discusses likely mechanisms of regulating Na^+ and Cl^- transport at the plasma membrane during early exposure to high NaCl and during the period when cells reach the quasi steady state. The energy required to drive various transport system is compared with the assessed energy available for the solute transport as deduced from increases in rate of catabolism and saving energy by reduction of growth, while the important role of changes in 'permeability' with time of exposure is also considered.

The initial period after a sudden increase in NaCl

The main demands for the energy expenditure on ion fluxes will be the Na^+ efflux, Cl- influx and or H^+ extrusion required if H^+ was co-transported on the Na^+/H^+ antiport and or Cl^-/H^+ symport. Therefore the huge differences between estimation of energy expenditure required and increase in energy measured (by extrapolating the measured rates of ethanol formation to ATP formation and growth reductions to re-allocation to other processes) in coleoptile tips at 0.3 and 50 mM NaCl can be used to suggest a Cl^- uptake scenario:

During early exposure to 50 mM NaCl, when the Na^+ influx and efflux were high, the possible involvement of Cl^-/H^+ symport in Cl^- uptake was unlikely, since the required H^+ extrusion would exceed the energy produced during anoxia. Instead, an initial entry of Na^+ along its free energy gradient may depolarize the plasma membrane and hence together with the concentration gradient into the cell would create a down-hill gradient for Cl^-, rather than the usual uphill gradient for Cl^- (Teakle and Tyerman, 2010) (scheme b Table 5.8). So, during the period of acclimation (0-36 h), Cl^- influxes would not cost energy. The possible transporter adopted during early hours are CLC (Chloride channels). In contrast, other transporter such as NKCC (1N1K2Cl) symporter is less likely as free energy produced by $1Na^+$ and $1K^+$ would not be sufficient to drag $2Cl^-$ against its electrochemical gradient. Later, during exposure to NaCl (e.g. 36-90 h), the plasma membrane needs to repolarize to resume other important transport, and then any further Cl^- uptake would cost energy, i.e. using H^+/Cl^- or $2H^+/Cl^-$ symporter.

Consideration for the quasi steady state

During longer time exposure to NaCl (36-90 h), since internal Cl^- has also risen, the free energy gradient for Cl^- will become uphill, by the combined effects of dissipation of the concentration gradient and repolarization. So, any further Cl^- uptake would cost energy, i.e. using H^+/Cl^- or $2H^+/Cl^-$ symporter. Presumably the Cl^- channels have a low permeability for Cl^- to prevent a large Cl^- efflux (Hochachka, 1986; Colmer et al., 2001). Otherwise, a high Cl^- influx would be needed and the likely Cl^-/H^+ co-transporter (Nguitragool and Miller, 2006; Jenning and Cui, 2008; Lisal and Maduke, 2009; Zifarelli and Pusch, 2009) would increase the energy requirements for H^+ extrusion, which is unlikely during an energy crisis.

Further Na^+ increases, which still would flow along a down-hill free energy gradient, would have to be mitigated, presumably via Na^+/H^+ antiport to extrude Na^+ (Table 5.8). Under anoxia, the expenditure of energy required for H^+ extrusion may be fairly small as long as there is a low permeability of the plasma membrane for energy independent Na^+ influx.

Another possible transporter for Na^+ and Cl^- efflux is the NKCC (1Na1K2Cl) transporter. This transporter is feasible since the free energy release of the Cl- efflux exceeds that of the energy required for K^+ and Na^+ extrusion of $2Cl > Na^+ + K^+$ during 0-90 h. Yet, this scenario is only useful if a substantial Cl^- efflux would be inevitable, i.e. when the membrane had a substantial permeability to Cl^-. Otherwise, any Cl^- efflux via this transporter would not save energy, since the Cl^- has to be reaccumulated via H^+/Cl^- or $2H^+/Cl^-$ symporter and hence involve more H^+ extrusion (scheme d Table 5.8).

Comparing the energy requirement in scheme a-d (Table 5.8) with the estimated energy available for ion transport (as discussed previously, the assessed maximum amount of energy available without jeopardizing survival in these coleoptile tips is assessed at 3.2 µmol ATP g^{-1} FW h^{-1}), so the energy available between 2-90 h would be more than sufficient to meet energy demands in all three schemes (b, c and d). Only preposition a (Table 5.8) would be very unlikely, i.e. transport is unlikely to be based on the conventional H^+/Cl^- symport, H^+/Na^+ antiport and a requirement of 1 ATP per mol H^+ extruded. Though at times later than 2 h after exposure, scheme b-d are all feasible, in an energy crisis the best economy in energy expenditure would be preferable, e.g. in the scheme c, proposing export of 2 rather than 1 H^+ for 1 ATP would be of substantial advantage.

Conclusion

The study demonstrated that excised coleoptile tips from rice seedlings have the ability to survive under the combination anoxia and 50 mM NaCl without significant injury, by producing extra energy as well as re-allocating energy diverted from growth which was of bigger magnitude than the extra ethanol production, to accomplish the higher energy requirement associated with Na^+ and Cl^- fluxes. This was indicated by the production of extra energy during the period when those ion fluxes increased prior to reach a quasi-steady state. Although Na^+ and Cl^- net uptake rates of anoxic coleoptile tips at 50 mM NaCl were much lower than those in aerated solution, which mean having less opportunity to employ 'cheap' osmoticum to maintain low osmotic potential, coleoptile tips were able to maintain ~ 70% organic solutes production of those at 0.3 mM NaCl as osmolytes. The discussion has argued that high NaCl during energy crisis may be partly coped with by transporter mechanisms which are efficient in energy consumption, but reductions in permeability to Cl^- and Na^+ of the plasma membrane might play an even more important role (not assessed in the present study).

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Appendix 1.

Coleoptile tips growth in aerated solution

The current study showed that the respiration and therefore the energy production per hour of these aerated rice coleoptiles in 50 mM NaCl was about 15% higher, which would produce ~12 µmol g^1 FW h^{-1} more ATP at 50 mM NaCl than at 0.3 mM NaCl (see Table 5.5). To assess whether this stimulation of respiration was associated with the increased growth, an assessment of energy requirements for maintenance in the coleoptiles at 0.3 mM NaCl is needed. Since then we can assess the energy requirement for growth by the difference between the energy produced by the observed rate of respiration and the assessed maintenance requirement. The minimum estimate of energy expenditure for growth would arise when we take the highest level of energy requirements for maintenance. These can be deduced from the data by de Visser (1991) and Penning de Vries (1975) as summarized by Colmer et al., (2001); Greenway and Gibbs (2003) (see Table 5.5). Taking the highest level of energy requirement for maintenance of 29 µmol ATP g^1 FW h^{-1}, would still mean that at 0.3 mM NaCl 39-85 µmol ATP g^1 FW h^{-1} was spent on growth. Yet, a 2 fold stimulation of growth at 50 mM NaCl was associated with an assessed increase of only ~12 µmol ATP g^{-1}FW h^{-1}. Energy cost for maintenance are unlikely to be lower at 50 than at 0.3 mM NaCl, so the most likely possibility is at 50 mM NaCl most of the increased growth was related to increased vacuolation, which will cost less energy than polymer synthesis. This could be an artifact of the coleoptile tips.

Respiration rates

In continuous aerated solution, there was also a 14-20% increase in catabolism, in this case measured by rate of O_2 uptake at 50 mM NaCl compared to 0.3 mM NaCl. The increase of respiration rate at 50 mM NaCl started from 24 h to the end of NaCl exposure. Different from anoxia, it seemed that there was no specific time-frame of salt-induced catabolism up to 90 h. During exposure to NaCl, more energy is required by cells to accomplish ion uptake and compartmentalization, cell maintenance and in this particular case increased growth. As with anoxia, in aeration, Na^+ and Cl^- in tissues also reached a quasi-steady state level following an initial rapid rise in Na^+ and Cl^- concentrations. However, the time needed to reach this quasi-steady state in aeration was less than that in anoxia (24 h and 72 h respectively), while internal Na^+ and Cl^- concentrations were approximately 30% higher in aeration than in anoxia.

Using Na^+ and Cl^- ions as osmoticum will be beneficial for rice coleoptiles to lower its osmotic potential to achieve osmotic adjustment. Na^+ and Cl^- when derived from high external concentrations may cost less energy than the alternatives of synthesis of organic solutes, leaving these metabolites for other metabolic purposes. Since Na^+ and Cl^- are cheap osmoticum compared to organic solutes, the faster and higher ion uptakes in aeration were efficient mechanisms for rice coleoptiles to survive under moderate salinity level.

Ionic balance

In aerated solution, the amount of ($Na^+ + K^+$) not balanced by Cl^- particularly at 0.3 mM NaCl was about 2-fold of those in anoxia. The malic acid concentrations at 0.3 mM NaCl (~ 17 μmol g^{-1} FW) which was about 3-fold of that at 50 mM NaCl and Pi concentration (41 and 21 μmol g^{-1} FW at 0.3 and 50 mM NaCl respectively) indicated that malate and Pi may served as counter-anion for the high K^+ concentration.

CHAPTER 6

Concluding Discussion

This thesis investigated the salt tolerance mechanisms of germinating rice seedlings during energy crisis in anoxia. Before discussing the results presented in the preceding three experimental chapters, some key characteristics associated with the growth system used in this study (i.e. non-transpiring plants) are contrasted with the one commonly used by many other studies (i.e. transpiring plants). Following this discussion, there will be a comparison of ethanol production by intact seedlings and excised coleoptile tips and the relevance to energy requirement for cell maintenance in both these materials (i.e. excised coleoptile tips and intact shoots of rice seedlings) used in this study.

Rice seedling resistance to NaCl in submerged aerated solution (non-transpiring plants) compared to other studies in aerial conditions (transpiring plants)

Plant responses to salinity in different growth systems

Salt tolerance mechanisms in transpiring plants have been reviewed elsewhere (Greenway and Munns, 1980; Drew and Dikumwin, 1985; Blumwald, 2000; Flowers, 2004; Castillo et al., 2007; Munns and Tester, 2008; Horie et al., 2012). In these transpiring non-halophyte plants, salinity damage is mainly due to the high amounts of Na^+ and Cl^- in leaves. The higher salt concentrations in the older leaves may entirely result from a cumulative transpiration stream to those older tissues, as only a small portion of Na^+ is re-translocated from shoots to roots (Greenway and Munns, 1980; Flowers and Yeo, 1986; Munns and Tester, 2008).

In the current study in submerged non-transpiring conditions, rice seedling responses to high NaCl exposure, i.e. growth and ion net uptakes, were different from some of those commonly reviewed in transpiring plants (Chapter 3). In this study, seedlings were grown submerged in aerated and anoxic nutrient solution (non-transpiring conditions). Rice germination and seedling growth tolerated 200 mM NaCl, much higher than generally reported for rice, where no germinating seeds were found at 80-200 mM NaCl (Heenan et al., 1988; Punyawerdana and Dharmasri, 1989; Lutts et al., 1995; Khan et al., 1997; Hakim et al., 2010). Furthermore, the pattern of Na^+ and Cl^- shoot concentrations which increased rapidly during initial hours and then remained in quasi-steady state or slightly decreased differed from that in transpiring plants which accumulate increasingly high Na^+ and Cl^- concentrations in the shoots with time. For example, young (14 days old) transpiring rice plants exposed to 200 mM NaCl for 4 days resulted in an accumulation of tissue Na^+ to ~260 μmol g^{-1} FW in roots and ~420

μmol g^{-1} FW in shoots, i.e. with a further Na^+ concentration increase with time (calculated from data in fresh weight basis by Cha-um et al., 2010). In a similar concentration and time range with that study, Na^+ concentration in roots and shoots of non-transpiring submerged rice seedlings was about the same as the external NaCl concentration (~200 mM) (Chapter 3). At 50 mM NaCl, the fresh weight reduction in shoots of transpiring rice seedling was 40-60% compared to those at non-saline condition (Yeo and Flowers, 1985), whereas no significant growth reduction was found in non-transpiring rice seedlings (Chapter 3).

Consideration of responses of non-transpiring plants can make use of the studies of arabidopsis that is traditionally used as a model species for molecular genetic aspects of salinity tolerance (e.g. by Zhu et al., 1998). The results from Zhu et al. (1998) for 4 d old arabidopsis growing on vertical agar plates then transferred to shaken liquid cultures containing 50 mM NaCl for 48 h in non-transpiring conditions (i.e. for Na^+ and Cl^- analysis) showed that in this non-transpiring condition, salinity tolerance in arabidopsis was unrelated to the extent of shoot Na^+ accumulation (Zhu et al., 1998; Moller and Tester, 2007). The low Na^+ concentration in non-transpiring arabidopsis shoots is presumably associated with the lack of transpiration and the more facilitated ion exclusion in this arabidopsis via exchange with external medium during the 48 h. Furthermore, comparing Na^+ concentration in arabidopsis genotypes is probably better to express in fresh weight rather than dry weight basis since different genotypes may perform various initial growth rate (i.e. turgor could develop faster in cultivar with more rapid ion uptakes) and therefore different water content. Adding to this, the sampling time is another crucial matter since at 48 h, Na^+ and Cl^- concentrations in shoots may have not yet reached the maximum levels in arabidopsis. These ions could be easily exchanged with those in external solutions, hence after certain period (~72 h in rice seedlings, Chapter 3) the net ion uptake is likely to become very small and possibly even declines in the tissue ion concentrations could occur under these non-transpiring conditions. Thus, assessing Na^+ concentrations in non-transpiring plants for salt tolerance might be best determined at steady state levels and use of this non-transpiring condition might even have advantages for some experimental objectives, such as possibly with better control of shoot tissue Na^+ concentrations over time enable better to assess possible Na^+ toxicity thresholds than for leaves of transpiring plants.

Different sensitivity between shoots and roots to NaCl exposure in the two systems (transpiring and non-transpiring plants)

It has been suggested elsewhere that shoot growth (i.e. in aerial condition) is intrinsically more sensitive to salinity than root growth (Sharp et al., 1988; Munns and Tester, 2008). The more vulnerability of shoots than roots growth to salinity is generally indicated by the ability of roots to continue to grow in salinity, while shoots growth are inhibited, thus leading to a higher root:shoot ratio under salinity as compared with non-saline control (Munns and Termaat, 1986; Katsuhara et al., 2003). Different from shoots, the root ion concentrations do not continue to increase with time and often have lower Na^+ and Cl^- concentrations than in the external solution, which rarely happens in leaves (Munns, 2002).

On the contrary, in non-transpiring plants, a larger root than shoot growth inhibitions demonstrated that roots are more sensitive than shoots to salinity. When in contact with the external solution, shoots and roots could both exchange ions with the external solution to reach equilibrium. However the less negative π_{sap} with less sugar accumulation in roots than in shoot at all external NaCl concentrations (Chapter 3) indicated that ions are the main contributor for root $\pi_{sap.}$ In the prolonged high salinity, the gradual decrease in internal ion concentrations (due to diminishing ion net uptakes and fresh weight increments) would dramatically reduce roots turgor pressure, and hence growth would be restricted.

Rice seedling resistance to NaCl in submerged anoxic solution

Growth responses of rice seedling

The ~2 days germination delay in salinity, as well as 20% shoot growth reduction in rice seedlings at 75 and 100 mM NaCl (Chapter 4) would be a disadvantage for rice seedlings in view of survival under saline flooded soil, since delayed growth would severely increase the time taken, as well as reduce possible emergence to reach the water surface. The estimated turgor pressure (P) of anoxic shoots at 100 mM NaCl was about 60% of that in non-saline anoxic solution (Chapter 4), much lower than the value found in aerated solution (~ 85% of value at aerated non-saline solution) (Chapter 3). Thus, the energy-dependent osmotic adjustment was less in anoxic than in aerated shoot tissues.

Ethanol production in intact seedlings and excised coleoptile tips in combined 50 mM NaCl and anoxia could be associated with Na^+ and Cl^- net uptake rate

During 24-70 h NaCl exposure, ethanol production from both intact seedlings and excised coleoptile tips at 50 mM NaCl showed 8-15% increase on FW basis compared to the production at 0.3 mM NaCl. However, this extra ethanol was not produced during the first hours in salinity by both intact seedlings and coleoptile tips at 50 mM NaCl. The higher ethanol at 50 mM NaCl than at 0.3 mM NaCl was still produced by excised coleoptile tips at 70-72 h after NaCl was imposed, while at this stage there was no more increase of ethanol production in intact seedlings.

The extra ethanol production by tissues at 50 mM NaCl than at 0.3 mM NaCl was getting less when approaching the quasi-steady state for tissue ion concentrations. Since Na^+ fluxes are almost cyclical, with very little of Na^+ accumulated in plant tissues compared to the entry due to large amounts being exported from the tissues (Cheeseman, 1982; Essah et al., 2003; Flowers and Colmer, 2008; Britto and Kronzucker, 2009), a low Na^+ uptake during quasi-steady state implies a low Na^+ influx and efflux, a notion supported by the downturn of K^+ fluxes in anoxic rice coleoptiles (Colmer et al., 2001). With low ion fluxes, less energy would be required for cell maintenance associated with Na^+ extrusion or Cl^- uptake, and so ethanol production might then have declined owing to a lower requirement for ATP after ion fluxes subsided.

Fig. 6.1 shows trends of ethanol production in intact seedlings and excised coleoptiles tips. In both materials, a 15-20% extra ethanol was produced by seedlings at 50 mM NaCl than at 0.3 mM NaCl from 24 h to 60-80 h after NaCl exposure. This time length in ethanol production is suggested to be associated with the time interval needed to reach quasi-steady state Na^+ tissues concentrations in both shoots of intact seedlings and excised coleoptile tips. That extra ethanol was no longer produced by tissues at quasi-steady state indicated that less energy was then required when Na^+ net uptakes were very low or close to zero.

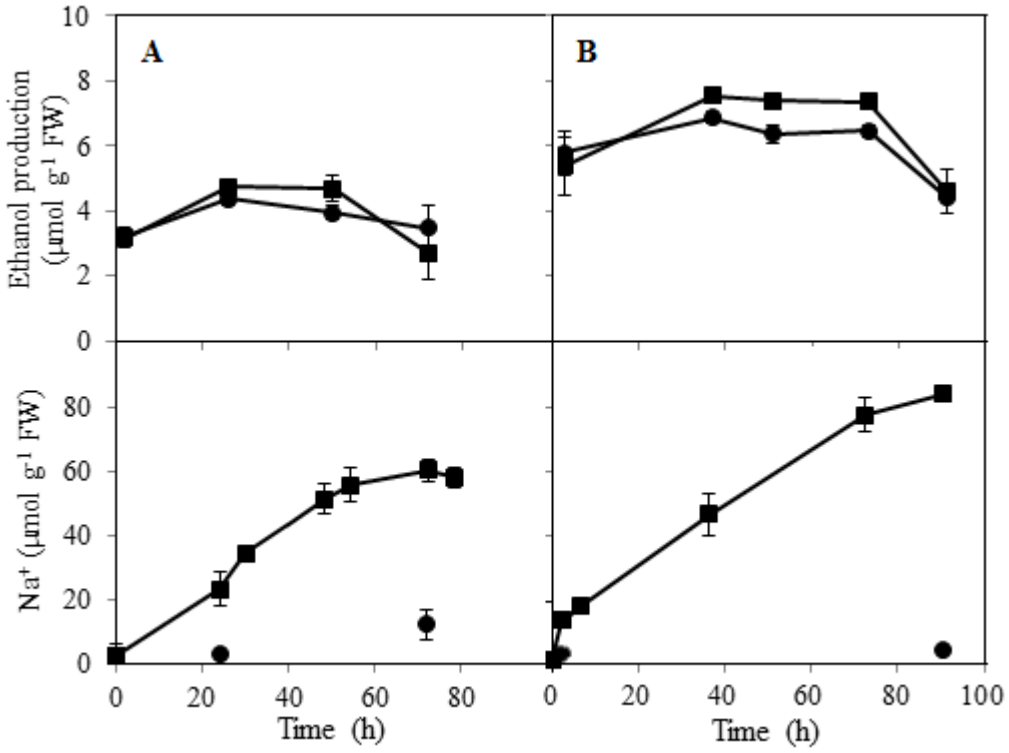

Figure 6.1. Ethanol production and Na^+ tissue concentrations in shoots of intact rice seedlings in Chapter 4 (A) and excised coleoptile tips in Chapter 5 (B) during NaCl exposure 0 – 90 h in anoxia. Tissues in non-saline solution, filled circles; tissues at 50 mM NaCl, filled squares.

Based on assumptions on the rates of Na^+ influx and efflux as stated in Chapter 5, the high energy spent on H^+ extrusion was likely the reason to induce more catabolism by rice seedlings in combined anoxia with salinity. If the Na^+ efflux rate during the first hour in anoxic-salinity (~ 15 µmol Na^+ g^{-1} FW h^{-1}) would be extruded via Na^+-H^+ antiport, possibly SOS1, provided the translocation stoichiometry of 1:1 (Malagoli et al., 2008) H^+/Na^+ for the antiporter, each mol Na^+ extruded would result in 1 mol of H^+ entry into the cytoplasm. The maximum energy needed to extrude this incoming H^+ especially during a rapid uptake of Na^+ (i.e. with cyclical fluxes, high Na^+ influx also means high Na^+ efflux) was estimated to have exceeded ~ 2-fold of the available energy produced by coleoptile tips at 50 mM NaCl (see Chapter 5).

Model of factors contributing to rice seedling tolerance to NaCl exposure in non-transpiring aerated and anoxic solution

The main objective of this thesis was to elucidate the salt tolerance mechanisms of germinating rice seedling during energy crisis in anoxia. Using what is known of rice seedling tolerance to anoxia and salinity individually, and the results presented in the

preceding three experimental chapters, the conceptual model of salt tolerance and anoxia tolerance mechanisms in rice seedlings presented earlier (Chapter 2; Fig. 2.1) has been extended to include rice seedling tolerance under a combination between salinity and anoxia (Fig. 6.2).

In non-transpiring submerged rice seedlings, the ability of shoots to extrude Na^+ and Cl^- by exchange with the external solution leads to a quasi-steady state in internal Na^+ and Cl^- concentrations (Chapter 3) and therefore mitigates accumulation of these ions in both shoots and roots. This pattern of ion exclusion in non-transpiring rice seedlings is more similar to roots than shoots in transpiring plant under salinity.

During anoxia, ATP produced by rice seedlings was much lower in anoxia than in air, implying an energy crisis (Greenway and Gibbs, 2003; Chapter 5). With an additional adverse factor of salinity (Chapter 4), any energy deficit in anoxic rice seedlings may be aggravated, since plants have to cope with additional cell maintenance associated with ion exclusion and compartmentalization. During anoxia, rice seedlings were able to grow and tolerate at least to 100 mM NaCl. However with less energy available, the maximum Na^+ and Cl^- tissue concentrations were ~30% less than those in aerated solution. The lower Na^+ concentration in shoots of non-transpiring rice seedlings under anoxia than that in seedlings submerged in aerated solution was quite different from transpiring rice which had higher Na^+ concentrations in the transpiring shoots of rice when the roots were in O_2-deficient solution than in controls with aerated solution around roots (John et al., 1976 in rice; Barrett-Lennard, 2003 in wheat). In those other studies, the substantial increase in Na^+ concentrations were argued to be associated with the susceptibility of Na^+ efflux, since the energy costs of Na^+ efflux are prohibitively high (Kriedemann and Sands, 1984 in sunflower; Drew and Dikumwin, 1985 in maize; Barrett-Lennard, 2003 in wheat). Overall, the results from the current study showed that the susceptibility of Na^+ efflux in submerged rice seedlings under anoxia could be mitigated by down-regulation of Na^+ uptake.

To further elucidate this energy production and consumption, homogenous tissues are required. Excised coleoptile tips provide a good system for evaluating mechanism of anoxia tolerance in plants (Huang et al., 2005). The reliability of excised coleoptile tips to study salinity and anoxia was investigated (Chapter 4) and the results indicated that under NaCl exposure up to 50 mM, excised coleoptile tips provide a suitable system to further investigate ion net uptakes and energy produced during anoxia.

Energy allocation has been assessed to different metabolic processes such as energy requirement for cellular maintenance associated with Na^+ exclusion and Cl^- uptake (Chapter 5). Compared to aerated solution, Na^+ and Cl^- uptake by excised coleoptile tips were much lower in anoxia, leading to about 30% lower tissue Na^+ and Cl^- concentrations at quasi steady state. During this rapid uptake of ions, higher ethanol production and growth inhibition in excised coleoptile tips at 50 mM NaCl than in 0.3 mM NaCl indicated that during this energy crisis, energy allocation for growth was shifted to cell maintenance (i.e. including regulation of tissue ion composition). Exposure to higher NaCl concentration, i.e. 100 mM NaCl, caused a remarkable K^+ leakage which lead to an extremely low K^+/Na^+ ratio and hence a very low turgor pressure (~0.1 MPa) and likely ion toxicity in the cells.

In contrast to anoxia, in aerated solution, the growth of excised coleoptile tips was stimulated with addition of 50 mM NaCl. Rapid Na^+ and Cl^- accumulation was presumably beneficial as cheap osmolytes to develop turgor and therefore stimulate volume expansion. More energy required during this period may be associated with faster growth. At higher NaCl concentrations up to 200 mM NaCl in aerated solution, however, growth was inhibited but seedlings survived (Chapter 3). Decrease of tissue ion concentrations during prolonged salinity to 186 h (i.e. due to low ion net uptakes and fresh weight increments) diminish the ability of rice seedlings to adjust osmotically, hence growth was likely inhibited due to the drop of turgor pressure.

Future research prospects

Given the lack of research attention focused on rice responses in a combined salinity and anoxia, this thesis raises several areas of research which would be essential to understand if we are to gain a more complete understanding of the responses of rice under this energy crisis.

a. Protein contents with time of the shoots and endosperms need to be assessed, especially during the rapid increase and quasi-steady state of ethanol production. Analysis using fresh weight basis raise further question: to what extent different salinity treatment affect protein synthesis, since ethanol formation would be best expressed on protein basis (Greenway and Gibbs, 2003).

b. Assessment of the energy requirement for cell maintenance during combined salinity and anoxia need measurements on the Na^+ and Cl^- influx and efflux,

especially during rapid ion uptakes in early hours of NaCl exposure and a period just prior to and during the quasi-steady state. To get a more accurate data, these ions fluxes would be best estimated using radio-isotope tracers.

c. In rice, functions for many HKT-like genes identified remains largely unknown. For example, OsHKT2;1 gene which is responsible for a major portion of Na^+ uptake, does not cause Na^+ toxicity, owing to a rapid down-regulation of the OsHKT2;1 transporter upon high internal Na^+ concentration to prevent Na^+ toxicity (Horie et al., 2007). It is suspected that these genes may be down-regulated when Na^+ concentrations reach quasi-steady state (Chapter 3, 4 and 5). Studies on the up and down regulation of these genes in relation with the dynamic in Na^+ concentrations in tissues of rice seedlings during NaCl exposure in non-transpiring system will give new insight on the roles of these genes under high NaCl exposure.

d. The possibility of Na^+ transporters (NKCC) down-regulation in hypoxia and anoxia is currently reported in human and animal cells, but no relevant information is available on plant cells. In the current study, evidence of much lower Na^+ net uptake in anoxic rice seedlings open a new prospect of Na^+ transporters down-regulation during energy starvation. Future molecular studies on the expression levels and functions of some Na^+ transporters during anoxia are required to address this issue.

e. In aerated condition, the measured π_{sap} of the shoots at 100 mM NaCl during the subsequent period (138-162 h after sowing) which decreased by~ -0.08 MPa (data not shown) might also indicate that turgor pressure was hard to be maintained during the prolong period at this high NaCl concentration, particularly when internal Na^+ and Cl^- concentrations kept decreasing with time. It is interesting to see in further studies whether longer exposure of salinity will again increase net ion uptake. In anoxia, the prolonged salinity could also be used to test the stimulation of ethanol production during rapid Na^+ uptake.

f. Different time length required by shoots and roots, and by shoots and excised coleoptile tips, to reach a quasi-steady state in internal Na^+ concentrations indicated that this time length is possibly organ specific. More detailed studies would be essential to determine the effect of cell vacuolation in different tissues on time needed to reach internal Na^+ maximum concentrations, as well as time required for removing tissue Na^+ content upon return to non-saline conditions.

g. Is it worthwhile to use other cultivars more tolerant to anoxia (and salinity) than Amaroo to check a few key points such as degree of tolerance, rate of ethanol formation and degree of growth stimulation by low salinity.

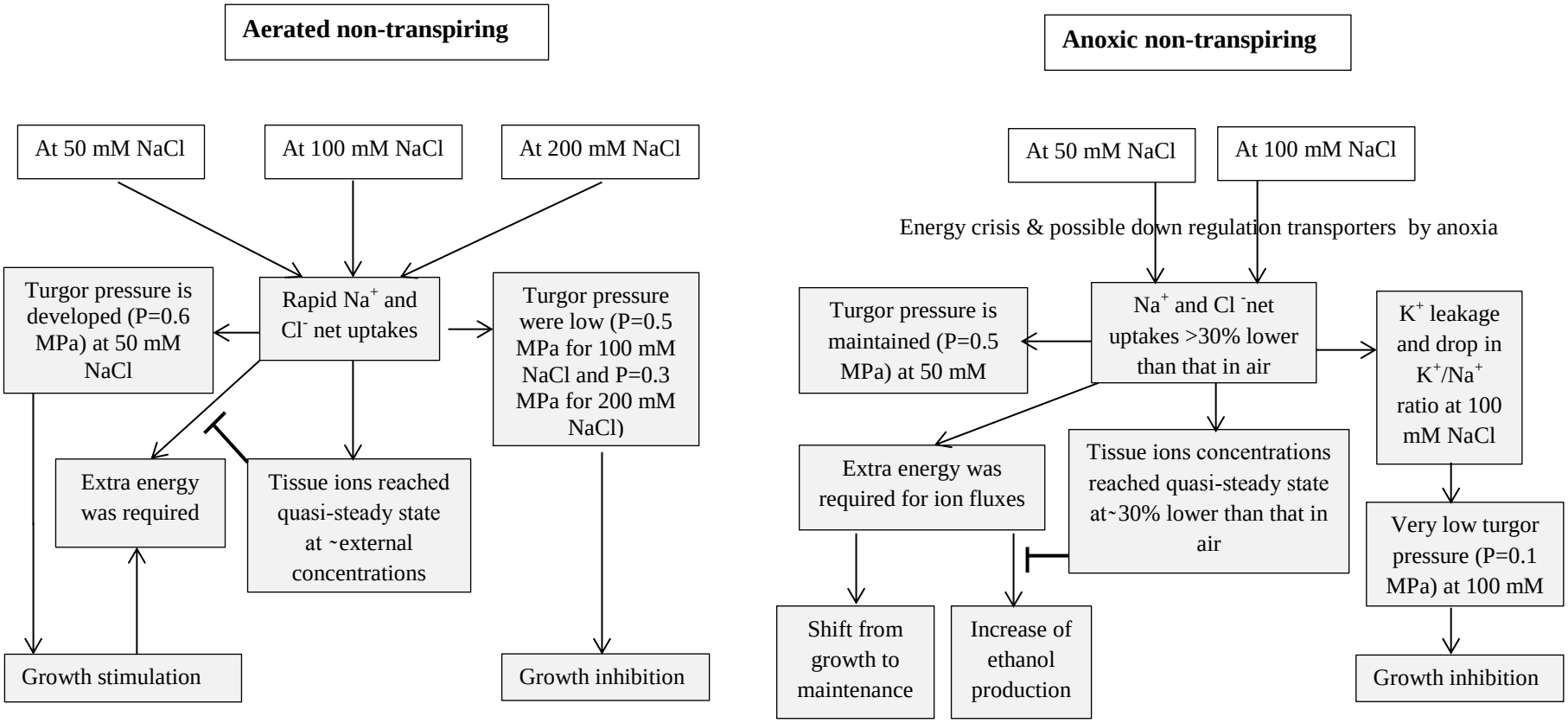

Figure 6.2. Conceptual model of factors contributing to rice seedling tolerance to NaCl exposure in non-transpiring (i.e. submerged) aerated solution (left) and anoxic solution (right). Shaded components indicate factors which were investigated to some extent in this thesis.

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