Physiological and Biochemical Mechanisms of Salt Tolerance in *Sesbania rostrata* Brem. & Oberm.

Division of Applied Biochemistry

Doctoral Degree Program in Agricultural Sciences

University of Tsukuba

Jarunee Jungklang

Contents

Chapter 6. General Discussion and Conclusion

Abbreviations

S. rostrata	*Sesbania rostrata* Brem. & Oberm.
P. vulgaris	*Phaseolus vulgaris* L. cv. Meal
AOS	Active oxygen species
$^{\bullet}OH$	Hydroxyl radicals
$^1O_2$	Singlet oxygen
O_2^-	Superoxide
H_2O_2	Hydrogen peroxide
CAT	Catalase
SOD	Superoxide dismutase
GR	Glutathione reductase
APX	Ascorbate peroxidase
MDHAR	Monodehydroascorbate reductase
DHAR	Dehydroascorbate reductase
GPX	Glutathione peroxidase
AsA	Ascorbate
MDA	Monodehydroascorbate
DHA	Dehydroascorbate
GSH	Reduced glutathione
GSSG	Oxidized glutathione
PSI	Photosystem I
PSII	Photosystem II

List of Tables

List of Figures

Chapter 1
General Introduction

1.1 Introduction

Saline soil is a serious problem which affects the yield of commercial crops in the Northeastern part of Thailand (Division of Soil Analysis 2002). This type of soil is scattered throughout most provinces in the Northeast and ranges from severe to low concentrations. The total area affected is estimated to be 17.8 million rai (2.8 million hectares) or approximately 16.7% of the total Northeastern land area. Most saline soils appear in areas of paddy fields (Land Development Department 1996a). The dominant accumulated salt is NaCl (Fitter and Hay 1987).

To reduce the devastating effects of salinity on crops, particularly in rice fields, many trials have been conducted. One of the biological approaches is to use halophytes as cover plants. Salty encrustations are generally found amongst soil texture in the affected areas (Division of Soil Analysis 2002). This is normally a consequence of strong dehydration of soil water containing high concentration of salts. This occurrence appears regularly, particularly in the dry season. By protecting the soil surface with cover plants, the loss of water is reduced. *Sesbania rostrata* Brem. & Oberm. has been used as a promising plant as this plant can tolerate salinity levels up to 0.4 – 0.5% of NaCl (Mahmood 1998, Wongwattana *et al.* 1998). *S. rostrata* has also been recommended by the Thailand Department of Land Development as the highly effective green manure to improve saline soil before growing rice, with a resultant increase of 20% in rice production (Land Development Department 1996b). This plant has symbiotic nodules of *Rhizobium*, which form on both the stems and roots fixing atmospheric N_2. Biomass production of *S. rostrata* is very rapid (Matoh *et al.* 1992), and the symbiotic N_2 fixation of this species is significant (Roger and Watanabe 1986).

However, a few studies have been conducted on physiological and biochemical responses induced by salt stress in *S. rostrata*. These

understandings are fundamental for use the species in saline environments. The effects of salt stress on many crop species have reported in numerous scientific papers and journals. At the relatively lower concentration of NaCl (100 mM), dry weight was significantly declined in salt-sensitive species (Flowers 1972, Warwick and Halloran 1992). Rate of photosynthesis was reduced in many species under salt stress (Chavan and Karadge 1986, Marler and Mickelbart 1993). Halophytic plants respond to salinity by accumulating Na and Cl, particularly in the older leaves (Pardossi *et al.* 1999). Moreover, sequestering excess Na and Cl ions into the vacuole (Barkla *et al.* 1995, Apse *et al.* 1999) and inducing compatible solutes such as proline, glycinebetaine in the cytoplasm (Marcum and Murdoch 1994, Girija *et al.* 2002) are well known as an important mechanism of salt tolerance in the cells. The total uptake of K, Ca and Mg decreased in salt-sensitive species with increasing salinity (Rabie and Kumazawa 1988). The production of active oxygen species (AOS) is one of the characteristic biochemical changes during salt stress (Vaidyanathan *et al.* 2003). When plants are subjected to the stress, the balance between the production of AOS and the quenching activity of the antioxidant is upset, often resulting in oxidative stress. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Shalata and Tal 1998, Dionisio-Sese and Tobita 1998, Bor *et al.* 2003).

1.2 Background

1.2.1 Saline soils and *Sesbania rostrata* in Northeast Thailand

The saline soils are a serious problem in many yields of economic crops in Thailand, particularly in northeastern part. As noted in the introduction, it was estimated by the Department of Land Development that the saline soils cover 17.8 million rai in the northeast (Land Development Department 1996a). Moreover, there are 19.4 million rai (3.1 million hectares) which is at increasing risk for the spread of soil salinity.

The saline soil can be divided into 4 types (Land Development Department 1996c).

1. High-level saline soil area is where salty dust is found amongst the soil texture in more than 10% of the area. Plants cannot be grown and the area is classified as un-arable.

2. Middle-level saline soil is in the area where salty dust is found among soil texture 1 – 10% of the area. Plants can be grown but crops yield is significantly reduced to the point of being un-economical.

3. Low-level saline soil is where salty dust is found among soil texture in less than 1% of the area. Subsurface water flow is brackish or briny with a depth of about 2 m from soil surface. Such areas are usually utilized as paddy field.

4. Areas susceptible to the spread of soil salinity. Such areas are located in the highlands where crops are planted. Salty dust is not found among the soil texture but below the soil, where crystalline salty stones are present. When rain falls, water from soil surface flows past the salty stone layers, becomes salty, and then flows to the adjoining cultivated area.

The Land Development Department has been trying to mitigate the salinity effects to allow the low-level and susceptible land to be utilized in an economic manner.

S. rostrata generally grows in water-logged soils in Senegal, Africa (Ndoye *et al*. 1996). Due to its vigorous growth, flood and salt tolerance, this plant has been used as a potential green-manure plant for lowland rice in Senegal, Japan and Philippines (Ndoye *et al*. 1996, Saraswati *et al*. 1992, Ladha *et al*. 1989). The research group of the Land Development Department, Thailand has also investigated the use of *S. rostrata* as a green manure in rice fields, particularly in the saline soils of the northeast, for 6 years. They have found that *S. rostrata* did not have a detrimental effect on the environment but seemed to be a significant factor in an increase of 20% in rice production for the area.

1.2.2 Plants and salt stress

Most of the salt stresses in nature are due to Na salts, particularly NaCl (Fitter and Hay 1987). The term *halophyte, salt-tolerant plant* literally means plants that can grow in the presence of high concentrations of Na salts. Plants that cannot grow in the presence of high salt concentration of Na salts are called *glycophytes, non-halophyte, non-tolerant plant, susceptible plant* or *salt sensitive plant*.

It is generally accepted as a fact that high salts can induce injury in plants. Levitt (1972) classified salinity effect in terms of primary stress and secondary stress. The primary stress includes membrane damage and metabolic disturbance. The secondary stress includes osmotic stress and nutrient deficiency stress. When plants are exposed to these stresses, some physiological and biochemical responses are induced to ameliorate such stresses and damage. Their capacities are different depending on plant species. Physiological mechanisms are usually complex and require the functions of many gene products. In contrast, the biochemical mechanisms are relatively simple, typically involving the action of only a few gene products.

1.2.2.1 Mechanism of salt tolerance

Under saline conditions, high salinity can affect the water and nutrient uptake in plants. When the solute concentration of the water surrounding a plant's roots is suddenly raised, the immediate effect is to reduce the water potential gradient between solution and root. Since water moves into roots down such a gradient, this will inhibit water uptake. If external water potential is lower (more negative) than the internal, water uptake will cease. In the short term this will reduce growth, in the long term it will cause wilting and ultimately death. Plant roots absorb ions from a complex medium, containing not only the dozen or so essential nutrient ions, but also a range of non-essential ions and organic compounds. If severe imbalances arise in this supply, the plant may not be able to take up nutrients efficiently. As a result, nutrient deficiency stress will take place in the plants.

Several plants are able to protect themselves against water and nutrient deficiency stresses by taking salts up from the solution in proximal root regions and accumulating them in the root cells. This phenomenon occurs until the water potential of internal cells is lower than the external, enough to drive water to the cells. However, higher accumulation of salts can directly be toxic to the cells. Halophytic plants posses some physiological and biochemical mechanisms to reduce the excess salts in the root cells.

1) *Ion inclusion*: Grasses, soybean and *Triticum* species (Rabie and Kumazawa 1988, Schachtman and Munns 1992, Kim *et al*. 1999) can basically tolerate salinity by including excessive salts in root cells. This plant group has ability to control translocation of Na and Cl from roots to shoots. In addition, ion localization within vacuole in root cells may accompany this system of the tolerance (Bohnert *et al*. 1999).

Some halophytes reduce the excessive salts from the root cells by transporting them to accumulate in shoots. In the shoots, there possess numerous mechanisms to withstand toxicity of the salts described as follows.

2) *Ion excretion*: *Diplachne fusca* (L.) Beauv. and *Avicennia marina* have salt glands in leaves which allow the salt to be excreted onto the surface

of the leaves, eventually to be removed by the action of wind and water (Rains and Epstein 1967, Warwick and Halloran 1992).

3) *Ion dilution*: *Atriplex* species (Greenway *et al*. 1966) overcome the detrimental effects of salts by an increase in succulence. The leaf cells (especially the parenchyma) enlarge due to an increase in water content, which prevents an excessive concentration of salts in the cell sap. Measured per unit leaf area, an increase in succulence will be accompanied by an increase in both water and dry matter content.

4) *Ion localization and accumulation of compatible solutes*: *Mesembryanthemum crystallinum* is reported as a strong salt accumulator that forms high contents of Na and Cl within the leaf cells by localizing them in the vacuole, balanced by compatible solutes within the cytoplasm (Adams *et al*. 1992, Bremberger and Luttge 1992). This aspect has been extensively reported as molecular mechanisms of salt tolerance in plants (Bohnert *et al*. 1999). The localization of Na salt into vacuoles is operated by a vacuolar Na/H antiport on tonoplast vesicles (Apse *et al*. 1999). This efficient mechanism averts the deleterious effects of Na (and Cl) in the cytoplasm and maintains osmotic balance by using Na accumulated in the vacuole to drive water into the cells. This Na/H antiport transports Na into the vacuole by using the electrochemical gradient of protons generated by the vacuolar H-translocating enzymes, H-adenosine triphosphatase (ATPase) and H-inorganic pyrophosphatase (PP_iase) (Bohnert *et al*. 1999). The major compatible solutes are K, proline, glutamate and quaternary ammonium compound. Some other molecules act as osmolytes such as betaines and glycinebetaine. Accumulation of compatible solutes in response to salt stress is generally accepted to prevent volume change and loss of water of plant cells (Verma 1999). The molecular mechanisms of salt tolerance are presented in Fig.1.

In plants, proline is synthesized from glutamate and ornithine. Proline biosynthesis from glutamate is functions of both gene encoding Δ^1-pyrroline-5-caroxylate reductase and Δ^1-pyrroline-5-caroxylate synthetase (Delauney and Verma 1993). Several studies (Rudolph *et al*. 1986, Smirnof and Cumbes 1989,

Venekamp 1989, Alia 1991, Rajendrakumar *et al.* 1994) suggested that the primary role of proline may not be solely as an osmolyte, but it also helps the cell to overcome oxidative stress. Other known attributes of proline are protecting enzymes from denaturation, interacting with membrane systems, regulating cytosolic acidity, scavenging free radicals, balancing the ratio of $NADH/NAD^+$, and acting as a energy source. They may be also important for the overall health of the plants under salt stress.

1.2.2.2 Active oxygen species and antioxidative enzymes

The accumulation of dioxygen (O_2) in Earth's atmosphere allowed for evolution of aerobic organisms that use O_2 as the terminal electron accepter, thus providing a higher yield of energy compared with fermentation and anaerobic respiration. In its ground state, molecular O_2 is relatively unreactive, yet it is capable of giving rise to lethal reactive excited states as the generation of active oxygen species (AOS). The AOS is considered to be a primary event under a variety of stress conditions such as drought stress, salinity stress or extreme temperature (Aroca *et al.* 2001, Egert and Tevini 2002, Vaidyanathan *et al.* 2003). It has been generally accepted that the AOS produced is a detrimental factor that causes lipid peroxidation, enzyme inactivation and oxidative damage to DNA (Davies 1987, Imlay and Linn 1988, Bor *et al.* 2003). AOS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^\bullet OH$), and singlet oxygen ($^1O_2$).

Superoxide is a nucleophilic reactant with both oxidizing and reducing properties (Elstner 1982). With regard to molecules of biological importance, O_2^- can oxidize sulfur compounds, *o*-diphenols, ascorbic acid, or NADPH, and has been shown to reduce cytochome *c*, metal ions and metal complexes. A particular importance is the dismutation of O_2^- which results in the formation of H_2O_2, a reaction which occurs spontaneously with a rate strongly dependent upon the pH, or is accelerated by several orders of magnitude via catalytic action of the enzyme superoxide dismutase (SOD).

Hydrogen peroxide has similar properties to O_2^- in that can act both as an oxidant and a reductant. H_2O_2 is the first stable compound among AOS

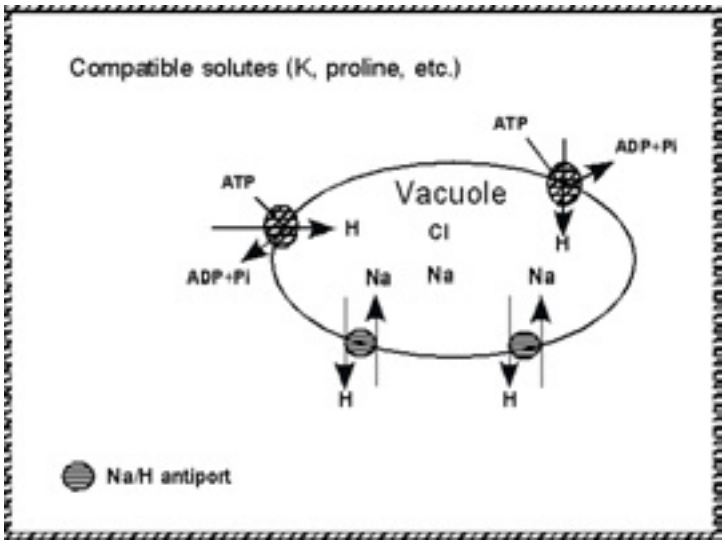

Figure 1. The molecular mechanisms of salt tolerance. Na and Cl are sequestered into the vacuole, and compatible solutes are presented in high concentrations in the cytoplasm (modified from Bohnert *et al.* 1999).

produced in the plant cell under normal conditions and as a result of stress. Hence, it is the most probable candidate for AOS-mediated signal transduction. This compound is relatively stable, and is able to penetrate the plasma membrane as an uncharged molecule and be transported to the site of action as a consequence of changes in the redox balance through the oxidation of metabolically active compounds leading to lipid peroxidation and degradation.

The formation of hydroxyl radicals is dependent on both of O_2^- and H_2O_2. The short lifetime and the strongly positive redox potential of "free" $^{\bullet}OH$ can react indiscriminately with all macromolecules (Elstner 1982). The detection of free $^{\bullet}OH$ is based mainly on the inhibition of a detector reaction by both SOD and catalase (CAT) as well as by (unspecific) $^{\bullet}OH$ scavengers such as benzoate, formate, manitol, ethanol, α-tocopherol and others, which also react at approximately equal rates with $^1O_2$.

Singlet oxygen is formed by photodynamic processes through interactions of several oxygen radicals (for example, during lipid peroxidation) and by electron donation from $^1O_2$ to certain electron acceptors. The detection of $^1O_2$ has been based on detector reactions for $^1O_2$ with the aid of nonspecific $^1O_2$ quenchers or scavengers, or by bioluminescence. None of the applied methods on its own is a reliable indicator for the detection of $^1O_2$ with the possible exception of 7-OH cholesterol formation from cholesterol in the presence of $^1O_2$ (Elstner 1982).

When plants are subjected to stresses, such as salt stress, high concentration of AOS is formed in plant cells (Gossett *et al.* 1994, Hernandez *et al.* 1995). Plants posses numerous defense mechanisms, both enzymatic and non-enzymatic, and these mechanisms are contributed to protect cells from oxidative injury (Shalata and Tal 1998, Dionisio-Sese and Tobita 1998, Gossett *et al.* 1994). The main non-enzymatic antioxidants are GSH, cysteine, hydroquinones, manitol, vitamins C and E, flavonoids, some alkaloids and β-carotene. The enzymatic antioxidant defenses include SOD, CAT, peroxidase. In addition, a whole array of enzymes in ascorbate-glutathione cycle is needed

for the regeneration of the active forms of the antioxidants (ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase(GR)). Related to the study in Chapter 5, the antioxidative enzymes to detoxify O_2^- and H_2O_2 will be discussed here. The main pathway to remove AOS in cells is shown in Fig. 2.

Superoxide dismutase is a major scavenger of O_2^- and its enzymatic results in the formation of H_2O_2 (Beauchamp and Fridovich 1971). CAT, APX and a variety of general peroxidases then catalyzed through the breakdown of H_2O_2. In an ascorbate-glutathione cycle, the enzymatic action of APX produces monodehydroascorbate (MDA) that can dismutase spontaneously or be enzymatically reduced to dehydroascorbate (DHA) by NADPH-dependent monodehydroascorbate reductase (MDHAR). DHA is reduced back to ascorbate non-enzymatically by reduced glutathione (GSH) or enzymatically in a reaction mediated by DHAR. The resulting oxidized glutathione (GSSG) is then converted back to the reduced from by NADPH-dependent GR.

The CAT and SOD are the most efficient antioxidative enzymes (Scandalios 1993). Their combined action converts the potentially dangerous O_2^- and H_2O_2 to water and molecular oxygen, thus averting cellular damage. SOD can be classified by its metal cofactors into three types: 1) FeSOD is found in aerobic and anaerobic bacteria 2) MnSOD is found in green algae and in motocondria 3) Cu/ZnSOD is presented in the cytoplasm and chloroplast of higher plants. All three enzymes are nuclear encoded, and SOD genes have been shown to be sensitive to environmental stresses, presumably as a consequence of increased AOS formation.

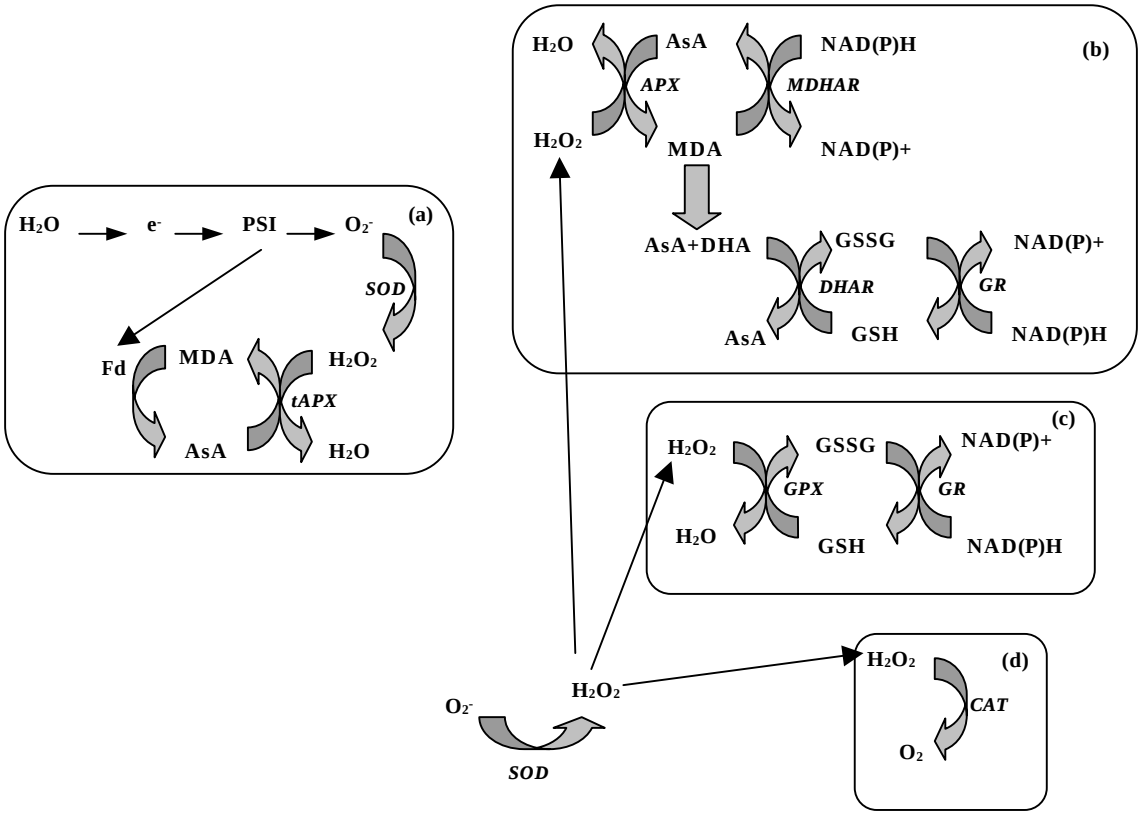

Figure 2. The main cellular pathways for AOS removal in plants. (a) SOD and thylakoid-bound APX (tAPX) in the chloroplast as part of the water-water cycle. (b) SOD and APX in the stroma, cytosol, mitochondria, and apoplast of plants, as part of the ascorbate-glutathione pathway. (c) Glutathione peroxidase (GPX) and its glutathione regenerating cycle. (d) Catalase in peroxisomes. AsA, ascorbate; GSH and GSSG, reduced and oxidized glutathione; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; MDA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; GR, glutathione reductase; PSI, photosystem I; Fd, ferredoxin; e^-, electron. (modified from Noctor and Foyer 1998).

1.3 Objective of this study

Until now, a few studies have been conducted on the mechanism of salt tolerance in *S. rostrata* (Ramani *et al.* 1989, Mahmood 1998, Wongwattana *et al.* 1998). The objective of this study was to obtain a better understanding on salt-tolerance mechanism of *S. rostrata* by determining the physiological and biochemical responses of the plant to NaCl comparing with *Phaseolus vulgaris* L. cv. Meal (kidney bean), salt sensitive species. Both of *S. rostrata* and *P. vulgaris* are grouped in dicotyledonous plant in the same leguminous family. The experiments explained in each Chapter were conducted in order to;

1. Determine growth and physiological responses to NaCl stress.
2. Investigate ions distribution and change of amino acid content under NaCl stress.
3. Clarify Na and Cl ions uptake and translocation due to NaCl stress to shoots.
4. Investigate effects of NaCl on antioxidative enzymes.

Chapter 2

Plant Growth and Physiological Responses to NaCl Stress

2.1 Introduction

It is generally accepted that salinity stress causes a decrease both in growth and in the photosynthesis of higher plant species. The subsequent evaluation of biomass production, which forms a majority of plant growth, will indicate the capacity with which each plant tolerates salinity. It was found that in general, the production of fresh and dry matter is severely reduced with the low concentration of salinity in glycophytes, but is still maintained in halophytes. Reduction in the photosynthesis rate as a result of NaCl exposure is observed in many species. The evaluation on the photosynthesis in leaves was extensively carried out by determining chlorophyll content, the main class of photosynthetic pigments that absorb light energy and initiate electron transport, and/or the kinetics of chlorophyll fluorescence that indicate the function of the photosynthetic electron transport (Smillie and Nott 1982, Belkhodja *et al.* 1994, Singh and Dubey 1995). Survey of tolerant varieties is often carried out by determining their growth and photosynthesis.

Sesbania rostrata was reported as a moderately salt-tolerant species (Mahmood 1998). The objective of this study was to investigate effects of NaCl on seed germination, fresh and dry matter production, chlorophyll content and chlorophyll fluorescence in *S. rostrata*.

2.2 Materials and Methods

2.2.1 Seed germination of *S. rostrata*

Seeds of *S. rostrata* (obtained from the Department of Land Development, Nakhonratchasima, Thailand) were soaked in concentrated H_2SO_4 for 30 min to break dormancy. The seeds were surface-sterilized with NaClO (1.25% active chlorine) under vacuum for 15 min and rinsed several times with sterile distilled water. Thereafter, they were soaked in sterile water at 25°C for one night. Fifteen seeds were placed in a petri dish (11 cm diameter) with two layer filter papers. Ten ml of 0, 50, 100, 150, 200, 250 and 300 mM NaCl solution was put into petri dish. They were then kept at room temperature. Germination rate were determined 7 days after treatment. The experiment was designed with three replications.

2.2.2 Seedling preparation and NaCl treatment

Seeds of *S. rostrata* were broken their dormancy and then surface-sterilized as described above. They were then soaked in sterile water at 25°C for one night. *Phaseolus vulgaris* L. cv. Meal (kidney bean) seeds were incubated on moist filter paper at 30°C for 2 days in darkness. Germinated seeds were planted in a container containing vermiculite and grown at 25°C for 2 days in darkness with aluminum foil cover. Then a half strength of modified Kasugai nutrient solution (Table 1) was added to the vermiculite. After 5 days, seedlings were transferred to a hydroponic culture with the same nutrient solution. *S. rostrata* was grown to the 3^{rd} to 4^{th} and *P. vulgaris* to the 1^{st} to 2^{nd} leaf stage. Salt treatment was started by adding 50 mM NaCl to the solution. For the treatments with higher concentrations, plants were transferred to 100 mM and then 150 mM at two-day intervals. Step of seedling preparation and NaCl treatment is shown in Fig. 3. The NaCl-solution containing nutrient was renewed every 4 days. Plants were grown in a growth chamber at 25/20°C day/night temperatures, with a 14 h photoperiod at 280-290 $\mu E/m^2/s$, and relative humidity of 70-80%. Seedlings were harvested 14 days after starting NaCl treatment. All experiments were designed with three replications.

2.2.3 Fresh and dry matter production

The test seedlings were harvested and separated into roots and shoots. Each part was then weighed for the determination of fresh matter mass. They were then dried at 80°C for 48 hours for the measurement of dry matter mass.

2.2.4 Measurement of chlorophyll contents

All leaves were excised from the test seedlings for the measurement of chlorophyll content. Chlorophyll contents were determined according to Chappelle *et al.* (1992). Chlorophyll was extracted by soaking excised intact leaves (1 g) into 5 ml of dimethyl sulfoxide (DMSO) at 30°C for 1 day in darkness. Absorbance at 648 and 664 nm was recorded using a spectrophotometer (DU640, Beckman Instruments Inc., Fullerton, CA, USA). The obtained absorbance values were used to calculate the chlorophyll contents using equations as described below;

$$\text{Chlorophyll } a = 12.25A_{664nm} - 2.79A_{648nm}$$

$$\text{Chlorophyll } b = 21.50A_{648nm} - 5.10A_{664nm}$$

2.2.5 Measurement of chlorophyll fluorescence

Leaves were excised for the measurement of chlorophyll fluorescence. The ratio of variable fluorescence (F_v) to the maximal one (F_m) was determined with a portable chlorophyll fluorometer (Photosynthesis Yield Analyzer Mini-PAM, Walz, Effecltrich, Germany) after 1 min dark adaptation. Changes in the intensity of fluorescence emission and its quenching are sensitive to changes in the photosynthetic apparatus. Therefore, this value was used in order to estimate the effect of NaCl, especially on photosynthetic electron transport. Detection was performed for 6 leaves (2^{nd} to 4^{th}) with 4 points in each leaf.

Table 1 The composition of modified Kasugai 's nutrient solution used in the study (Ohta *et al.* 1970)

Elements	Reagents	Concentration of element in nutrient solution (ppm)
NH_4^+	$(NH_4)_2SO_4$	10
NO_3	$NaNO_3$	10
P_2O_5	$Na_2HPO_4.12H_2O$	40
K_2O	KCl	40
MgO	$MgSO_4.7H_2O$	10
CaO	$CaCl_2.2H_2O$	40
Fe^{2+}	EDTA-Fe	7
Mn	$MnCl_2.4H_2O$	0.5
B	H_3BO_3	0.05
Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.05
Cu	$CuSO_4.5H_2O$	0.02
Zn	$ZnSO_4.7H_2O$	0.05

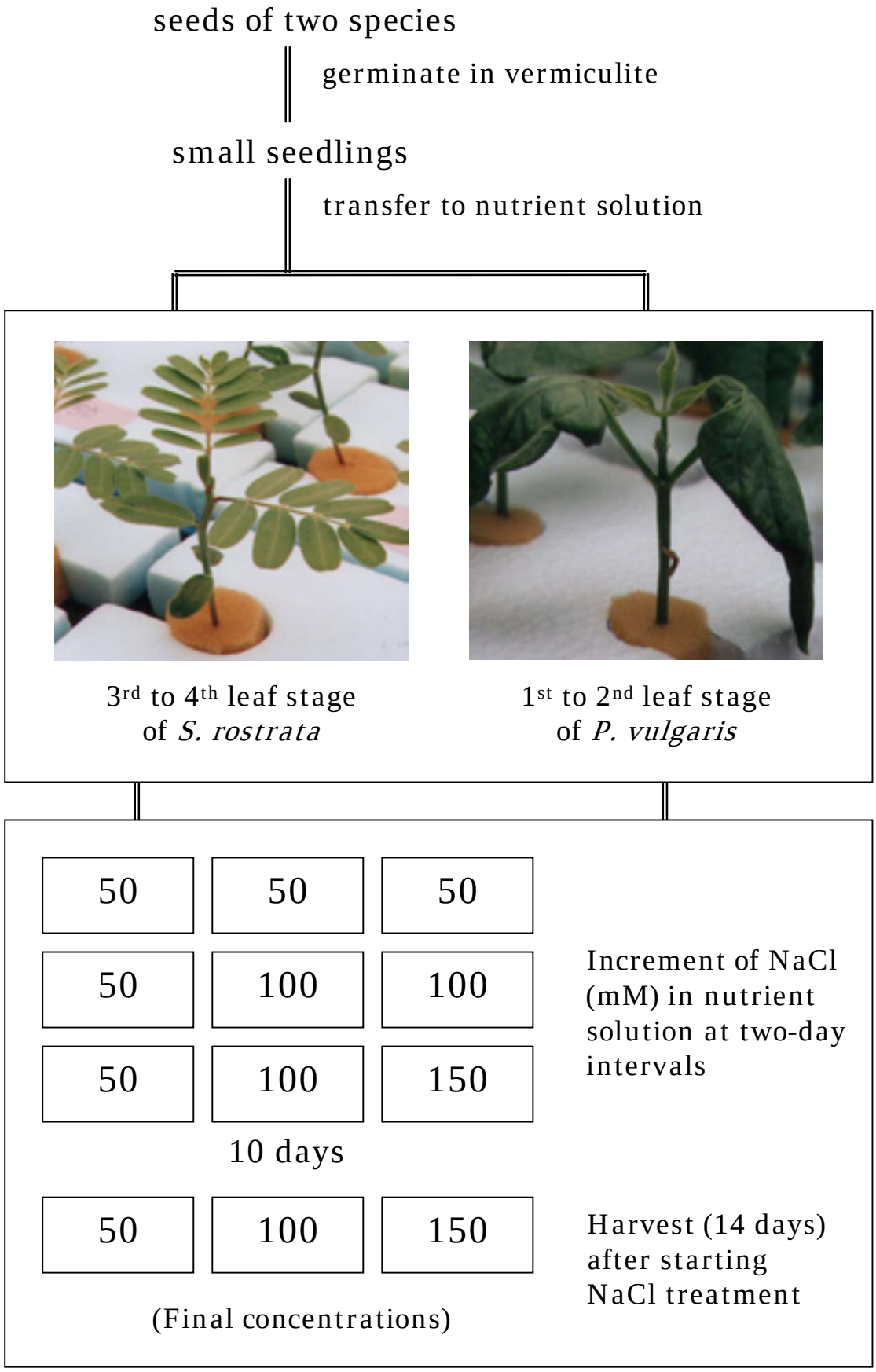

Figure 3. Step of seedling preparation and NaCl treatment

2.3 Results

Germination rate of *S. rostrata* did not reduce much up to 200 mM NaCl. Their germination rate showed 88% of the control at 200 mM. At higher concentrations (250 and 300 mM), the germination was less than 50% of the control. Moreover, the plant's root became shorter and hypocotyls turned yellow (Table 2).

The effect of salinity on growth of two species is shown in Table 3. Increase of NaCl concentration reduced biomass production in both species. However, at the highest concentration of NaCl (150 mM), salinity affected the fresh and dry matter slightly in shoots and roots of *S. rostrata*. In contrast, the shoots of *P. vulgaris* were markedly inhibited by more than 50% of the control value at the lowest concentration of NaCl (50 mM). Visible observation also showed that leaf injury (chlorosis and necrosis) were very apparent even at fairly low concentrations of NaCl in the 1st and 2nd leaves of *P. vulgaris*, while this level caused no symptoms in *S. rostrata* (Fig. 4). Furthermore, the highest dose of NaCl (150 mM) caused *P.vulgaris* to die.

The effect of salinity on the chlorophyll content and chlorophyll fluorescence of the two species are shown in Table 4. Decrease of total chlorophyll content was observed with increasing NaCl concentration in both species but its reduction was more obvious in *P. vulgaris*. It showed that both of chlorophyll *a* & *b* decreased and consequently affected chlorophyll *a+b* and *a/b* ratio. For *S. rostrata*, increase of NaCl concentration decreased the content of chlorophyll *a* slightly, but the loss of chlorophyll *b* was evident. This resulted in the enhancement of the chlorophyll *a/b* ratio. The determination of quantum yield (F_v/F_m) showed that chlorophyll fluorescence was unaffected in *S. rostrata* but very severe in *P. vulgaris* at lowest concentration of NaCl (50 mM). Furthermore, their leaves were undetectable in the quantum yield at the higher concentrations of NaCl (100 and 150 mM) due to necrosis.

Table 2 Percentage of germination in *S. rostrata* 7 days after treatment.

NaCl (mM)	Germination rate	
	Germinated seed	Percent of control
0	15 ± 0.3	100 ± 1.9
50	14 ± 0.3	98 ± 1.9
100	14 ± 0.3	93 ± 1.9
150	14 ± 0.3	93 ± 1.9
200	13 ± 0.5	88 ± 3.2
250*	7 ± 0.5	48 ± 3.2
300*	6 ± 0.3	43 ± 1.9

* Roots were shorter and hypocotyls became yellow.

Table 3 Effect of NaCl on fresh and dry weights (g/plant) of *S. rostrata* and *P. vulgaris* 14 days after starting NaCl treatment.

	NaCl Concentrations			
	0 mM	**50 mM**	**100 mM**	**150 mM**
Roots of *S. rostrata*				
Fresh weight	4.2 ± 0.6	4.4 ± 0.5	2.5 ± 0.4	2.2 ± 0.2
% of control	100 ± 14.3	105 ± 11.9	60 ± 9.5	52 ± 4.8
Dry weight	0.4 ± 0.05	0.4 ± 0.03	0.3 ± 0.02	0.3 ± 0.02
% of control	100 ± 12.5	100 ± 7.5	75 ± 5.0	75 ± 5.0
Shoots of *S. rostrata*				
Fresh weight	3.7 ± 0.6	4.1 ± 0.5	2.7 ± 0.4	2.5 ± 0.2
% of control	100 ± 16.2	111 ± 13.5	73 ± 10.8	68 ± 5.4
Dry weight	0.7 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
% of control	100 ± 14.3	100 ± 14.3	57 ± 14.3	57 ± 14.3
Roots of *P. vulgaris*				
Fresh weight	8.3 ± 0.1	6 ± 0.3	4.7 ± 0.2	D
% of control	100 ± 1.2	72 ± 3.6	57 ± 2.4	D
Dry weight	0.6 ± 0.01	0.4 ± 0.02	0.3 ± 0.01	D
% of control	100 ± 1.7	67 ± 3.4	50 ± 1.7	D
Shoots of *P. vulgaris*				
Fresh weight	7.3 ± 0.2	1.8 ± 0.1	1.2 ± 0.1	D
% of control	100 ± 2.7	25 ± 1.4	16 ± 1.3	D
Dry weight	0.9 ± 0.04	0.4 ± 0.07	0.4 ± 0.03	D
% of control	100 ± 4.4	44 ± 7.7	44 ± 3.3	D

- Data are the mean of three replicates ± S.E.
- D indicates the seedling died.

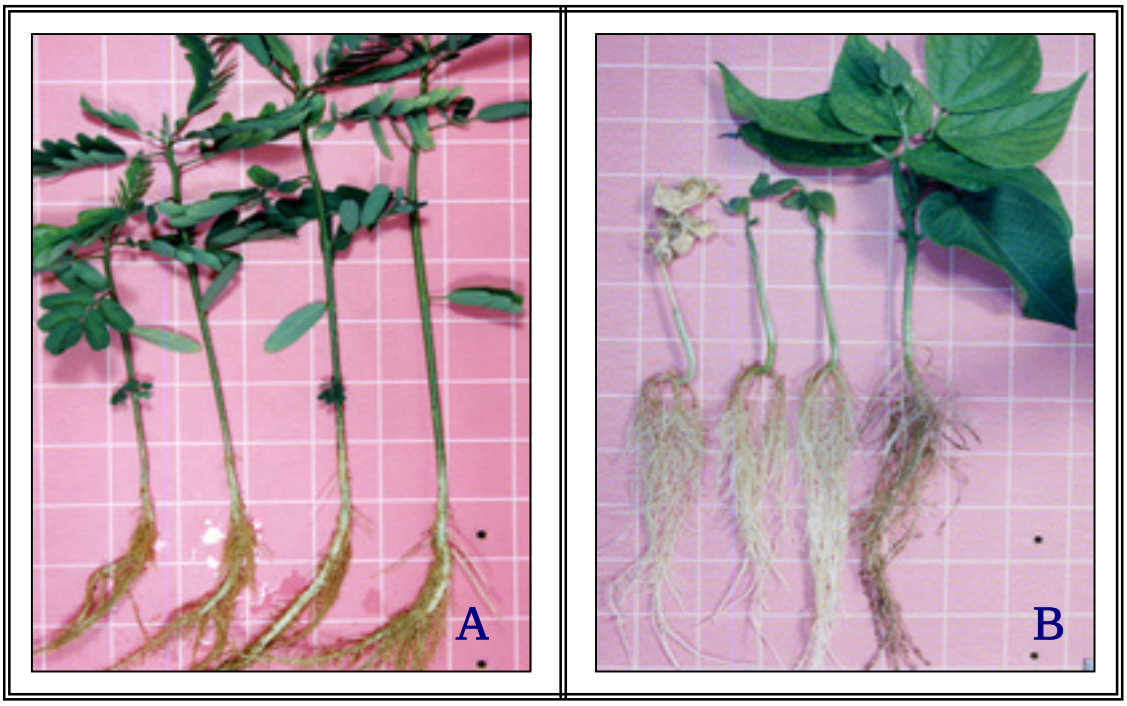

Figure 4. *S. rostrata* (A) and *P. vulgaris* (B) grown hydroponically 14 days after starting NaCl treatment (Left to right 150, 100, 50 and 0 mM NaCl, respectively). The injury on the 1st and 2nd leaves of *P. vulgaris* at 50 mM NaCl (C).

Table 4 Effect of NaCl on chlorophyll content and fluorescence in the leaves of *S. rostrata* and *P. vulgaris* 14 days after starting NaCl treatment.

NaCl (mM)	Chlorophyll content (μg/g FW)				Chlorophyll fluorescence (F_v/F_m)
	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *a*+*b*	Chlorophyll *a*/*b*	
S. rostrata					
0	156.5 ± 1.9	233.1 ± 3.1	389.6 ± 2.6	0.7 ± 0.02	0.78 ± 0.001
50	157.7 ± 1.0	145.8 ± 13.9	303.5 ± 12.9	1.1 ± 0.12	0.78 ± 0.004
100	118.6 ± 5.4	40.1 ± 1.9	158.7 ± 7.2	3.0 ± 0.05	0.76 ± 0.013
150	135.4 ± 3.0	45.2 ± 1.6	180.6 ± 4.4	3.0 ± 0.06	0.79 ± 0.002
P. vulgaris					
0	79.9 ± 4.4	34.2 ± 1.5	109.1 ± 5.9	2.2 ± 0.04	0.70 ± 0.012
50	62.2 ± 8.3	38.5 ± 3.9	100.7 ± 12.1	1.6 ± 0.06	0.04 ± 0.021
100	31.3 ± 3.3	22.0 ± 2.2	53.3 ± 5.4	1.4 ± 0.06	0
150	10.2 ± 1.3	11.1 ± 0.9	21.3 ± 2.2	0.9 ± 0.06	0

The data are the mean of 3 replicates ± S.E.

2.4 Discussion

Decrease in fresh and dry weights of shoots undoubtedly indicated the difference of salt tolerance between the two species. *S. rostrata* was more tolerant to salt than *P.vulgaris*. *S. rostrata* could tolerate 150 mM NaCl or about 0.8% NaCl (w/v). Moreover, this concentration of NaCl was unaffected to their seed germination. This finding is similar to the case of previous studies which classified this plant as a moderately salt tolerant species (Ramani *et al.* 1989, Mahmood 1998, Wongwattana *et al.* 1998). On the other hand, *P. vulgaris* was very sensitive to the salt. This species could survive under 0.2-0.3% NaCl (w/v), as previously reported (Gouia *et al.* 1994). In the investigation on salt tolerant mechanism in *S. rostrata*, comparison of its characteristics to *P. vulgaris* seemed to be useful.

The decreasing tendency of chlorophyll content and chlorophyll fluorescence were parallel to the biomass production in shoots of both species. In *S. rostrata*, the decrease in Chl *a+b* content was mainly attributed to the destruction of Chl *b*, which is more sensitive to salinity than Chl *a* (Ma *et al.* 1997). In *P. vulgaris*, chlorophyll *a+b* decreased drastically as reported in the previous study (Singh and Dubey 1995). This can be attributed that NaCl stress decrease total chlorophyll content of the plant by increasing the activity of Chl degrading enzyme cholorophyllase (Rao and Rao 1981), inducing the destruction of chloroplast structure and instability of pigment-protein complexes (Singh and Dubey 1995). Results obtained from this study indicate that chlorophyll *b* is more susceptible to NaCl stress than chlorophyll *a* and it will be a good indicator of salt stress. The quantum yield (F_v/F_m) is an indicator of potential yield of photochemical reaction of Photosystem II (Krause and Weis 1991). Moreover, the reduction of chlorophyll fluorescence is associated with increased Na accumulation (Dionisio-Sese and Tobita 2000). Under NaCl stress, the quantum yields were unaffected in *S. rostrata*, but greatly reduced in *P. vulgaris*. This indicates that photosynthetic electron transport normally works in *S. rostrata* but disorders in *P. vulgaris*. The evaluation of chlorophyll fluorescence could be used for screening of the salt-sensitive and tolerant species.

2.5 Conclusion

NaCl with 50, 100, 150 and 200 mM did not affect germination of *S. rostrata* seeds. Fresh and dry weights were decreased with increasing NaCl in both *S. rostrata* and *P. vulgaris*, however, the decrease was less pronounced in *S. rostrata*. At the highest concentration of NaCl (150 mM), shoot fresh and dry weights of *S. rostrata* were more than 50% of the control. In contrast, NaCl affected severely on shoot fresh and dry weights of *P. vulgaris*. At the lowest concentration of NaCl (50 mM), their values were less than 50% of the control. At the highest concentration (150 mM) caused the seedling death. These findings indicate that *S. rostrata* was more tolerant to NaCl than *P. vulgaris*.

In *S. rostrata*, NaCl caused the reduction of chlorophyll *b* content, but it did not affect chlorophyll *a* content and chlorophyll fluorescence in leaves, while NaCl greatly reduced chlorophyll *a* & *b* contents and chlorophyll fluorescence in those of *P. vulgaris*. These results suggest that under NaCl stress chlorophyll *b* is more susceptible than chlorophyll *a* and photosynthetic electron transport normally works in *S. rostrata,* but disorders in *P. vulgaris*.

Chapter 3

Ions Distribution and Change of Amino Acids Content under NaCl Stress

3.1 Introduction

Plants need to take essential elements for the use of physiological and biological processes which are necessary for growth and development. It is accepted in general that a high concentration of potassium (K) is needed to neutralize the soluble and macromolecular anions of cytoplasm. In this role, K contributes much to the osmotic potential. Magnesium (Mg) has a structural role in chlorophyll, is required for ribosome integrity, and undoubtedly contributes to the structural stability of nucleic acids and cell membranes. Calcium (Ca) bonds formed give stability to a variety of cytoplasmic structures and enzymes and have apoplastic importance in cell membranes, cell walls, and their adhesion. Phosphorus (P) supplied as phosphate (PO_4^{3-}) resists polarization and nucleophilic attack except in metal-enzyme complex, thus providing water stable anhydrides and ester. This stability provides for the mechanism of energy capture, transfer and recovery (Clarkson and Hanson 1980).

Under saline conditions, replacing K by Na on the uptake may occur in proximal root regions. This phenomenon is called “K deficiency” (Fitter and Hay 1987). Additionally, the excessive accumulation of Na and Cl may be toxic to the root cell, resulting in wilting and death of the whole plant. In several plants, however, an ability to maintain a high level of Na and Cl ions in the cell is well known as an important mechanism to alleviate stress. In this case, some elements such as K, Ca or organic solutes such as proline and glycinebetaine are also induced to function as the osmoprotectants to keep osmotic balance in the cells (Bohnert *et al.* 1999).

The studies in this Chapter aim to investigate effects of NaCl on distribution of essential elements (K, Ca, Mg, P), non-essential elements (Na,

Cl) in roots and shoots, accumulation of Na and Cl in stems and leaves, and on content of total amino acids and proline in roots and shoots of *S. rostrata*.

3.2 Materials and Methods

Seedlings of *S. rostrata* and *P. vulgaris* were prepared and then treated with 0, 50, 100 and 150 mM NaCl as described in Chapter 2. The seedlings were harvested 14 days after starting NaCl treatment and measured as follows.

3.2.1 Determination of amino acid contents

The test seedlings were harvested and separated into roots and shoots. Five hundred milligrams of the fresh roots and shoots were ground and homogenized with 4 ml of 15 mM HCl. The homogenate was centifuged at 2,000*x g* for 2 min at 4 °C. Five hundred microliters of the supernatant was mixed with 100 µl 5-sulfosalicylic acid dihydrate (10% w/v) and kept on ice for 15 min. The mixture was then centrifuged at 2,000*x g* for 15 min at 4°C. Five hundred microliters of the supernatant was taken and its pH was adjusted to 2.2–2.3 according to the modified method of Desmaison *et al.* (1984). Amino acids in the solution were analyzed with an amino acid analyzer (JIC-300, JOEL Ltd., Tokyo, Japan).

3.2.2 Ions analyses

Ion contents were measured according to the method described by Kim *et al.* (1999). Roots, shoots, stem and leaves of the test seedlings were divided and dried at 80°C for 2 days. Dried samples were then ground into a fine powder for wet digestion and dry ashing. For the wet digestion, 10 ml of 1.4 N HNO_3 was added to 0.1 g of each ground sample and the mixture was kept for 1 day at room temperature. The samples were then heated at 100-150°C until fully dried. Deionized water (500 ml) was added to the sediment and filtrated with 0.2 µm membrane filter. Na, K, Ca, Mg and P contents were analyzed by a plasma atomic emission spectrophotometer (ICAP-757V, Nippon Jarrell-Ash, Kyoto, Japan). For the dry ashing, 0.1 g of ground sample was mixed with 0.1 g CaO and 1 ml deionized water and then combusted at 500°C for 3 h. The ash was dissolved with 500 ml deionized water and filtrated with 0.2 µm

membrane filter. Cl content was analyzed by an ion chromatographic analyzer (IC 7000 Series II, Yokokawa Analytical System, Tokyo, Japan).

3.3 Results

Ions distribution of *S. rostrata* and *P. vulgaris* is shown in Table 5 and 6, respectively. Na and Cl contents increased with increasing salinity in roots and shoots of *S. rostrata*. However, their accumulations were noted as being greater in shoots than in roots. In roots of *P. vulgaris*, the Na and Cl contents also increased similarly as in the shoots of *S. rostrata*. A difference of Na distribution in the shoots of *P. vulgaris* was observed. At the high concentration of NaCl (100 and 150 mM), Na content tended to decrease, but Cl content increased slightly. Furthermore, these doses of NaCl caused wilting on the shoots of this plant and eventual death.

Instability of Potassium (K) content in shoots and roots was observed in *P. vulgaris,* while it slightly reduced by salinity in *S. rostrata*. Phosphorus (P) content tended to increase in roots and shoots with the increasing concentration of NaCl, but there was no difference in concentrations of this element between the two species. The change of Mg and Ca content by salinity in the roots and shoots of the two species was not observed.

In *S. rostrata*, the distribution patterns of Na and Cl were further investigated in stem and each leaf (Fig. 5). The result also showed that the high level of NaCl treatment caused greater accumulation of Na and Cl ions in leaves and stem of *S. rostrata* (Figs.5 A and B). The ion analyses in individual leaf showed that Na tended to accumulate in the older leaves with higher concentration. The chlorine ion showed the same tendency although it was not clear when compared with Na ion (Figs.5 C and D).

Total amino acid and proline contents in *S. rostrata* and *P. vulgaris* are shown in Table 7. The amount of soluble amino acids increased in both plants with increasing NaCl concentration. At the highest concentration, although the total amino acid and proline were clearly built up in both the shoots and roots of *S. rostrata*, their accumulation was more obvious in shoots. Similarly, their accumulation was also found in *P. vulgaris* at the higher concentration (100 and 150 mM).

Table 5 Ion contents (μmol/g DW) in NaCl-treated *S. rostrata* 14 days after starting NaCl treatment.

Elements	NaCl (mM)			
	0	50	100	150
Roots				
Na	404 ± 29.4	528 ± 17.2	668 ± 18.0	736 ± 31.8
K	519 ± 5.5	306 ± 10.7	366 ± 7.8	312 ± 4.8
Mg	130 ± 8.7	114 ± 0.8	138 ± 3.4	139 ± 4.4
Ca	50 ± 8.7	32 ± 3.1	35 ± 3.9	35 ± 3.1
P	341 ± 16.5	332 ± 11.7	384 ± 5.4	407 ± 7.3
Cl	366 ± 65.5	399 ± 5.7	543 ± 14.2	528 ± 5.7
Shoots				
Na	443 ± 4.2	1621 ± 19.4	2055 ± 85.9	2713 ± 334.5
K	1037 ± 33.2	617 ± 10.1	639 ± 19.1	627 ± 10.1
Mg	202 ± 10.4	220 ± 2.3	215 ± 3.6	208 ± 3.1
Ca	179 ± 5.1	134 ± 4.8	162 ± 5.3	233 ± 11.7
P	452 ± 12.5	671 ± 17.3	820 ± 8.3	874 ± 18.1
Cl	409 ± 40.0	1154 ± 28.7	1415 ± 33.2	2138 ± 33.1

The data are the means of 3 replicates ± S.E.

Table 6 Ion contents (μmol/g DW) in NaCl-treated *P. vulgaris* 14 days after starting NaCl treatment.

Elements	NaCl (mM)			
	0	50	100	150
Roots				
Na	197 ± 6.9	1780 ± 42.6	2366 ± 98.7	2782 ± 133.5
K	713 ± 29.0	643 ± 25.9	569 ± 43.3	514 ± 34.0
Mg	172 ± 1.2	289 ± 19.5	262 ± 10.6	197 ± 15.2
Ca	72 ± 1.8	133 ± 1.9	118 ± 8.4	99 ± 2.2
P	364 ± 22.5	708 ± 27.0	630 ± 58.0	659 ± 44.1
Cl	426 ± 11.0	1940 ± 103.9	2505 ± 25.0	2709 ± 190.0
Shoots				
Na	103 ± 1.5	1474 ± 189.0	876 ± 39.4	981 ± 43.3
K	718 ± 25.2	1090 ± 62.3	784 ± 38.1	761 ± 39.9
Mg	209 ± 4.6	203 ± 8.9	187 ± 9.4	190 ± 8.7
Ca	202 ± 6.6	359 ± 4.9	400 ± 30.5	400 ± 10.2
P	420 ± 11.2	551 ± 12.7	568 ± 28.0	594 ± 22.5
Cl	591 ± 8.5	1178 ± 12.8	1486 ± 73.9	1718 ± 29.6

- The data are the means of 3 replicates ± S.E.
- At 100 and 150 mM NaCl, shoots of *P. vulgaris* wilted and then died, respectively.

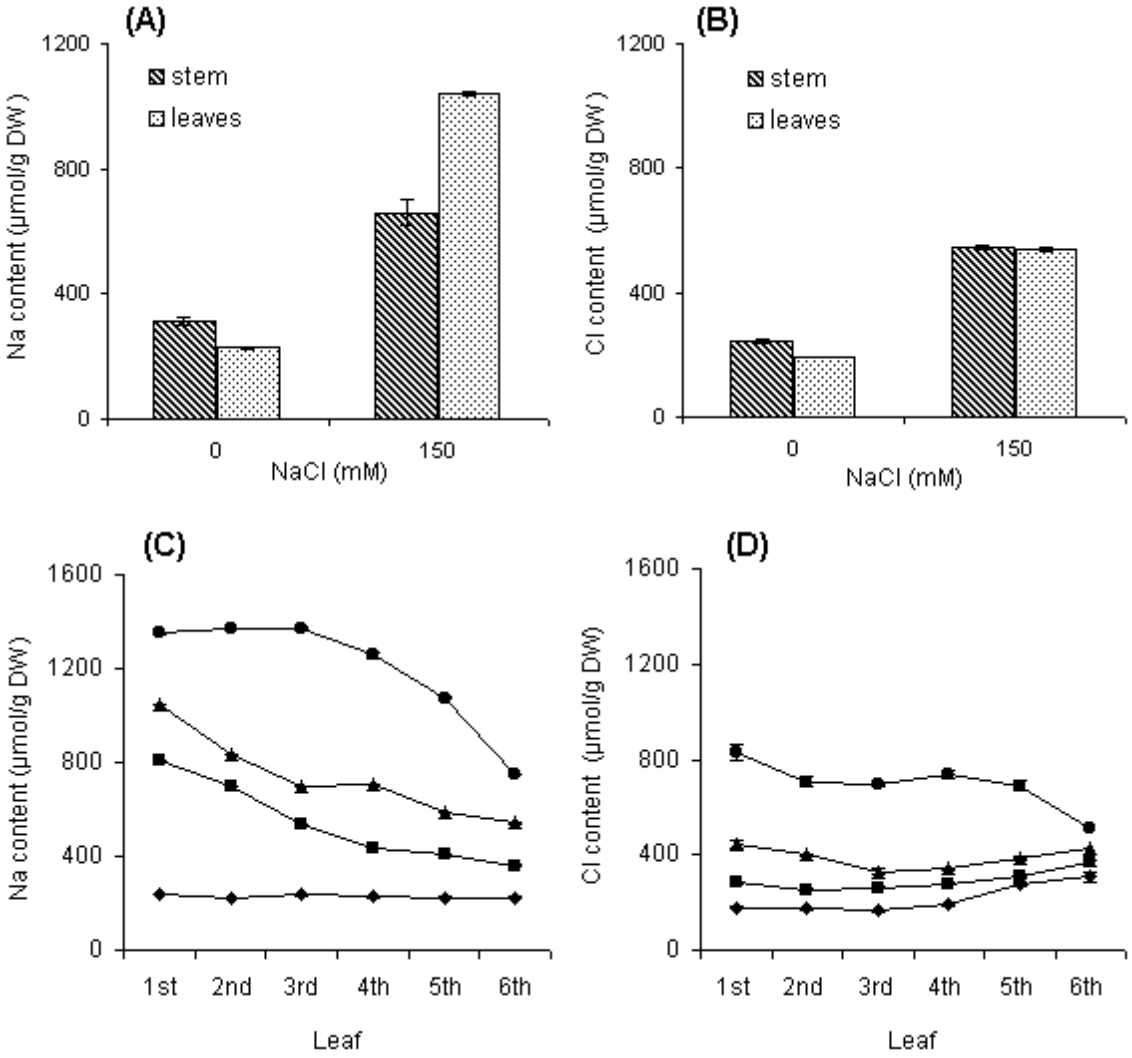

Figure 5. Distribution of Na and Cl in stem and leaves (A and B), and 1st – 6th leaves (C and D) of *S. rostrata* 14 days after starting NaCl treatment. (C and D, • 150 mM, ▲ 100 mM, ■ 50 mM and ◆ 0 mM of NaCl). Vertical bars indicate standard errors (n = 3).

Table 7 Amino acid contents (μmol/g DW) in NaCl-treated *S. rostrata* and *P. vulgaris* 14 days after starting NaCl treatment.

NaCl (mM)	Roots		Shoots	
	Total amino acid	Proline	Total amino acid	Proline
S. rostrata				
0	11.2 ± 1.27	0.32 ± 0.03	19.7 ± 0.87	0.75 ± 0.03
50	14.7 ± 0.63	0.63 ± 0.03	32.3 ± 1.40	1.89 ± 0.12
100	23.7 ± 1.36	4.52 ± 0.51	57.1 ± 5.52	5.68 ± 1.15
150	34.2 ± 0.67	9.85 ± 0.40	117.0 ± 6.62	16.62 ± 1.62
P. vulgaris				
0	10.6 ± 0.92	0.18 ± 0.04	15.7 ± 1.87	1.53 ± 0.66
50	14.6 ± 1.18	0.32 ± 0.04	25.6 ± 2.69	1.97 ± 0.30
100	22.4 ± 1.93	0.85 ± 0.28	113.4 ± 8.33	24.73 ± 1.08
150	42.8 ± 3.77	1.01 ± 0.23	90.1 ± 11.81	26.16 ± 4.49

- The data are the means of 3 replications ± S.E.

- At 100 and 150 mM NaCl, shoots of *P. vulgaris* wilted and then died, respectively.

3.4 Discussion

In some plant species, higher accumulation of Na and Cl requires compatible solutes for osmotic adjustment under salinity stress (Apse 1999, Zhu 2003). In this experiment, accumulation of the ions was also found in *S. rostrata* and *P. vulgaris* as a response to changing salinity stress levels. However, a different pattern of Na and Cl distribution in each part of the two species was clearly seen with increasing salinity. *S. rostrata* has a higher accumulation of the ions in shoots, but *P. vulgaris* in roots. It may likely indicate that *S. rostrata* has an ability to translocate the ions and hold them in the shoots. This physiological process may be important to reduce the salt toxicity in and away from the root cells. As a result the plant could survive even at the higher salinity levels to which was subjected during this study.

Some halophytes are known to accumulate the salts in their leaves under salinity stress (Black 1956, Scholander *et al.* 1966). Glycophytes, on the other hand, respond to salinity basically by ion inclusion. The majority of these species accumulate high levels of Na in their roots and stems (Flowers *et al.* 1977, Flowers 1985). At lower NaCl concentration, an ability to localize Na and Cl ions in root cells seemed to be important to alleviate salinity stress in *P. vulgaris*. At higher doses of NaCl, however, they could not resist toxicity of salt resulting in wilting and death of the plant. The higher Na and Cl accumulation in shoot of *S. rostrata* than *P. vulgaris* may indicate that shoot cells of *S. rostrata* has mechanism to tolerate higher ions concentration.

The replacement of K by Na observed in *P. vulgaris* was considered to be similar with that reported previously in tomato in which the replacement occurred progressively with increasing absorption of Na, and absorbed Na and was transported to the stem (Besford 1978). NaCl did not decrease P uptake in roots and shoots of both species as was found by Tattini *et al.* (1995) in aeroponically grown olive plants. As plant nutrients (Clarkson and Hanson 1980), calcium (Ca) and magnesium (Mg) play the same role by acting as a buffer system of the plant cells. NaCl did not affect on the Ca and Mg uptake in roots and shoots of *S. rostrata* as was found in *S. grandiflora* (Chavan and

Karadge 1986).

From this study, using 50, 100 and 150 mM NaCl concentrations (see also in Chapter 2), it was difficult to discuss the pattern of ion distribution in *P. vulgaris*. The highest concentration of NaCl (150 mM) is not a lethal dose of *S. rostrata*, whilst the lowest concentration (50 mM) proved too strong for the shoots of *P. vulgaris*. Furthermore, 14 days of NaCl exposure might be too long for the seedlings to discuss the effect of NaCl on the uptake of these ions because this species is very susceptible to the stress.

More precise determinations of the distribution of the ions in shoot parts showed that *S. rostrata* accumulated more Na and Cl ions particularly in the old leaves (Fig. 5). Greater accumulation of these ions in the older leaves has been reported in several other plant species and this function is considered to be effective in avoiding huge accumulations of the toxic salts in the growing young leaves (Lutts *et al*. 1996, Pardossi *et al*. 1999, Wahome *et al*. 2001).

In some plant species, salt tolerance associates with the capacity of a species to accumulate proline, which acts as a compatible solute involved in osmotic adjustment at the plant cell level (Delauney and Verma 1993). Proline accumulates in the cytoplasm without having any detrimental effects on cytosolic enzymes activities (Stewart and Lee 1974), while toxic ions, mainly Na, are sequestered in vacuole (Barkla *et al*. 1995). It has been suggested that the proline accumulation is due primarily to the function of both gene encoding Δ^1-pyrroline-5-carboxylate reductase, and Δ^1-pyrroline-5-carboxylate synthetase (Delauney and Verma 1993). Eder *et al*. (1977) reported that decreased protein synthesis and/or increased protein hydrolysis in pearl millet seedling by salinity could lead to the accumulation of free amino acids and proline. In the present study, proline accumulation was observed in the roots and shoots of both species. However, at higher NaCl (100 and 150 mM) seedlings of *P. vulgaris* wilted or died; this suggests proline does not help reducing salt damage in this plant. On the contrary, these concentrations had no detrimental effect on the seedlings of *S. rostrata*; this indicates that higher

proline accumulation may contribute to the alleviation of NaCl stress in the plant. Another compatible solute, glycinebetaine also suggests to act as an important osmoprotectant between the cytoplasm and vacuole (Venkatesan and Chellappan 1998). Furthermore, this compound can reduce lipid peroxidation and protect mitochondria electron transport reactions from salt damage (Chen and Murata 2002). Previous studies has been reported that increased glycinebetaine contributed to overcome water and salt stress in leguminous plants (Xing and Rajashekar 1999, Girija *et al*. 2002). Until now, the accumulation of glycinebetaine in *S. rostrata* has not been reported. For a better understanding the role of this compatible solute in osmotic maintenance in *S. rostrata*, further studies are needed.

3.5 Conclusion

S. rostrata and *P. vulgaris* had different patterns of Na and Cl distribution responding to NaCl stress. *S. rostrata* had a higher accumulation of the ions in shoots, with *P. vulgaris* having the accumulation in the roots. The high accumulation in shoots may be an important mechanism to alleviate the salt toxicity in *S. rostrata*.

Accumulation of Na and Cl in the old leaves appears to be part of the mechanism with which *S. rostrata* derives its salt tolerance.

Other elements, P, Mg and Ca showed no noteworthy change in either two species.

The higher accumulation of proline with increasing NaCl was found in the roots and shoots in both species. According to biomass production in Chapter 2, the involvement of NaCl alleviation is considered only in *S. rostrata*.

Chapter 4

Sodium and Chlorine Ions Uptake and Translocation

4.1 Introduction

Under salinity conditions, the increasing accumulation of Na and Cl is of primary importance in physiological action of plants. Excess of these ions can be extremely toxic to plant cells. The restriction of the ion accumulation in roots and shoots is important in the salt-tolerance mechanisms. Ion distribution within entire plant can generally be described two patterns. The first one, these plant have the ability to accumulate high levels of Na and Cl in shoots without any detrimental effect (Flowers 1972, Flowers *et al.* 1977). In this pattern, ion translocation is an important factor for the tolerance of the plants. It is found that on average more than 90% of absorbed Na is translocated to shoots and at least 80% accumulated in the leaves– frequently in the old leaves, thus preventing young photosynthetically active leaves (Umezawa *et al.* 2000, Ghoulam *et al.* 2002). In some plants, their leaves can avoid the injurious effect of salts by compartmentalization, succulence or excretion (Greenway *et al.* 1966, Adams *et al.* 1992, Warwick and Halloran 1992). The second one, this plant group can basically tolerate salinity by including excessive salts in root cells. These plants have ability to restrict translocation of Na (and Cl) from roots to shoots. Examples of these plants include rice, beetle grass and soybean (Ramani and Kannan 1986, Rabie and Kumazawa 1988, Warwick and Halloran 1992).

From the data obtained in Chapter 3, the distribution of Na and Cl responding to NaCl stress seemed to be important in the salt tolerance of *S. rostrata*. To obtain a better understanding, more concentrations of NaCl treatment were used. Sodium and chlorine uptake and translocation from roots to shoots and percentage of their accumulation in roots of this plant were investigated.

4.2 Materials and Methods

Seeds of *S. rostrata* and *P. vulgaris* were germinated and grown following the methods described in Chapter 2. When their seedlings reach the 1st to 2nd (*P. vulgaris*) and 3rd to 4th (*S. rostrata*) leaf stage, they were placed in a solution of 20 mM NaCl. For the treatment with higher concentrations, some plants were transferred to 50, 100, 150 mM and then 200 mM at two-day intervals. Step of NaCl treatment is shown in Fig. 6. After reaching the final concentrations of NaCl, the seedlings were kept in a growth chamber. NaCl-solution containing nutrients were renewed every 4 days. Eighteen days after starting NaCl treatment, the seedlings were harvested for the following assays.

4.2.1 Bioassay

The test seedlings were divided into roots and shoots, and dried at 80°C for 48 h for the determination of dry weight.

4.2.2 Ions analyses

The dried sample (0.1 g) of roots and shoots were ground into a fine powder for wet digestion and dry ashing (as described in Chapter 2). The solution obtained by wet digestion was analyzed for Na content by a plasma atomic emission spectrophotometer (ICAP-757V, Nippon Jarrell-Ash, Kyoto, Japan). The solution prepared from dry ashing was analyzed for Cl content by an ion chromatographic analyzer (IC 7000 Series II, Yokokawa Analytical System, Tokyo, Japan).

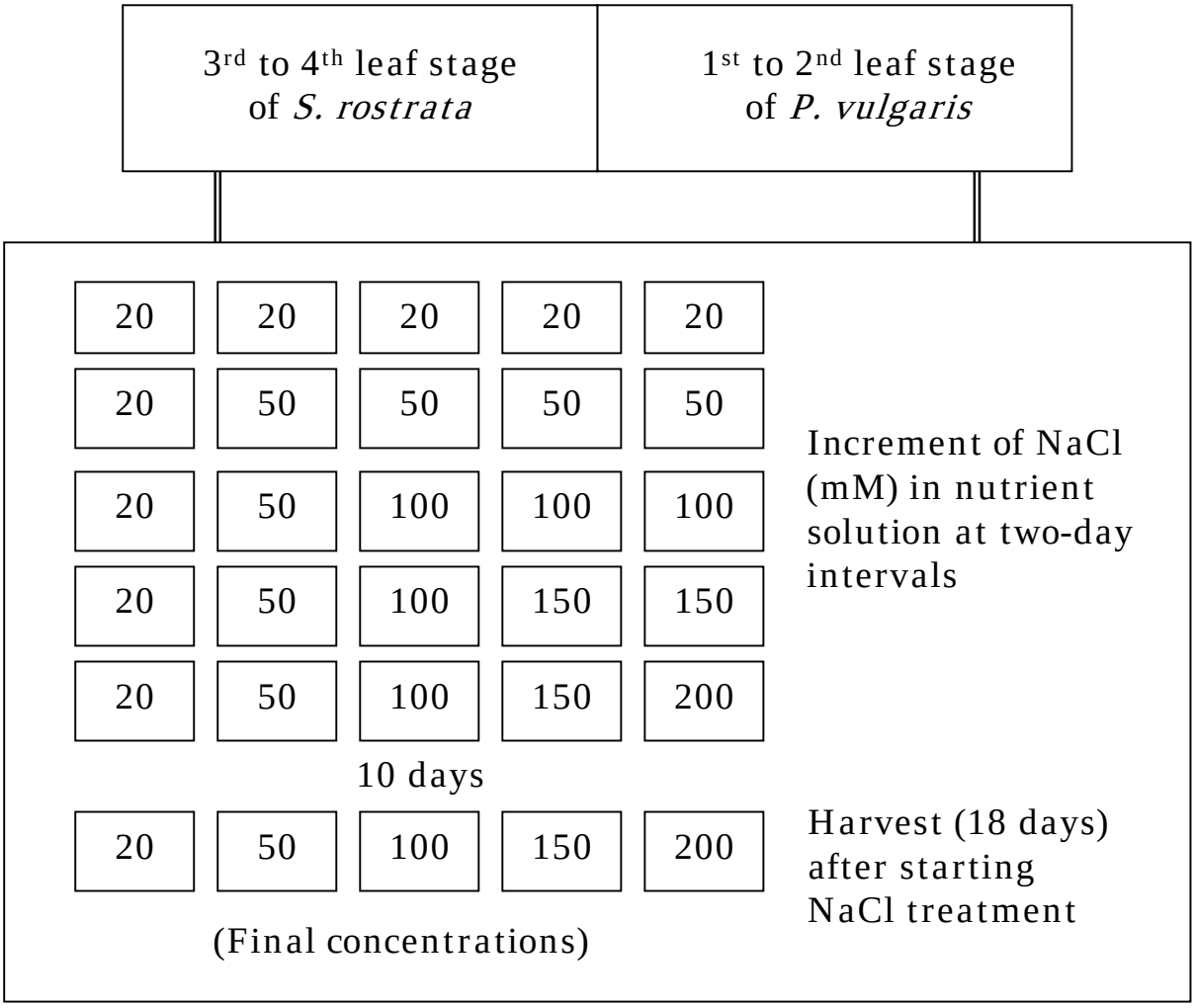

Figure 6. Step of NaCl treatment

4.3 Results

The effects of NaCl on shoots and roots dry weight are shown in Fig.7. Increase of NaCl concentration decreased shoots and roots dry weight in both species. Comparing two plants, *S. rostrata* was less affected by the salinity. At low concentrations (20 and 50 mM), NaCl had minimal effect on *S. rostrata* dry weights. At the highest concentration (200 mM NaCl), although the shoot dry weight was reduced to 46% of the control, the seedlings still survive. In contrast, NaCl affected *P. vulgaris* more severely. Twenty mM NaCl caused strong growth inhibition. At 50 mM, the dry weight value was less than 50% of the control. Hundred mM NaCl caused the shoot to wilt and the seedlings could not survive at the 150 and 200 mM NaCl concentrations.

Tables 8 and 9 show the Na and Cl distribution in *S. rostrata* and *P. vulgaris*, respectively. In *S. rostrata*, Na and Cl contents in roots and shoots were enhanced with increasing NaCl concentrations and those ion concentrations were higher in shoots than in roots. An accumulation of Na and Cl in *P. vulgaris* was also induced with increasing NaCl. However, the pattern of their accumulation was different from *S. rostrata*. The accumulations were greater in roots than in shoots.

Translocation rates of Na and Cl in *S. rostrata* and *P. vulgaris* are shown in Table 10 and 11, respectively. The translocation rate of ions in *S. rostrata* increased slightly with increasing NaCl concentration. In contrast, the rate tended to decrease at fairly low concentrations of NaCl treatment in *P. vugaris*.

Percentage of Na and Cl remaining in roots of *S. rostrata* and *P. vulgaris* is shown in Table 12 and 13, respectively. At 20 – 200 mM NaCl treatment, 29 – 41% of Na and Cl remained in *S. rostrata*. The percentages in root of *P.vulgaris* were more than 58% at low concentration of NaCl (20 and 50 mM), whereas Cl content was about 46 – 47%.

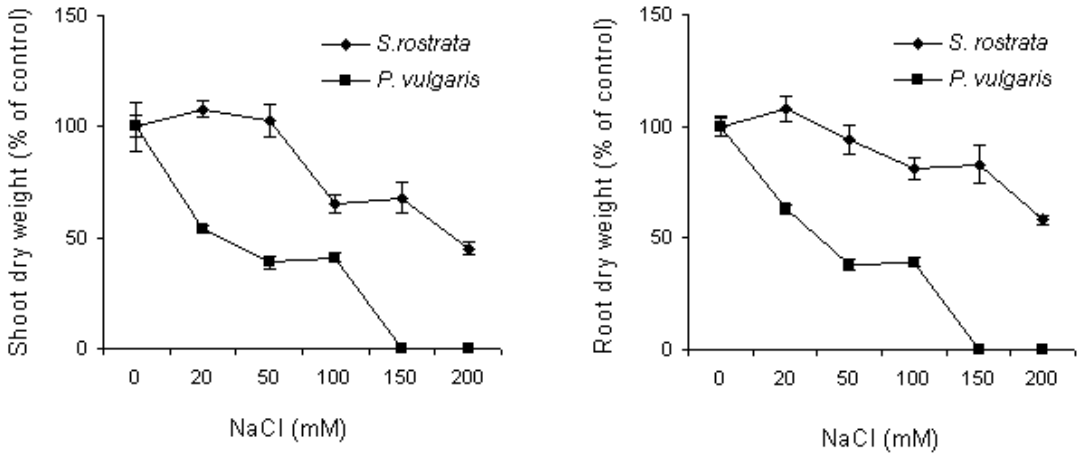

Figure 7. Effect of NaCl on growth of *S. rostrata* and *P. vulgaris* 18 days after starting NaCl treatment. The dry weights of shoots in non-treated control were 0.58 g and 0.65 g and those of roots were 0.30 g and 0.34 g for *S. rostrata* and *P. vulgaris*, respectively. Vertical bars indicate standard errors (n=3).

Table 8 Ions distribution (μmol/g DW) in NaCl-treated *S. rostrata* 18 days after starting NaCl treatment.

NaCl (mM)	Elements	
	Na	Cl
Roots		
0	308 ± 3.1	261 ± 9.8
20	522 ± 7.0	327 ± 18
50	658 ± 10.1	454 ± 7.2
100	781 ± 53.9	519 ± 19.7
150	838 ± 28.1	561 ± 37.6
200	832 ± 5.0	615 ± 11.9
Shoots		
0	266 ± 3.4	243 ± 6.5
20	441 ± 7.3	277 ± 5.7
50	561 ± 11.1	300 ± 4.3
100	815 ± 19.6	468 ± 16.7
150	966 ± 22.0	686 ± 33.9
200	1324 ± 20.5	873 ± 30.5

The data are the means of 3 replicates ± S.E.

Table 9 Ions distribution (μmol/g DW) in NaCl-treated *P. vulgaris* 18 days after starting NaCl treatment.

NaCl (mM)	Elements	
	Na	Cl
Roots		
0	198 ± 1.7	737 ± 19.1
20	1090 ± 37.4	1364 ± 26.7
50	1408 ± 95.2	1741 ± 41.3
100	1245 ± 3.8	1746 ± 26.4
150	1186 ± 61.1	1886 ± 13.4
200	2152 ± 21.8	2138 ± 38.4
Shoots		
0	125 ± 2.1	572 ± 12.6
20	285 ± 10.5	923 ± 63.1
50	507 ± 35.4	1007 ± 101
100	757 ± 107	980 ± 54.1
150	991 ± 50.2	1239 ± 156
200	1321 ± 17.8	1205 ± 80.1

- The data are the means of 3 replicates ± S.E.
- At 100, 150 and 200 mM NaCl, shoots of *P. vulgaris* wilted and died, respectively.

Table 10 Uptake and translocation of Na and Cl in *S. rostrata*.

NaCl (mM)	Plant part	Na (μmol/plant)	TR (%)	Cl (μmol/plant)	TR (%)
0	Shoots	154	63	141	64
	Roots	92		78	
	Total	246		219	
20	Shoots	278	62	174	62
	Roots	167		105	
	Total	445		279	
50	Shoots	337	65	180	59
	Roots	184		127	
	Total	521		307	
100	Shoots	310	62	178	59
	Roots	188		125	
	Total	498		303	
150	Shoots	377	64	268	66
	Roots	210		140	
	Total	587		408	
200	Shoots	344	71	227	68
	Roots	141		105	
	Total	485		332	

- Values are the means of 3 replications.

- Na and Cl contained in nutrient solution are 1.84 and 2.31 mM, respectively.

- TR : translocation rate (amount of each ion in shoots/total amount x 100)

Table 11 Uptake and translocation of Na and Cl in *P. vulgaris*.

NaCl (mM)	Plant part	Na (μmol/plant)	TR (%)	Cl (μmol/plant)	TR (%)
0	Shoots	81	55	372	60
	Roots	67		251	
	Total	148		622	
20	Shoots	100	30	323	53
	Roots	229		286	
	Total	329		610	
50	Shoots	132	42	262	54
	Roots	183		226	
	Total	315		488	
100	Shoots	197	55	255	53
	Roots	162		227	
	Total	359		482	
150	Shoots	278	63	347	57
	Roots	166		264	
	Total	444		611	
200	Shoots	383	64	350	62
	Roots	215		214	
	Total	598		563	

- Values are the means of 3 replications.
- Na and Cl contained in nutrient solution are 1.84 and 2.31 mM, respectively.
- TR : translocation rate (amount of each ion in shoots/total amount x 100)
- At 100, 150 and 200 mM NaCl, shoots of *P. vulgaris* wilted and died, respectively.

Table 12 Percentage of Na and Cl remaining in roots of *S. rostrata*.

NaCl (mM)	Plant part	Na		Cl	
		(μmol/plant)	(%)	(μmol/plant)	(%)
0	Roots	92	37	78	36
	Total	246		219	
20	Roots	167	38	105	38
	Total	445		279	
50	Roots	184	35	127	41
	Total	521		307	
100	Roots	188	38	125	41
	Total	498		303	
150	Roots	210	36	140	34
	Total	587		408	
200	Roots	141	29	105	32
	Total	485		332	

- Values are the means of 3 replications.
- Na and Cl contained in nutrient solution are 1.84 and 2.31 mM, respectively.

Table 13 Percentage of Na and Cl remaining in roots of *P. vulgaris*.

NaCl (mM)	Plant part	Na		Cl	
		(μmol/plant)	(%)	(μmol/plant)	(%)
0	Roots	67	45	251	40
	Total	148		622	
20	Roots	229	70	286	47
	Total	329		610	
50	Roots	183	58	226	46
	Total	315		488	
100	Roots	162	45	227	47
	Total	359		482	
150	Roots	166	37	264	43
	Total	444		611	
200	Roots	215	36	214	38
	Total	598		563	

- Values are the means of 3 replications.
- Na and Cl contained in nutrient solution are 1.84 and 2.31 mM, respectively.
- At 100, 150 and 200 mM NaCl, shoots of *P. vulgaris* wilted and died, respectively.

4.4 Discussion

The bioassay of this Chapter indicates clearly again that *S. rostrata* has a greater tolerance to NaCl than *P. vulgaris*. *S. rostrata* could resist NaCl up to 200 mM. Same patterns in Na and Cl distribution and translocation with the former experiments (Table 8-11) were observed. During NaCl stress, roots of *S. rostrata* responded to the stress by increasing Na and Cl accumulation, but the quantity of these ions were greater in the shoots. Calculation of the translocation rate of Na and Cl (Table 10-11) from roots to shoots and percentage of their remains in roots (Table 12 and 13) also confirmed that *S. rostrata* has an ability to limit Na and Cl accumulation in roots and translocate higher amount of the ions to shoots. The ability to absorb Na and Cl from roots and transfer them to shoots is considered to be one of the mechanisms of salt tolerance gifted in *S. rostrata*. High accumulation of the excessive salts in shoots indicates there has some mechanisms to reduce detrimental effect of the ions in the shoot cells. *Mesembryanthemum crystallinum* resists salinity stress by accumulating high contents of Na and Cl within the leaf cells. Localizing Na and Cl in the vacuole and balancing by compatible solutes within the cytoplasm was reported as the important salt tolerant mechanisms at cellular level (Adams *et al*. 1992, Bremberger and Luttge 1992). The mechanisms of salt tolerance in the shoot cells of *S. rostrata* are needed to clarify.

P. vulgaris was very sensitive to salinity. Their shoot biomass decreased more than 50% of the control when exposed to 50 mM NaCl as previously reported by Gouia *et al*. (1994). Results from this study showed that *P. vulgaris* responded to the low concentrations of NaCl (20-50 mM) by higher accumulation of Na in the roots (Table 13). These observations are also in agreement with a previous study (Jacoby 1979) suggesting that salt-sensitive *P. vulgaris* was a non-accumulator plant responding to salinity stress by including Na in the roots.

The results obtained from this study are quite clear that the salt tolerance of *S. rostrata* does not occur with ion inclusion in the roots but there

is a true tolerance of high ion contents in the shoot cells. This is accepted as a characteristic of some halophytes as previously reported (Black 1956, Scholander *et al.* 1966, Rains and Epstein 1967).

4.5. Conclusion

S. rostrata has a greater tolerance to NaCl than *P. vulgaris* that could resist NaCl up to 200 mM.

High salt tolerance of *S. rostrata* is probably related with the ability to restrict Na and Cl content in roots and translocate higher amount of the ions to hold in shoots.

P. vulgaris responds to the fairly low concentration of NaCl (20-50 mM) by including Na in roots.

Chapter 5

Antioxidative Enzymes Response to NaCl Stress

5.1 Introduction

In recent years, biochemical responses of plants to salt stress have been studied intensively. One of the biochemical changes possibly induced by salt stress is the increased production of active oxygen species (AOS) (Gossett *et al.* 1994, Vaidyanathan *et al.* 2003). The AOS are also produced during normal aerobic metabolism by the interaction between O_2 and electrons leaked from electron transport chains in the chloroplasts and mitochondria (Halliwell and Gutteridge 1989). AOS include superoxide (O_2^-) and hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Excess AOS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Fridovich 1986, Davies 1987, Imlay and Linn 1988). To escape from the toxic action of AOS, plants possess a number of antioxidant systems (Sies 1997). Superoxide dismutase (SOD) is a major scavenger of $O_2^-$and its enzymatic action leads to the less effective form, hydrogen peroxide (H_2O_2). The breakdown of H_2O_2 is then catalyzed to the non-toxic form such as oxygen and water by other antioxidative enzyme activities. Catalase (CAT) is a catalyst which acts in peroxisomes to convert H_2O_2 to water and molecular oxygen. In plant cells, the most important reducing substrate for H_2O_2 detoxification is ascorbate (Noctor and Foyer 1998). Ascorbate peroxidase (APX) uses two molecules of ascorbate to reduce H_2O_2 to water. This reaction also works together with other antioxidative enzymes, such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR). It is well klnown as an ascorbate-glutathione cycle (Noctor and Foyer 1998).

When plants are subjected to various oxidative stresses, high concentration of AOS is formed in plant cells (Scandalios 1993). Plants with high levels of antioxidants, either constitutive or induced, have been reported

to produce greater resistance to this oxidative damage in plant cells (Shalata and Tal 1998, Dionisio-Sese and Tobita 1998, Bor *et al.* 2003).

Paraquat is a redox-active compound wildly used to control existing vegetation (Suntres 2002). The mechanisms of paraquat toxicity involve: 1) the generation of the O_2^- in the light, which can lead to the formation of more toxic AOS, such as $^{\bullet}OH$ 2) lipid peroxidation which results in the oxidative degeneration of cellular polyunsaturated fatty acids. In addition, the effects of paraquat are much pronounced by light irradiation (Bowler *et al.* 1992). Paraquat has been used for production of AOS in studies of oxidative tolerance and cross-tolerance in plants (Kawashima *et al.* 2000, Lascano *et al.* 2003).

The objective of this study was to investigate effects of NaCl on the activity of antioxidative enzymes, CAT, GR, APX and SOD and the tolerance to AOS generator paraquat in the old and young leaves of *S. rostrata*. Non-tolerant *P. vulgaris* was used for comparison.

5.2 Materials and Methods

Seedlings of *S. rostrata* and *P. vulgaris* were grown and treated by 0, 50 and 100 mM NaCl as described in the previous Chapter. Twelve days after starting NaCl treatment (step for NaCl treatment is shown in Fig. 8), the test seedlings were then processed as follows.

5.2.1 Shoot fresh weight

Seedlings of the two species were excised and divided into young (4^{th} to 6^{th}) and old (1^{st} to 3^{rd}) leaves and their fresh shoots were immediately weighted.

5.2.2 Enzyme extraction and assay

Old and young leaves (0.5 g) were homogenized in 25 mM K-phosphate buffer (pH7.8) containing 0.4 mM EDTA-4H, 1 mM ascorbate and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was then centrifuged at 15,000*x g* for 20 min at 4°C. The filtered supernatant was used as an enzyme extract for APX, CAT and GR. For SOD assay, the extract was dialyzed against Seamless Cellulose Tubing® (Wako Pure Chemical Industries, Osaka, Japan) overnight with 10 mM K-phosphate buffer (pH7.8) at 4°C. The dialyzed extract was centrifuged at 15,000*x g* for 20 min at 4 °C. The supernatant was filtered, and the filtrate was used as an enzyme extract for SOD assay.

In superoxide dismutase assay, the reaction mixture consisted of 500 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA-4H (0.1 ml), 1 mM xanthine dissolved in 10 mM NaOH (0.1 ml), 0.1 mM cytochrome c from horse heart (0.1 ml), distilled water (0.66 ml), xanthine oxidase diluted 30 times in 2 M $(NH_4)_2SO_4$ containing 1 mM EDTA-4H (0.02 ml) and enzyme extract (0.02). The reaction was started by adding xanthine oxidase and the reduction rate of cytochome c was measured by the initial rate of increase of absorbance at 550 nm. SOD assay activity was calculated following the formula below;

Assay SOD units = (V_b/V_s)-1, where V_b is the reaction rate of the blank and V_s is the reaction rate of the sample.

In catalase assay, the reaction mixture consisted of 50 mM K-phosphate buffer (pH 7.0) containing 10 mM H_2O_2 (0.95 ml) and enzyme extract (0.05 ml). Immediately after adding the enzyme to the buffer, the initial rate of absorbance at 240 nm was determined. The molar absorption coeffient of H_2O_2 (0.04/mM/cm) was used to calculate the enzyme activity.

In glutathione reductase assay, the reaction mixture consisted of 100 mM K-phosphate buffer (pH 7.8) (0.25 ml), 10 mM oxidized glutathione (GSSG) (0.05 ml), distilled water (0.48 ml), 1 mM NADPH (0.12 ml) and enzyme extract (0.1 ml). The assay was started by addition of GSSG. GR activity was determined from the rate of NADPH oxidation measuring the decrease of absorbance at 340 nm. The molar absorption coefficient of NADPH (6.1/mM/cm) was used to calculate the enzyme activity.

In ascorbate peroxidase assay, the reaction mixture consisted of 100 mM K-phosphate buffer (pH 7.0) (0.25 ml), 1 mM ascorbate (0.25 ml), 0.4 mM EDTA-4H (0.25 ml), distilled water (0.19 ml), 10 mM H_2O_2 (0.01 ml) and enzyme extract (0.05 ml). The reaction was started by adding H_2O_2 and the oxidation rate of ascorbate was measured by the initial rate of decrease of absorbance at 290 nm. The molar absorption coefficient of ascorbate (2.8/mM/cm) was used to calculate the enzyme activity.

The enzyme activity determination was carried out according to the modified methods of Yanagida *et al.* (1999).

5.2.3 Paraquat sensitivity

Ten disks with 4 mm diameter were excised from the leaves and soaked into 3 ml of 0.3 μM of paraquat (1,1-dimethyl-4,4-bipiridinium dichloride salt) solution with their adaxial side up. They were preincubated in the dark for 1 h and then irradiated with fluorescent light (260-280 $\mu E/m^2/s$) at 25°C for 2 days. Chlorophyll contents in the leaf disks were determined with the modified method described by Chappelle *et al.* (1992).

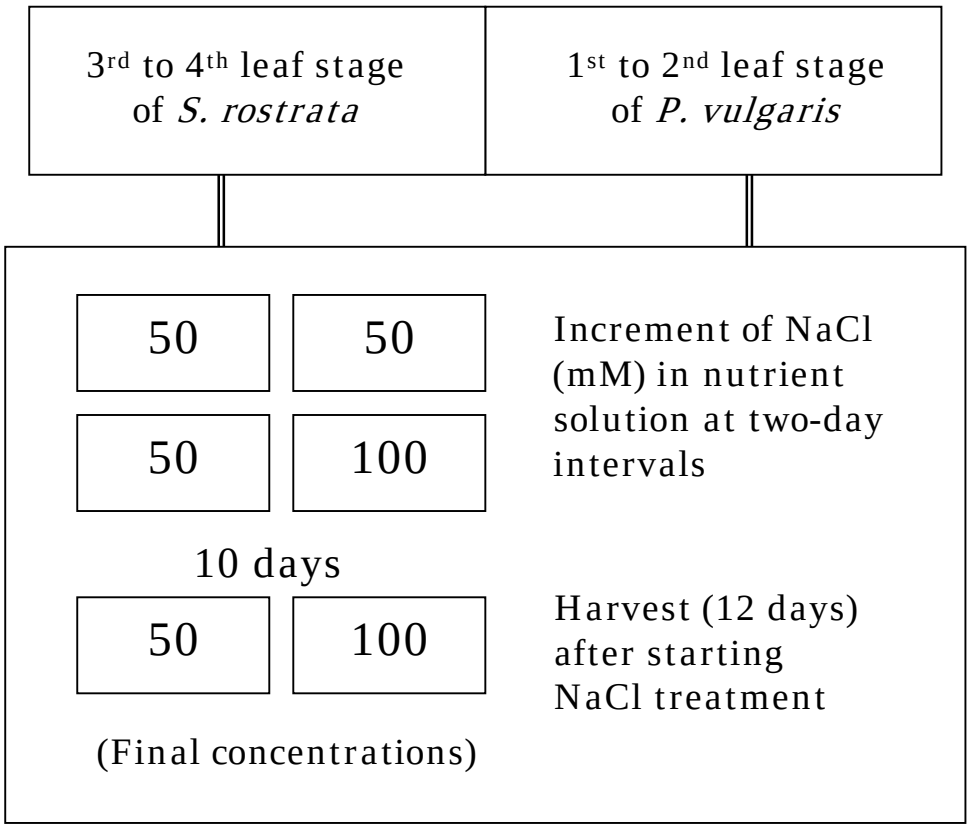

Figure 8. Step of NaCl treatment

5.3. Results

The fresh weights of shoots were reduced with increasing NaCl concentration in both species (Table 14). However, those of *S. rostrata* were less affected by the salinity; their values were more than 70% of the control at 50 and 100 mM NaCl treatment. In contrast, *P. vulgaris* was severely affected; the fresh weight of shoots reduced to 46 and 16% of the control at 50 and 100 mM NaCl, respectively.

Antioxidative enzymes activities in the two species and effect of NaCl on them were shown in Table 15 and 16. In non-treated control, the greater activity of antioxidative enzymes was found in younger leaves than in older leaves in both species. Constitutive levels of the enzyme activities in young and old leaves were much higher in *S. rostrata* than the leaves of *P. vulgaris.* In *S. rostrata*, SOD, APX and CAT activities in the young and old leaves were induced with 50 and 100 mM NaCl treatment at 10 days, while the activity of GR did not changed. In the old leaves of *P.vulgaris*, increasing salinity caused the reduction of all enzyme activities. In the young leaves of *P. vulgaris*, the enzyme activities except CAT were stimulated with 50 mM NaCl, but the higher dose of NaCl (100 mM) caused wilting of the part.

Effect of paraquat on leaf disks preparing from the seedlings treated with NaCl were determined by measuring chlorophyll contents (Fig. 9). Chlorophyll contents in the young and old leaf disks of *S. rostrata* both of non-treated and treated with 0.3 μM paraquat slightly increased with increasing NaCl concentration. In contrast, chlorophyll contents in the young and old leaf disks of *P. vulgaris* were greatly reduced with increasing NaCl concentration.

Table 14 Effects of NaCl on shoot fresh weight (g/plant) of *S. rostrata* and *P. vulgaris* 12 days after starting NaCl treatment.

	NaCl treatment		
	0 mM	**50 mM**	**100 mM**
S .rostrata			
Fresh weight	3.7 ± 0.6	4.1 ± 0.5	2.7 ± 0.4
% of control	100 ± 15.5	93 ± 5.3	73 ± 10.9
P. vulgaris			
Fresh weight	7.3 ± 0.2	1.8 ± 0.1	1.2 ± 0.1
% of control	100 ± 2.6	46 ± 5.1	16 ± 1.4

Data are the means of three replicates ± S.E.

Table 15 The effect of NaCl on activities of antioxidative enzymes in the leaves of *S. rostrata*

Activities of antioxidative enzymes	12 days after starting NaCl treatment		
	0 mM	50 mM	100 mM
Young leaves			
APX (μmol AsA decomposed/gFW/min)	6.1 ± 0.5	8.8 ± 0.8	9.5 ± 0.5
% of control	100 ± 8.2	144 ± 13.1	156 ± 8.2
CAT (μmol H_2O_2 decomposed/gFW/min)	1098 ± 44.1	1152 ± 34.2	1294 ± 64.5
% of control	100 ± 4.0	105 ± 3.1	118 ± 5.9
GR (μmol NADPH oxidized/gFW/min)	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.0
% of control	100 ± 10.0	90 ± 10.0	90 ± 0.0
SOD (Unit/gFW)	965 ± 119	896 ± 11.9	1415 ± 195
% of control	100 ± 12.3	93 ± 1.2	147 ± 20.3
Old leaves			
APX (μmol AsA decomposed/gFW/min)	3.1 ± 0.4	4.0 ± 0.5	5.4 ± 0.3
% of control	100 ± 12.9	129 ± 16.1	174 ± 9.7
CAT (μmol H_2O_2 decomposed/gFW/min)	554 ± 125	966 ± 142	1207 ± 39.0
% of control	100 ± 22.6	174 ± 25.6	218 ± 7.0
GR (μmol NADPH oxidized/gFW/min)	1.2 ± 0.1	1.1 ± 0.0	1.2 ± 0.0
% of control	100 ± 8.3	92 ± 0.0	100 ± 0.0
SOD (Unit/gFW)	529 ± 19.3	691 ± 53.3	751 ± 58.0
% of control	100 ± 3.6	131 ± 10.1	142 ± 11.0

The data are the means of three replicates ± S.E.

Table 16 The effect of NaCl on activities of antioxidative enzymes in the leaves of *P. vulgaris*

Activities of antioxidative enzymes	12 days after starting NaCl treatment		
	0 mM	50 mM	100 mM
Young leaves			
APX (μmol AsA decomposed/gFW/min)	2.8 ± 0.3	16.4 ± 1.7	N
% of control	100 ± 10.7	586 ± 60.7	
CAT (μmol H_2O_2 decomposed/gFW/min)	333 ± 41.2	172 ± 23.7	N
% of control	100 ± 12.4	52 ± 7.2	
GR (μmol NADPH oxidized/gFW/min)	0.7 ± 0.1	1.3 ± 0.1	N
% of control	100 ± 14.3	186 ± 14.3	
SOD (Unit/gFW)	201 ± 18.5	479 ± 12.4	N
% of control	100 ± 9.2	238 ± 6.2	
Old leaves			
APX (μmol AsA decomposed/gFW/min)	1.7 ± 0.1	0.4 ± 0.3	0
% of control	100 ± 5.9	24 ± 18.0	0
CAT (μmol H_2O_2 decomposed/gFW/min)	177 ± 25.6	18 ± 9.0	20 ± 13.5
% of control	100 ± 14.5	10 ± 5.0	11 ± 7.4
GR (μmol NADPH oxidized/gFW/min)	0.6 ± 0.0	0.3 ± 0.1	0.4 ± 0.2
% of control	100 ± 0.0	50 ± 16.7	67 ± 33.5
SOD (Unit/gFW)	162 ± 57.7	16 ± 10.6	40 ± 15.9
% of control	100 ± 35.7	10 ± 6.6	25 ± 10.0

- The data are the means of four replicates ± S.E.
- N indicates that the shoots of *P. vulgaris* wilted and the young parts did not develop.

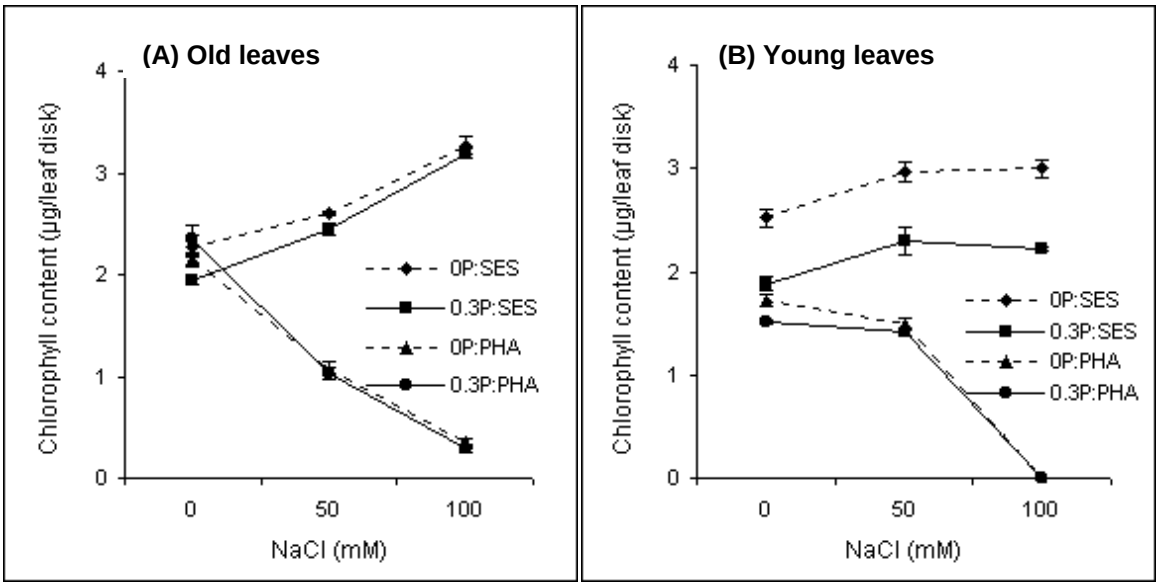

Figure 9. Effects of paraquat on chlorophyll contents in leaf disks from old leaves (A) and young leaves (B) of *S. rostrata* (SES) and *P. vulgaris* (PHA) treated by 0, 50 and 100 mM NaCl for 12 days. Vertical bars indicate standard errors (n=3). 0P, 0 µM paraquat; 0.3P, 0.3 µM paraquat.

5.4 Discussion

It has been frequently reported that salt stress induces oxidative damage in plant tissue (Hernandez *et al*. 1995, Uchida *et al*. 2002). The oxidative stress is considered to be due to increased production of O_2^-, $^{\bullet}OH$, H_2O_2 and $^1O_2$. Hence, constitutive and/or induced antioxidative enzymes such as SOD, APX, CAT and GR are needed to prevent plant tissue from the oxidative damage. The young and old leaves of *S. rostrata* have higher constitutive levels of SOD, APX, CAT and GR activities than the leaves of *P. vulgaris* (Table 15 and 16). This indicates that *S. rostrata* has a better protection system against oxidative damage caused by salinity stress. Several previous studies also reported that salt-tolerant cultivars of tomato and beet had higher constitutive levels of antioxidant enzymes (Shalata and Tal 1998, Bor *et al*. 2003). By NaCl treatment, SOD, CAT and APX activities were enhanced in the young and old leaves of *S. rostrata*. The activities of SOD, APX and GR also induced in the young leaves of *P. vulgaris* although their constitutive activities were lower than those in *S. rostrata*. These data suggest that these enzymes fulfill the important role for the tolerance to NaCl especially in *S. rostrata*.

To investigate whether these higher enzyme activities in *S. rostrata* contribute to overcome oxidative stress caused by AOS, sensitivity of leaf disks to paraquat was examined. Chlorophyll contents in the disks were measured to determine the decomposition of the pigment caused by generated AOS. With increasing NaCl concentrations, chlorophyll contents in the disks from young and old leaves of *S. rostrata* did not decrease. On the contrary, tolerance to paraquat was slightly higher in NaCl-treated plants. This finding indicates that this species has greater tolerance to the toxicity of active oxygen species. This antioxidative enzyme system in *S. rostrata* seemed to be effective for elimination of AOS which arises from salt stress. In case of *P. vulgaris*, it was difficult to determine whether the treated paraquat caused the reduction of chlorophyll content with the 50 and 100 mM NaCl treatment because the plant showed chlorosis and necrosis of the leaves.

In plant cell, chloroplast, mitochondria and peroxisomes are important intracellular generators of active oxygen species like O_2^- and H_2O_2 (Scandalios 1993). In enzymatic scavenging systems, SOD is well known as O_2^- scavenger, CAT as H_2O_2 scavenger. The combined action of SOD and CAT converts the potentially dangerous O_2^- and H_2O_2 to water (H_2O) and molecular oxygen (O_2). Results from this study showed the high constitutive and induced levels of SOD and CAT in the leaves of *S. rostrata*. This may indicate that their combined action is an effective scavenging mechanism to abate the toxic of O_2^- and H_2O_2 in the leaf cells.

In plant cells, the most important substrate for H_2O_2 detoxification is reduced ascorbate. APX uses reduced ascorbate as the electron donor for the reduction of H_2O_2 and also acts together with the strong catalysts, MDHAR, DHAR and GR (Noctor and Foyer 1998). The removal of H_2O_2 through the reaction is known as the ascorbate-glutathione cycle (Noctor and Foyer 1998). The result from this study, a higher activity of APX in the leaves of *S. rostrata* also seemed to be responsible for scavenging of H_2O_2. Because GR activity was not changed, however, it was not clear about involvement of the reaction to detoxify H_2O_2 through the ascorbate-glutathione cycle. To obtain a better understanding of antioxidant defense system in this cycle, the activity of MDHAR and DHAR enzymes should be clarified.

5.5 Conclusion

NaCl affected the growth of *P. vulgaris* much more than in *S. rostrata*. At 50 and 100 mM, shoot fresh weight of *S. rostrata* was more than 70% of the control, whereas it was less than 50% in *P. vulgaris*.

The young and old leaves of *S. rostrata* had a higher constitutive level of SOD, APX, CAT and GR activities than the leaves of *P. vulgaris*.

With increasing NaCl concentrations, *S. rostrata* induced the activity of SOD, APX and CAT both in the young and old leaves. At 50 mM NaCl, *P. vulgaris* was also induced the SOD, APX and GR activities in the young ones.

In paraquat treatment, total chlorophyll content in the young and old leaf disks from the seedlings treated with NaCl was slightly increased in *S. rostrata*, but the content was severely reduced in *P. vulgaris*.

These findings can conclude that *S. rostrata* has higher levels of antioxidative enzymes both constitutive and induced resulting in greater resistance to oxidative damage caused by NaCl stress.

Chapter 6

General Discussion and Conclusion

6.1 Growth and chlorophyll content

The results in Chapter 2 and 4 showed that young seedlings of *S. rostrata* could grow with 150 – 200 mM (0.9 – 1.2%) NaCl. The germination rate of *S. rostrata* seeds was not affected by the NaCl treatment (Table 2). These NaCl concentrations are approximately equal to those in the low-level saline soils in Northeast Thailand classified by the Department of Land Development, Thailand (as noted in the background). Most of the low-level saline soil areas are used as paddy fields. The results obtained from this study confirm that the young seedlings of *S. rostrata* are capable to grow in the affected areas before planting of paddy rice. The Department of Land Development has suggested the use of *S. rostrata* as the green manure to farmer with plowing and mixing the plants with the soil after growing 30-60 days. This practice can keep moisture in the soil, resulting in reduction of salty patches on the soil surface. It also increases soil productivity for the following rice or any other viable crops as a consequence of increases organic matters and nitrogen (Rinaudo *et al*. 1983, Halepyati and Sheelavantar 1990).

In photosynthesis, light energy utilized by higher plants is absorbed by a number of photosynthetic pigments. The most prominent pigments that absorb light are chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* is a green pigment whose absorbs maximally at wavelengths of 430 and 662 nm. Chlorophyll *b* is a bluish-green pigment that absorbs maximally at wavelength of 453 and 642 nm. These photosynthetic systems contain reaction center chlorophylls for the conversion of absorbed energy to drive an electron transport. There are two kinds of the reaction center chlorophylls. One of these chlorophylls absorbed maximally at 700 nm is called P700. The complex containing P700 is Photosystem I (PSI). The second one whose absorbs maximally at 680 nm is called P680. The complex containing P680 is Photosystem II (PSII). In the both of P700 and P680,

chlorophyll *a* is the electron acceptor that participates directly in photosynthesis. Chlorophyll *b* functions as an accessory pigment to transmit absorbed energy to chlorophyll *a*. In plants, the photochemical reactions oxidize water, produce ATP, reduce $NADP^+$ through the series of two photosystems. The ATP and reduced $NADP^+$ (NADPH) made in the photochemical reactions are used in reduction of carbon dioxide to carbohydrate (Moore *et al*. 1995). Results from this study showed that NaCl stress reduced total chlorophyll in both species. However, the effect of NaCl on chlorophyll *a*/*b* ratio showed clear difference between two species; it increased in the leaves of *S. rostrata*, but reduced in *P. vulgaris*. Furthermore, the quantum yield of photochemical reaction of PSII was unchanged in *S. rostrata*, but greatly reduced in *P. vulgaris* (Table 4). These results were also parallel with the biomass production in shoot of both species (Table 3). These findings indicate that chlorophyll *b* is more susceptible to NaCl stress and it will be a good indicator of salt stress. In *P. vulgaris*, increasing Na content in shoots (Table 6) caused the reduction of total chlorophyll as previously reported in another susceptible species (Yeo and Flowers 1983). The reduction of shoot biomass production of the plant may be attributed to these chlorosis and necrosis of the leaves that reduces the photosynthetically active area (De Herralde *et al*. 1998).

6.2 Ion distribution and translocation, proline accumulation and salt tolerance

Concerning biomass production, results of this study indicated that *S. rostrata* was a moderately salt tolerant species that could tolerate about 1% NaCl. By investigating the responses of *S. rostrata* comparing with the salt sensitive *P. vulgaris*, the following physiological characteristics possibly related to salt tolerance were found;

(1) *Root-shoot translocation*: Salt tolerant *S. rostrata* had an ability to limit Na and Cl accumulation in roots (30 – 40%) and translocate higher amount of the ions (60 – 70%) to hold in shoots (Table 10 and 12). This characteristic has been reported in some halophytes (Black 1956, Scholander *et al*. 1966). In *S. rostrata*, the shoot cells may have some mechanisms to reduce detrimental effect of the excessive salts.

(2) *Localization of Na and Cl in old leaves*: Results of this study showed that *S. rostrata* accumulated higher amount of Na and Cl in leaves, particularly in the old ones (Fig. 5). High Na concentration causes metabolic damages and cessation of growth (Schachtman and Munns 1992). The ability to localize Na and Cl in the old leaves may be one of important mechanisms of the plant to protect the relatively new and actively transpiring leaves.

(3) *Proline accumulation*: Results from this study showed that proline content was enhanced in roots and shoots of both species when exposed to salt stress (Table 7). However, at high-level concentrations of NaCl (100 and 150 mM), the salts injured *P. vulgaris* seedlings drastically (Table 3). This finding suggests that the higher accumulation of proline may not help reducing salt damage in this plant. Proline accumulation mainly helps plant cell to withstand osmotic stress (Ghoulam *et al*. 2002). Moreover, its accumulation can protect plants by maintaining protein structure or increasing scavenging of active oxygen species (Tester and Davenport 2003). Increased proline content in roots and shoots of *S. rostrata* (Table 7) is possibly related with NaCl tolerance.

In some halophytes, more than 80% of salts is transported to shoots and accumulated into leaves (Flowers 1972). The shoot cells may accompany with another physiological mechanism(s) to resist the toxic salts, for example, ion excretion in *Diplachne fusca* and *Avicennia marina* (Rains and Epstein 1967, Warwick and Halloran 1992), or ion dilution in *Atriplex* species (Greenway *et al*. 1966). In case of *S. rostrata*, 60 – 70% of Na and Cl were transported to accumulate into shoots, particularly in leaves (Table 10, Fig. 5). How the shoot cells cope with high accumulation of salt is still obscure. Visible observation of leaves and the decrease of shoot fresh weight (Table 3 and Fig. 4) suggested that ion dilution in the leaves may not be associated with the salt tolerance of *S. rostrata*. The possibility of ion excretion from leaves was also tested in *S. rostrata*. The seedlings of *S. rostrata* were treated with 200 mM NaCl for 2 weeks. The test leaves were harvested and washed. Na and Cl contents in the obtained solutions were then analyzed. The results showed that the Na and Cl contents were not different between the control and treatment. This suggests that ion excretion from the leaf surface is not associated with the salt tolerance of *S. rostrata*.

Therefore, involvement of the other molecular mechanisms such as sequestering of Na and Cl into the vacuole, and synthesizing and accumulating some compatible solutes in the cytoplasm for intracellular osmotic adjustment is suggested in the leaf cell (Verma 1999). Proline has been known as the compatible solutes which protect the cells from salt injury of halophytic plants (Stewart and Lee 1974, Zhu 2003). In this study, higher accumulation of Na, Cl and proline in the shoots of *S. rostrata* (Table 7 and 10) are likely involved in the alleviation of salt stress.

6.3 Activity of antioxidative enzymes and salt tolerance

Salt stress often induces oxidative damage in plant tissue. Oxidative stress is caused by increased production of active oxygen species (AOS), such as O_2^-, $^{\bullet}OH$, H_2O_2 and $^1O_2$. The AOS produced raised lipid peroxidation, enzyme inactivation and oxidative damage to DNA (Davies 1987, Imlay and Linn 1988, Bor *et al.* 2003). Plants have antioxidant defense systems to protect against the production and action of the active oxygen species. Antioxidants can be divided into three classes: (1) lipid soluble and membrane-associated compounds α-tocopherol and β-carotene (2) water soluble reductants such as ascorbate (AsA) and glutathione (GSH) (3) scavenging enzymes such as SOD, CAT, APX and GR.

Results of this study (Table 15 and 16 and Fig. 9) clarified the differences of antioxidative enzyme activities between *S. rostrata* and *P. vulgaris*. A high level of one or more antioxidative enzymes, either constitutive or induced, has been reported to correlate with plant resistance to salt stress (Gossett *et al.* 1994, Sreenivasulu *et al.* 1999). In this study, constitutive CAT, GR, SOD, APX and induced CAT, SOD activities in the leaves of *S. rostrata* were higher than those in *P. vulgaris* leaves. The high constitutive and induced levels of SOD and CAT in the leaves of *S. rostrata* (Table 15) might indicate that the combined action of them was an effective scavenging mechanism to detoxify O_2^- and H_2O_2 in the leaf cells. These results suggest that there has relationship between antioxidative enzyme activities and salt tolerance in *S. rostrata*. A better protection capacity in

S. rostrata against increased O_2^- and H_2O_2 is considered to be one of the mechanisms of salt-tolerance.

For the studies of physiological and biochemical responses to NaCl between *S. rostrata* and *P. vulgaris* (Table 17), the salt tolerant mechanism of *S. rostrata* was found to be related with an ability to transport and sequester Na and Cl in shoot cells, localization of the ions in the old leaves, high proline accumulation and high constitutive and induced levels of antioxidative enzymes (Fig.10).

Table 17 Differences in physiological and biochemical responses to NaCl stress between *S. rostrata* and *P. vulgaris*

1. Growth
- *S. rostrata* can grow with 0.9 – 1.2% NaCl-solution
- *P. vulgaris* can grow with ≤ 0.3% NaCl-solution
* *S. rostrata* is more salt-tolerant than *P. vulgaris*
2. Effect of NaCl to chlorophyll content and fluorescence
- Reduction of chlorophyll *b* in *S. rostrata*
- Reduction of chlorophyll *a*, *b* and fluorescence in *P. vulgaris*
* Activity of photosynthesis in the NaCl-treated leaves is higher in *S. rostrata* than *P. vulgaris*
3. Na and Cl accumulation in roots and translocation them to shoots
- Restriction of Na and Cl accumulation in roots and translocation of higher amount of the ions to hold in shoots of *S. rostrata*
- Higher accumulation of Na in roots of *P. vulgaris*
* *S. rostrata* has an ability to translocate and sequester Na and Cl in shoots (leaves)
4. Proline accumulation
- Higher accumulation of proline in shoots and roots was found in both species, but total content is higher in *S. rostrata*
* Proline is an effective compatible solute to alleviate NaCl stress in *S. rostrata*.
5. The activity of antioxidative enzymes and paraquat sensitivity
- Constitutive levels of APX, CAT, GR and SOD activities in young and old leaves of *S. rostrata* are higher than the leaves of *P. vulgaris*
- Total induced activities of CAT and SOD in young and old leaves of *S. rostrata* is greater than the leaves of *P. vulgaris*.
- NaCl-treated leaf disks of *S. rostrata* showed greater tolerance to an active-oxygen generating herbicide paraquat
* *S. rostrata* has higher levels of antioxidative enzymes, either constitutive and induced, resulting in greater resistance to oxidative damage caused by NaCl stress

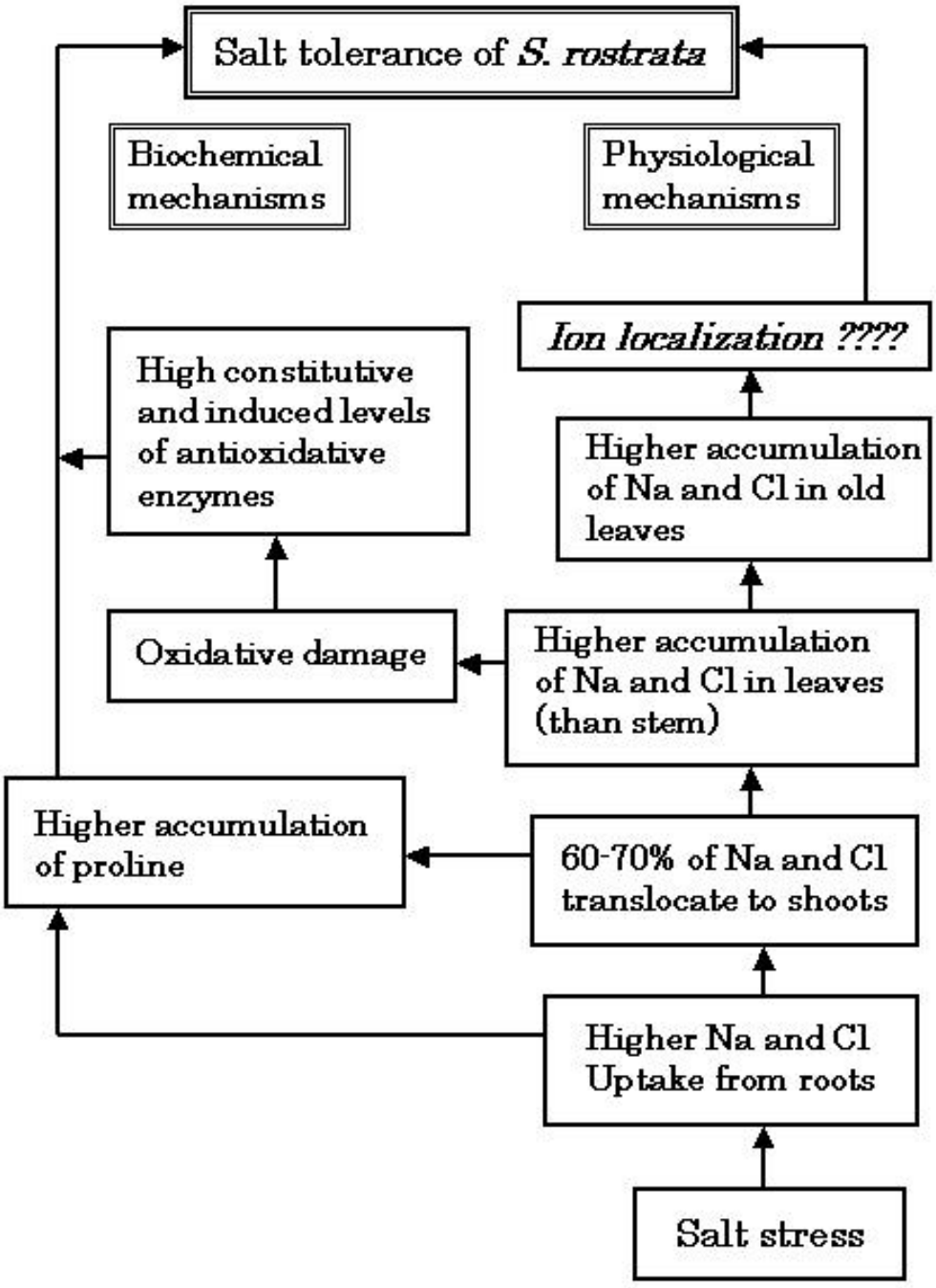

Figure 10. The possible biochemical and physiological mechanisms of salt tolerance in *Sesbania rostrata*

Physiological and Biochemical Mechanisms of Salt Tolerance in *Sesbania rostrata* Brem. & Oberm.

Abstract

Saline soil is a serious problem which affects the yields of commercial crops in the Northeastern part of Thailand. To reduce the devastating effects of salinity on crops, many trials have been conducted. One of the biological approaches is to use halophytes as cover plant. *Sesbania rostrata* Brem. & Oberm., a member of leguminous family, has been used as a promising plant to ameliorate the affected area. Until now, very little is known about mechanisms of salt tolerance in this plant. To obtain more information on the salt-tolerant mechanisms, the physiological and biochemical responses of *S. rostrata* to NaCl were investigated comparing with those of salt-sensitive species, *Phaseolus vulgaris* L. cv. Meal (kidney bean). In this study, all experiments were conducted in a growth chamber at 25/20°C day/night temperatures, with a 14 h photoperiod at 280-290 $\mu E/m^2/s$, and relative humidity of 70 – 80%. The experiments were designed with three replications. Seedlings of both species were grown hydroponically with 0, 20, 50, 100, 150 and 200 mM NaCl (the concentrations used differed in each Chapter) and harvested 12-18 days after starting NaCl treatment. Effects of NaCl on seed germination, biomass production, chlorophyll content, fluorescence yield (F_v/F_m), ions distribution, change of amino acid and proline contents, ions uptake and translocation, change of antioxidative enzyme activity and paraquat sensitivity were determined.

The results showed that tolerance to NaCl was clearly different between two species. *S. rostrata* could normally grow in 0.9 – 1.2% of NaCl-solution, while 0.3% NaCl-solution caused drastic leaf injury in *P. vulgaris*. The concentrations of 0-200 mM NaCl were not affected to germination rate of *S. rostrata* seeds. Increasing NaCl concentrations reduced the content of chlorophyll *b* only in the leaves of *S. rostrata*, but severely reduced total chlorophyll content and chlorophyll fluorescence in *P. vulgaris*. Under salinity stress, a higher accumulation of Na and Cl was found in shoots of *S. rostrata*. It showed that 60-70% of Na and Cl were translocated to accumulate in the shoots. Moreover, the

high accumulation of these ions tended to accumulate in the older leaves of *S. rostrata*. While, *P. vulgaris* responded to fairly low salinities by accumulating Na in the roots. Phosphorus content increased with increasing NaCl in both species but potassium content was reduced. Magnesium and calcium contents showed no noteworthy changes in either two species. Higher accumulation of amino acids and proline contents was observed with increasing NaCl in both species. The young and old leaves of *S. rostrata* were higher constitutive level of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities than those of *P. vulgaris*. NaCl stress induced the activities of CAT and SOD in the young and old leaves of *S. rostrata* with higher total activities than those of *P. vulgaris*. NaCl-treated leaf disks of *S. rostrata* showed greater tolerance to an active oxygen generating herbicide paraquat.

The results obtained from this study indicate that physiological and biochemical mechanisms of salt tolerance in *S. rostrata* are related with an ability to transport and sequester Na and Cl in shoots, higher accumulation of Na and Cl in the old leaves, higher proline accumulation and high constitutive and induced level of antioxidative enzymes.

Acknowledgements

I would like to express my grateful appreciation to all people who greatly contributed to the success of my study and the completion of this dissertation.

Firstly, I would like to express my sincerest appreciation to Prof. Dr. Hiroshi Matsumoto, my advisor and Chairman of the Advisory Committee, who kindly welcomed me to study at the University of Tsukuba and continually provided me with full guidance and supervision throughout my tenure.

I express my sincere appreciation to the members of my Advisory Committee: Prof. Dr. Kenji Usui, Assoc. Prof. Dr. Katsuichiro Kobayashi and Prof. Dr. Masao Yokoo for their support throughout the period and the improvement of the manuscript.

I am grateful appreciation to Assoc. Prof. Dr. Srisom Suwanwong for her guidance and moral support throughout my graduated studies and extremely grateful to Asst. Prof. Dr. Yukari Sunohara for her helpful comments and suggestions in my research work.

My study would not have even commenced without the financial support from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) to which I would like to express my grateful acknowledgement.

My special thanks express to Land Development Department, Thailand for supplying seeds of *Sesbania rostrata* used in the experiments.

My deep thankfulness is extended to Mr. Alan Richardt who kindly contributed his time to correct my English in the dissertation.

I am thankful to Ms. Ayumi Hachinohe, Ms. Hoai Nguyen Thi Thu, all of the members in Laboratory of Plant Function Regulation, Institute of Applied Biochemistry and my friends in the Thai community in the University of Tsukuba for their kindness and warm friendship during my entire stay in Japan.

Finally, I would like to express my deepest gratitude to Mr. Jan, Ms. Tanomsin and Ms. Jariya Jungklang for their endless love, encouragements and understanding of my whole education.

References

Adams P., Thomas J.C., Vernon D.M. Bohnert H.J. and Jensen R.G. 1992. Distinct cellular and organismic responses to salt stress. *Plant Cell Physiol.* **33**, 1215-1223.

Alia S.P.P. 1991. Suppression in mitochondrial electron transport is the primary cause behind stress induced proline accumulation. *Biochem. Biophys. Res. Commun.* **193**, 54-58.

Apse M.P., Aharon G.S., Snedden W.A. and Blumwald E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. *Science* **285**, 1256-1258.

Aroca R., Irigoyen J.J. and Sanchez-Diaz M. 2001. Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity. *Plant Sci.* **161**, 719-726.

Barkla B.J., Zingarelli L., Blumwald E. and Smith J.A.C. 1995. Tonoplast Na^+/H^+ antiport activity and its energization by the vacuolar H^+-ATPase in the halophytic plant *Mesembryanthemum crystallinum* L. *Plant Physiol.* **109**, 549-556.

Beauchamp C. and Fridovich I. 1971. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**, 276-287.

Belkhodja R., Morales F., Abadia A., Gomez-Aparisi J. and Abadia J. 1994. Chlorophyll fluorescence as a possible tool for salinity tolerance screening in barley (*Hordeum vulgare* L.). *Plant Physiol.* **104**, 667-673.

Besford R.T. 1978. Effect of replacing nutrient potassium by sodium on uptake and distribution of sodium in tomato plants. *Plant Soil.* **50**, 399-409.

Black R.F. 1956. Effect of NaCl in water culture on the ion uptake and growth of *Atriplex hastata* L. *Aust. J. Biol. Sci.* **9**, 67-80.

Bohnert H.J. and Su H. and Shen B. 1999. Molecular mechanisms of salinity tolerance. In: Biotechnology intelligence unit1: molecular responses to cold, drought, heat and salt stress in higher plants (ed. by Shinozaki K. and Yamaguchi-Shinozaki K.). R.G. Landes Company, pp. 29-60.

Bor M., Ozdemir F. and Turkan I. 2003. The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Sci.* **164**, 77-84.

Bowler C., Montagu M.V. and Inze D. 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 83-116.

Bremberger C. and Luttge U. 1992. Dynamics of tonoplast proton pumps and other tonoplast proteins of *Mesembryanthemum crystallinum* L. during the induction of Crassulacean acid metabolism. *Planta* **188**, 575-580.

Chappelle E.W., Kim M.S. and McMurtrey J.E. 1992. Ratio analysis of reflectance spectra (RARS): an algorithm for the remote estimation of the concentrations of chlorophyll *a*, chlorophyll *b* and carotenoids in soybean leaves. *Remote Sens. Environ.* **39**, 239-247.

Chavan P.D. and Karadge B.A. 1986. Growth, mineral nutrition, organic constituents and rate of photosynthesis in *Sesbania grandiflora* L. grown under saline conditions. *Plant Soil.* **93**, 395-404.

Chen T.H.H. and Murata N. 2002. Enhancement of tolerance of abiotic stress

by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* **5**, 250-257.

Clarkson D.T. and Hanson J.B. 1980. The mineral nutrition of higher plants. *Ann. Rev. Plant Physiol.* **31**, 239-298.

Davies K.J.A. 1987. Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **262**, 9895-9901.

De Herralde F., Biel C., Save R., Morales M.A., Torrecillas A., Alarcon J.J. and Sanchez-Blanco M.J. 1998. Effect of water and salt stresses on the growth, gas exchange and water relations in *Argyranthemum coronopifolium* plants. *Plant Sci.* **139**, 9-17.

Delauney A.J. and Verma D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. *Plant J.* **4**, 215-223.

Desmaison A.M., Marcher M.H. and Tixier M. 1984. Changes in the free and total amino acid composition of ripening chestnut seeds. *Phytochemistry* **23,** 2453-2456.

Dionisio-Sese M.L. and Tobita S. 1998. Antioxidant responses of rice seedlings to salinity stress. *Plant Sci.* **135**, 1-9.

Dionisio-Sese M.L. and Tobita S. 2000. Effects of salinity on sodium content and photosynthetic responses of rice seedlings differing in salt tolerance. *J. Plant Physiol.* **157**, 54-58.

Division of Soil Analysis. 2002. Improving the management of salted-affected soils. Case of saline patches in rained paddy fields in Northeast Thailand. Land Development Department, Bangkok, Thailand. (http://www.th.ird.fr/activities/salted01.htm, accessed on May14, 2002)

Eder A., Huber W. and Sankhla N. 1977. Interaction between salinity and ethylene in nitrogen metabolism of *Pennisetum typhoides* seedlings. *Biochem. Physiol. Pflanzen.* **171**, 93-100.

Egert M. and Tevini M. 2002. Influence of drought on some physiological parameters symptomatic for oxidative stress in leaves of chives (*Allium schoenoprasum*). *Environ. Exp. Bot.* **48**, 43-49.

Elstner E.F. 1982. Oxygen activation and oxygen toxicity. *Ann. Rev. Plant Physiol.* **33**, 73-96.

Fitter A.H. and Hay R.K.M. 1987. Environmental physiology of plants. Academic Press, 423p.

Flowers T.J. 1972. Salt tolerance in *Suaeda maritima* (L.) Dum. *J. Ext. Bot.* 23, 310-321.

Flowers T.J. 1985. Physiology of halophytes. *Plant Soil.* **89**, 41-56.

Flowers T.J., Troke P.F. and Yeo A.R. 1977. The mechanism of salt tolerance in halophytes. *Ann. Rev. Plant Physiol.* **28**, 89-121.

Fridovich I. 1986. Biological effects of the superoxide radical. *Arch. Biochem. Biophys*. **247**, 1-11.

Ghoulam C., Foursy A. and Fares K. 2002. Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environ. Exp. Bot*. **47**, 39-50.

Girija C., Smith B.N. and Swamy P.M. 2002. Interactive effects of sodium

chloride and calcium chloride on the accumulation of proline and glycinebetaine in peanut (*Arachis hypogaea* L.). *Environ. Exp. Bot.* **47**, 1-10.

Gossett D.R., Millhollon E.P. and Cran Lucas M. 1994. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop Sci.* **34**, 706-714.

Gouia H., Ghorbal M.H. and Touraine B. 1994. Effects on NaCl on flows of N and mineral ions and on NO_3^- reduction rate within whole plants of salt-sensitive bean and salt-tolerant cotton. *Plant Physiol.* **105**, 1409-1418.

Greenway H., Gunn A. and Thomas D.A. 1966. Plant response to saline substrates: VIII. Regulation of ion concentrations in salt-sensitive and halophytic species. *Aust. J. Biol. Sci.* **19**, 741-756.

Halepyati A.S. and Sheelavantar M.N. 1990. *Sesbania rostrata* – a new green manure for rice. *Indian J. Agron.* **35**, 279-282.

Halliwell. B and Gutteridge J.M.C. 1989. Free radicals in biology and medicine. Clarendon Press, Oxford.

Hernandez J.A., Olmos E., Corpas F.J., Sevilla F. and del Rio L.A. 1995. Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Sci.* **105**, 151-167.

Imlay J.A. and Linn S. 1988. DNA damage and oxygen radical toxicity. *Science* **240**, 1302-1309.

Jacoby B. 1979. Sodium recirculation and loss from *Phaseolus vulgaris* L. *Ann. Bot.* **43**, 741-744.

Kawashima M., Takeda K. and Kondo N. 2000. Enhancement of oxidative stress tolerance and antioxidative systems in UV-B irradiated cucumber (*Cucumis sativus* L.) seedlings. *Environ. Sci.* **13**, 539-548.

Kim Y.H., Shim I.S., Kobayashi K. and Usui K. 1999. Relationship between Na content or K/Na ratio in shoots and salt tolerance in several Gramineous plants. *J. Weed Sci. Tech.* **44**, 293-299.

Krause G.H. and Weis E. 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Ann. Rev. Plant Physiol. Mol. Biol.* **42**, 313-349.

Ladha J.K., Miyan S. and Garcia M. 1989. *Sesbania rostrata* as a green manure for lowland rice: Growth, N_2 fixation, *Azorhizobium* sp. inoculation, and effects on succeeding crop yields and nitrogen balance. *Biol. Fertil. Soils.* **7**, 191-197.

Land Development Department. 1996a. Land use pattern and land suitability in Thailand. Bangkok, Thailand. (http://www.ldd.go.th/EFiles_html/land%20resource/ed0300.htm, accessed on Mar17, 1996).

Land Development Department. 1996b. Planting *S. rostrata* in order to increase rice production. Bangkok, Thailand. (http://www.ldd.go.th/EFiles_project/article/article03.html, accessed on May3, 1996).

Land Development Department. 1996c. Reclamation of saline soil in the Northeast. Bangkok, Thailand. (http://www.ldd.go.th/EFiles_project/ld-technique/Technique/k07.htm, accessed on Jan4, 1996)

Lascano H.R., Melchiorre M.N., Luna C.M. and Trippi V.S. 2003. Effect of photooxidative stress induced by paraquat in two wheat cultivars with differential tolerance to water stress. *Plant Sci.* **164**, 841-848.

Levitt J. 1972. Responses of plants to environmental stresses. Academic Press, London, 697p.

Lutts S., Kinet J.M. and Bouharmont J. 1996. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Plant Growth Regul.* **19**, 207-218.

Ma H.C., Fung L., Wang S.S., Altman A. and Huttermann A. 1997. Photosynthetic response of *Populus euphratica* to salt stress. *For. Ecol. Manage.* **93**, 55-61.

Mahmood K. 1998. Effects of salinity, external K^+/Na^+ ratio and soil moisture on growth and ion content of *S. rostrata*. *Biol. Plant.* **41**, 297-302.

Marcum K.B. and Murdoch C.L. 1994. Salinity tolerance mechanisms of six C_4 turfgrasses. *J. Amer. Soc. Hortic. Sci.* **119**, 779-784.

Marler T.E. and Mickelbart M.V. 1993. Growth and chlorophyll fluorescence of *Spondias purpurea* L. as influenced by salinity. *Trop. Agric.* **70**, 245-247.

Matoh T., Saraswati R., Phupaibul P. and Sekiya J. 1992. Growth characteristics of *Sesbania* species under adverse edaphic conditions in relation to use as green manure in Japan. *Soil Sci. Plant Nutr.* **38**, 741-747.

Moore R., Clark W.D., Stern K.R. and Vodopich D. 1995. Botany:

photosynthesis. Wm. C. Brown Publishers, Inc. Chicago, USA. 824p.

Ndoye I., Dreyfus B. and Becker M. 1996. *Sesbania rostrata* as green manure for lowland rice in Casamance (Senegal). *Trop. Agric*. **73**, 234-237.

Noctor G. and Foyer C.H. 1998. Ascorbate and glutathione: Keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 249-279.

Ohta Y., Yamamoto K. and Deguchi M. 1970. [Efeect of calcium supply and plant age on the distribution of calcium salts in rice.] *Jpn. J. Soil Sci. Plant Nutr.* **41**, 19-26 (in Japanese).

Pardossi A., Malorgio F. and Tognoni F. 1999. Salt tolerance and mineral relations for celery. *J. Plant Nutr.* **22**, 151-161.

Rabie R.K. and Kumazawa K. 1988. Effect of NaCl salinity on growth and distribution of sodium and some macronutrient elements in soybean plant. *Soil Sci. Plant Nutr.* **34**, 375-384.

Rains D.W. and Epstein E. 1967. Preferential absorption of potassium by leaf tissue of the mangrove, *Avicennia marina*: an aspect of halophytic competence in coping with salt. *Aust. J. Biol. Sci.* **20**, 847-857.

Rajendrakumar C.S., Reddy B.V. and Reddy A.R. 1994. Proline-protein interaction: Protection of structural and functional integrity of M4 lactate dehydrogenase. *Biochem. Biophys. Res. Commum.* **201**, 957-963.

Ramani S. and Kannan S. 1986. Absorption and transport of Na and Cl in rice cultivars differing in their tolerance to salinity: an examination of the effects of ammonium and potassium salts. *J. Plant Nutr.* **9**,1553-1564.

Ramani S., Joshua D.C. and Shaikh M.S. 1989. Response of three *Sesbania* species to salinity when grown hydroponically. *J. Plant Nutr.* **12**, 1447-1455.

Rao G.G. and Rao G.R. 1981. Pigment composition & chlorophyllase activity in pigeon pea (*Cajanus indicus* Spreng) & Gingelley (*Sesamum indicum* L.) under NaCl salinity. *Indian J. Exp. Bio.* **19**, 768-770.

Rinaudo G., Dreyfus B. and Dommergues Y. 1983. *Sesbania rostrata* green manure and the nitrogen content of rice crop and soil. *Soil Biol. Biochem.* **15**, 111-113.

Roger P. and Watanabe I. 1986. Technologies for utilizing biological nitrogen fixation in wetland rice: potentialities, current usage, and limiting factors. *Fert. Res.* **9**, 39-77.

Rudolph A.S., Crowe J.H. and Crowe L.M. 1986. Effects of three stabilizing agents-proline, betaine, and trehalose on membrane phospholipids. *Arch. Bioche. Biophys.* **245**, 134-143.

Saraswati R., Matoh T. and Sekiya J. 1992. Nitrogen fixation of *Sesbania rostrata*: Contribution of stem nodules to nitrogen acquisition. *Soil Sci. Plant Nutr.* **38**, 775-780.

Scandalios J.G. 1993. Oxygen stress and superoxide dismutases. *Plant Physiol.* **101**, 7-12.

Schachtman D.P. and Munns R. 1992. Sodium accumulation in leaves of *Triticum* species that differ in salt tolerance. *Aust. J. Plant Physiol.* **19**, 331-340.

Scholander P.F., Bradstreet E.D., Hammel H.T. and Hemmingsen E.A. 1966. Sap concentrations in halophytes and some other plants. *Plant Physiol.* **41**, 529-532.

Shalata A. and Tal M. 1998. The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plant.* **104**, 169-174.

Sies H. 1997. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.* **82**, 291-295.

Singh A.K. and Dubey R.S. 1995. Changes in chlorophyll *a* and *b* contents and activities of photosystems 1 and 2 in rice seedlings induced by NaCl. *Photosynthetica* **31**, 489-499.

Smillie R.M. and Nott R. 1982. Salt tolerance in crop plants monitored by chlorophyll fluorescence *in vivo*. *Plant Physiol.* **70**, 1049-1054.

Smirnof N. and Cumbes Q.J. 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* **28**, 1057-1060.

Sreenivasulu N., Ramanjulu S., Ramachandra-Kini K., Prakash H.S., Shekar-Shetty H., Savithri H.S. and Sudhakar C. 1999. Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. *Plant Sci.* **141**, 1-9.

Stewart G.R. and Lee J.A. 1974. The role of proline accumulation in halophytes. *Planta* **120**, 279-289.

Suntres Z.E. 2002. Role of antioxidants in paraquat toxicity. *Toxicology* **180**, 65-77.

Tattini M., Gucci R., Coradeschi M.A., Ponzio C. and Everard J.D. 1995. Growth, gas exchange and ion content in *Olea europaea* plants during salinity stress and subsequent relief. *Physiol. Plant.* **95**, 203-210.

Tester M. and Davenport R. 2003. Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.* **91**, 503-527.

Uchida A., Jagendorf A.T., Hibino T., Takabe T. and Takabe T. 2002. Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. *Plant Sci.* **163**, 515-523.

Umezawa T., Shimizu K., Kato M. and Ueda T. 2000. Enhancement of salt tolerance in soybean with NaCl pretreatment. *Physiol. Plant.* **110**, 59-63.

Vaidyanathan H., Sivakumar P., Chakrabarty R. and Thomas G. 2003. Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.)—differential response in salt-tolerant and sensitive varieties. *Plant Sci.* **165**, 1411-1418.

Venekamp J.H. 1989. Regulation of cytosol acidity in plants under conditions of drought. *Physiol. Plant.* **76**, 112-117.

Venkatesan A. and Chellappan K.P. 1998. Accumulation of proline and glycine betaine in *Ipomoea pes-caprae* induced by NaCl. *Biol. Plant.* **41**, 271-276.

Verma D.P.S. 1999. Osmotic stress tolerance in plants: role of proline and sulfer metabolisms. In: Biotechnology intelligence unit1: molecular responses to cold, drought, heat and salt stress in higher plants (ed. by Shinozaki K. and Yamaguchi-Shinozaki K.) R.G. Landes Company, pp. 153-168.

Wahome P.K., Jesch H.H. and Grittner I. 2001. Mechanisms of salt stress tolerance in two rose rootstocks: *Rosa chinensis* 'Major' and *R. rubiginosa*. *Sci. Hortic.* **87**, 207-216.

Warwick N.W.M. and Halloran G.M. 1992. Accumulation and excretion of sodium, potassium and chloride from leaves of two accessions of *Diplachne fusca* (L.) Beauv. *New Phytol.* **121**, 53-61.

Wongwattana C., Na Nakorn M., Surawattananon S. and Kobayashi K. 1998. Effect of sodium chloride on growth and root nodulation in some leguminous plants. *J. Weed Sci. Tech.* **43**, 129-133.

Xing W. and Rajashekar C.B. 1999. Alleviation of water stress in beans by exogenous glycine betaine. *Plant Sci.* **148**, 185-195.

Yanagida M., Matsumoto H. and Usui K. 1999. Responses of antioxidative systems to oxyfluorfen and their role in herbicidal tolerance of plants. *J. Weed Sci. Tech.* **44**, 67-76.

Yeo A.R. and Flowers T.J. 1983. Varietal differences in the toxicity of sodium ions in rice leaves. *Physiol. Plant.* **59**, 189-195.

Zhu J.K. 2003. Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.* **6**, 441-445.