Master thesis

Nutrio-physiological studies on salinity tolerance of New Zealand spinach (*Tetragonia tetragonioides*)

Yousif Basim S. Yousif

Graduate School of Biosphere Science
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We hereby recommend that the dissertation by Mr. Yousif Basim S. Yousif entitled Nutrio-physiological studies on salinity tolerance of New Zealand spinach (*Tetragonia tetragonioides*) be accepted in partial fulfillment of the requirements for the degree of MASTER OF AGRICULTURS

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Contents

Abbreviations

Abbrev. /symbol	Word/ unit
DW	Dry weight
FW	Fresh weight
H_2O_2	Hydrogen peroxide
C_i	Intercellular CO_2 concentration
ψ_1	Leaf water potential
MDA	Malondialdehyde
OA	Osmotic adjustment
ψ_π	Osmotic potential
$\psi_{\pi\,(100)}$	Osmotic potential at full turgor
P_0	Photosynthetic rate
RGR	Relative growth rate
RWC	Relative water content
NaCl	Sodium chloride
G_s	Stomatal conductance
T_r	Transpiration ratio

Chapter 1

Introduction

Salinity in soil is one of the major stresses, and more than 800 million hectare of world land surface area is salt-affected (about 6%) (Turkan & Demiral, 2009; Munns & Tester, 2008). In Iraq, soil salinity affects approximately 75% of the area of central and southern parts, and very often occurs together with drought and these are the major constrains that limit crop production in the arid regions (Halim *et al.*, 1988; Lennard, 2003). The ability of vegetation to survive under higher salinity conditions is important for the distribution of plants and agriculture around the world. Enhancing the salt tolerance of plants is an important breeding objective in areas which are affected by soil salinity (Flowers & Flowers, 2005; Greenway & Munns, 1983).

A plant's ability of acclimatization to salt stress includes alterations at the leaf level, associated with morphological, physiological and biochemical characteristics whereby many plants adjust to high salinity and the consequent low soil water availability (Munns, 2002; Ashraf, 2004). One of the major effects of salt stress in plants is induced nutritional disorders; these disorders may result from the effect of salinity on nutrient availability, competitive uptake, and transport or partitioning within the plant; salinity

dominated by Na^+ salts reduces Mg^{2+} and Ca^{2+} availability and K^+ uptake; salinity can also cause a combination of complex interactions that affect plant metabolism, susceptibility to injury or internal nutrient requirement (Grattan & Grieve, 1999; Bartels & Sunkar, 2005; Munns & Tester, 2008).

Under saline conditions, halophytic plants tend to take up and accumulate Na^+ in their vacuoles and use it as an osmoticum, halophyte membrane lipids may also be adapted to prevent salt leakage (Glenn & Brown, 1999); however non-halophytic monocotyledons tend to exclude Na^+ to maintain a high K^+/Na^+ ratio, which seems to be crucial for salt tolerance, but may consequently be deficient in solutes for osmotic regulation (Greenway & Munns, 1983; Grattan & Grieve, 1999). Salt stress also induces a decrease in stomatal conductance and transpiration. Under saline conditions, stomatal closure helps to maintain higher leaf water content; however this leads to a decrease in the leaf CO_2 assimilation rate (Maggio *et al.*, 2000; Parida *et al*., 2004).

Osmotic adjustment is recognized as an important adaptive mechanism for poor water availability as it helps maintain growth in many plants, and there would be little advantage in selecting for plants with a higher capacity for osmotic adjustment under stress (Flowers *et al*., 1990; Morgan, 1995). Osmotic adjustment involves the regulation of the intracellar levels of

organic and inorganic elements; many of organic compounds are compartmentalized principally in the cytoplasm, whereas inorganic ions are sequestered in the vacuole, the vacuoles are the major sequestration site of NaCl in leaves of halophyte (Matoh *et al*., 1987). Under saline conditions, the osmotic adjustment which occurs through the accumulation of inorganic compounds (mainly Na^+ and Cl^-) in plants is less energy and carbon-demanding than the adjustment by organic solutes; the synthesis of organic solutes for osmotic regulation would require less than 10% of the carbohydrate consumed in respiration (Greenway & Munns, 1983).

The mechanisms of salt tolerance include osmotic adjustment by accumulation of compatible solutes such as proline and soluble sugars (Yancy, 2005; Hasegawa *et al.*, 2000) and lowering the toxic concentration of ions in the cytoplasm by restriction of Na^+ influx or its sequestration into vacuole and/or its extrusion (Blumwald, 2000). The contributory role of proline to osmotic adjustment was reported by many researchers (Meloni *et al.,* 2003; Tani & Sasakawa, 2006; Ashraf & Foolad, 2007; Lee *et al.,* 2004). Proline has also been considered as a carbon and nitrogen source for growth, a stabilizer for membranes and some macromolecules and also a free radical scavenger under stress condition (Lutts *et al.,* 1995; Mansour, 1998; Molinari *et al.,* 2007).

It is well known that free radical-induced peroxidation of lipid membrane is a reflection of stress-induced damage at cellular level (Parida & Das, 2005). Therefore, the level of MDA (malondialdhyde), produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. Mandhania *et al.* (2006) reported that a salt sensitive wheat (*Triticum aestivum*) line suffered greater damage to cellular membranes due to lipid peroxidation, as indicated by higher accumulation of H_2O_2 and MDA compared to a salt tolerant line.

New Zealand spinach (*Tetragonia tetragonioides* Pall.) is a member of Tetragoniaceae and is distributed widely from tropical and subtropical to temperate areas (Wilson *et al.,* 2000; Matraszek, 2008). This plant is used as a vegetable, ground-cover cum ornamental and medicinal sources with, anti-ulcerogenic and anti-inflammatory activities as indicated by the compounds isolated from New Zealand spinach (Kato *et al.,* 1985). However, there is very little information about the physiological characteristics of this plant (Wilson *et al.,* 2000).

Water spinach (*Ipomoea aquatica* L.) is a member of Convolvulaceae and is distributed in humid areas from subtropical to temperate zones, and is used as a green vegetable, food for livestock, and is useful for the sequestration of environmental pollutants (Harwood & Sytsma, 2003; Yao *et*

al., 2009). The physiological responses to salt tolerance of New Zealand spinach and water spinach have not been reported.

Chard (*Beta vulgaris* L.) is a glycophytic member of the Chenopodiaceae and is distributed all over the world, and is used as a green vegetable (Shannon & Grieve, 1998).

The objectives of this thesis were to compare salinity tolerance among New Zealand spinach, water spinach and chard in terms of plant growth, water relations, osmotic adjustment, photosynthetic activity and accumulations of ions, soluble sugars, proline and malondialdhyde (MDA), and discuss the salinity tolerance of New Zealand spinach to salinity.

Chapter 2

Comparative physiological responses of New Zealand spinach and water spinach to salinity

2.1. Introduction

Physiological criteria such as growth characters, water relations, photosynthesis, and accumulation of various inorganic ions and organic metabolites in plants are able to attribute as selection criteria for improving salt tolerance through selection and breeding programs (Ashraf, 2004). Therefore, understanding mechanisms of salt tolerance will be an important first step in the search for physiological characters that contribute to the resistance in salt sensitive agricultural crops (Parida & Das, 2005).

The objectives of this chapter were to assess the influence of salt stress on the absorbance of inorganic ions, photosynthesis, and water relations in New Zealand spinach and water spinach and discussed the physiological responses and adaptive strategies of New Zealand spinach to salt stress.

2.2. Materials and Methods

Plant material and culture conditions

This experiment was conducted at the Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan, from April to June, 2008. Seeds of New Zealand spinach and water spinach were germinated in seedbeds with a soil mixture containing granite regosol soil, perlite and peat moss (2:1:1 v/v/v). Pots were kept under greenhouse conditions. Plants were irrigated with nutrient solution at each watering using an irrigation system. The basal nutrient solution contained 8.3 mM NO_3-N, 0.8 mM NH_4-N, 0.5 mM P_2O_5, 2.2 mM K_2O, 0.7 mM MgO, 2.1 mM CaO, 11μM MnO, 5μM B_2O_3 and 13μM Fe. At six weeks after transplanting, the plants were subjected to three levels of salinity treatment through irrigation with a nutrient solution containing 0, 50, 100, and 200 mM NaCl twice (at 10:00 a.m. and 15:00 p.m.) every day until water drained from the bottom of the pot for 14 days. Each treatment was applied to three replicates located randomly in the greenhouse in order to avoid positional effects. Three plants per treatment were collected for analysis at two weeks after salinity treatment**.**

Measurement of growth

Two weeks after salt treatments, three plants were harvested, and each was separated into the leaves, stems and roots. Plant parts were washed gently with tap water for a few minutes, wiped with paper, and their fresh weight was measured. The fresh samples were frozen in liquid nitrogen, then freeze-dried, and the dry weight was measured. Dry samples were ground into fine powder using a vibrating sample mill (Model T1-100, Heiko Co., Ltd., Japan) for chemical analysis.

Measurement of water potential, osmotic potential and photosynthetic rate in leaf

The leaf water potential (ψ_1) was measured according to the method described by Saneoka *et al.*(1995), using the uppermost fully expanded leaf employing a pressure chamber (Daiki-Rika Instruments, Tokyo, Japan) at 14 days after the initiation of the salt treatment. After the water potential was measured, the leaves were frozen in liquid nitrogen and thawed, centrifuged at 3000×g for 10 min to extract cell sap, and the osmotic potential (ψ_π) (of the sap was measured using a Wescor 5500 vapor pressure osmometer (Wescor Inc., Logan, UT. USA). Turgor potential was calculated by

subtracting ψ_π from ψ_1. Osmotic adjustment (OA) was calculated as the difference in ψ_π between salinized and control plants.

The photosynthetic rate was simultaneously measured for the attached and uppermost fully expanded leaves using a portable open gas exchange system (Li6400, Li-Cor, Lincoln, NE, USA). The photosynthetic photon flux density was maintained at 1,500 μmol m^{-2} s^{-1} and the relative humidity was 60%. The temperature of the leaf was 25°C and the ambient CO_2 concentration was 370 μmol mol^{-1} while measurements were taken.

Measurement of Na^+, K^+, Ca^{2+}, Mg^{2+} and N concentrations

After the measurement of leaf water potential, the other half of the same leaf samples were frozen in liquid nitrogen, freeze-dried, and ground into a fine powder using a vibratory mill and passed through a 1 mm mesh. The Na^+ and K^+ concentrations were determined after digestion by nitric acid–hydrogen peroxide, using a flame photometer (ANA 135, Eiko Instruments Inc., Tokyo).The $Ca^{2+}$and Mg^{2+} concentrations were determined using an inductively coupled argon plasma method (ICAP-575, Nippon Jarrel Ash, Kyoto, Japan). The N concentration was determined by the Kjeldahal method after digestion with sulfuric acid.

Measurement of sugar concentration

Dried ground leaves samples were boiled with 80% (v/v) ethanol, in hot water (80^0C/20min), this mixture was then centrifuged at 2000 rpm for 5 min, the supernatant was collected, and the precipitate was subjected to two more times of the same extraction process. The sugar concentration in the ethanol-soluble extract was determined using anthrone reagent method by spectrophotometer (U-2001, Hitachi, Japan) using glucose solution as standard, according to the method of Yemm and Willis (1954).

Statistical analysis

Data (n=3) were examined by one-way ANOVA analysis of variance. Multiple comparisons of the means between different salinity treatments within a plant species were performed using Duncan's test at the 0.05 significance level (all tests were performed with SPSS Version 16.0 for Windows).

2.3. Results

Plant growth

The dry weight of roots of New Zealand spinach increased at low salt level (50 mM) and then decreased with increasing salinity; however the dry

weights of leaves and roots of this plant were still higher under 100 and 200 mM NaCl treatment compared to the control (Fig. 1). The dry weight of stems of New Zealand spinach was not affected by the salinity. That of leaves, stems and roots of water spinach was markedly decreased ($P<0.05$) with increaseing salinity. Increasing concentration of NaCl in treatment solution significantly reduced ($P<0.05$) the leaf area of both species, but the reduction level of water spinach was higher than that of New Zealand spinach, especially, under high salinity (200 mM NaCl) (Fig.2).

Leaf water potential (ψ_1), osmotic potential (ψ_π) and water content (WC)

The ψ_1 of both species was decreased ($P<0.05$) with increasing salinity. However, New Zealand spinach exhibited a lower ψ_1 than water spinach under each treatment. The ψ_π also decreased with increasing salinity, and the ψ_π of New Zealand spinach was lower than that of water spinach. The WC of both species was decreased with increasing salinity (Table 1).

Ion concentrations

Both species accumulated Na^+ ($P<0.05$) with increasing salinity in all plant tissues (leaves, stems and roots) (Table 2). The concentration of Na^+ in

leaves of New Zealand spinach was higher than that of water spinach, but the concentration of Na^+ in the stems and roots of water spinach was higher than that of New Zealand spinach.

The uptake of Na^+ by both species rose with increasing salinity (Table. 2). The uptake of Na^+ by New Zealand spinach was twice that of water spinach at 50 and 100mM NaCl treatment, and 1.5fold at 200mM NaCl. The percentage distributions of Na^+ in the leaves of New Zealand spinach was 81% and 90% of the control at 50 and 100mM NaCl treatments respectively, and 70% at 200mM NaCl treatment. In the leaves of water spinach, they were 43% and 58% of control at 50 and 100mM NaCl treatments, respectively.

The K^+ concentration in the leaves of both species was markedly decreased with increasing salinity (Table 2). The Na^+/K^+ and Na^+/ Ca^{2+} ratios in leaves of both species rose with increasing salinity. The Na^+/K^+ ratio in the leaves of New Zealand spinach was slightly higher than that of water spinach (Table 3).

The Ca^{2+} and Mg^{2+} concentrations in the leaves of New Zealand spinach were markedly decreased with increasing salinity. These elements in the leaves of water spinach were slightly increased, but there was no difference between the treatments (Table 2).

The uptake of N by New Zealand spinach at 50mM treatment was increased compared to the control, and that at 100mM and 200mM NaCl treatments was not altered compared to the control (Fig 3). The uptake of N by water spinach was markedly decreased at 50mM NaCl treatment. The uptake of N by New Zealand spinach was higher than that of water spinach for all treatments.

Photosynthesis

Salinity levels strongly influenced leaf gas exchange (Table 4). The photosynthetic rate (P_o), stomatal conductance (g_s), intercellular CO_2 concentration (Ci), and transpiration rate (T_r) of both species decreased ($P<0.05$) with increasing salinity, but the reduction of these parameters was lower in New Zealand spinach compared to water spinach. The T_r of both species also decreased with increasing salinity; however the T_r of New Zealand spinach was higher than that of water spinach for all treatments.

Sugar concentration

Salt treatment decreased the leaf and stem sugar concentrations of both species with increasing salinity stress, except that in leaves of New Zealand spinach under (50 mM NaCl) was increased (Table 5).

2.4. Discussion

In the present study, the two plant species, New Zealand spinach and water spinach were exposed to salt stress by increasing the NaCl concentration (0, 50, 100 and 200mM NaCl) of irrigation water. When the salinity increased, the plant dry weight of water spinach markedly decreased; however, the growth of New Zealand spinach increased under salt conditions (Fig. 1). The dry weight of New Zealand spinach increased 1.7, 1.4, and 1.3 times at 50, 100 and 200mM NaCl treatments, respectively, compared with the control. Generally, the growth of glycophytes decreases with increasing salinity, while that of halophytes improves. In the present study, the growth of New Zealand spinach increased under salt stress, agreeing with previous data reported on the halophytes *Salicornia europaea* and *Suaeda maritina* (Moghaieb *et al*., 2004) and *Alhagi pseudoalhagi* (Kurban *et al*., 1999), in which salt treatment at low levels improved plant growth. These results indicated that New Zealand spinach is a halophyte, and so the salt tolerance of this plant was higher than that of water spinach.

Table 1 shows that the leaf water potential and osmotic potential of New Zealand spinach were lower than those of water spinach under salt stress. These results suggest that New Zealand spinach could absorb more water from the saline soil compared to water spinach. In fact, in the present

study, the water content of New Zealand spinach was 74%, 73% and 64% at 50, 100 and 200mM NaCl treatments, respectively; however, in water spinach, the water content was markedly decreased by 63%, 56% and 44% at 50, 100, and 200mM NaCl treatments, respectively. Similar result found on *Atriplex halimus* (Martinez *et al.*, 2004) and *Plantago coronopus* (Koyro, 2006) under salt stress.

In the present study, the Na^+ concentration of leaves of New Zealand spinach was higher than that of water spinach at all salt treatments (Table 2). In New Zealand spinach, 80% of Na^+ taken up by plants was distributed to the leaves. The marked decline in the osmotic potential of New Zealand spinach was mainly due to a large accumulation of Na^+ ions in the leaves, as observed in many reports, whereby halophytic plants accumulate and compartmentalize a large amount of Na^+ in vacuoles to lower the osmotic potential (Cheeseman, 1988; Munns, 2002; Ashraf, 2004; Song *et al*., 2009).

The relationship between the salinity and mineral nutrition of plants is extremely complex. Salinity can adversely affect plant growth by inducing nutritional disorders, which may result from the effect of salinity on nutrient availability, competitive uptake, transport, or partitioning within plant organs (Greenway & Munns, 1983). In the present study, salt stress reduced K^+ uptake (Table 2). Table 3 showed that the Na^+/K^+ and Na^+/Ca^{2+} ratios in

leaves, stems and roots increased with increasing salt stress in both species, but Na^+/K^+ ratio was higher in roots of New Zealand spinach than in leaves and stems, indicating that the roots in New Zealand spinach have ability to select K^+ to keep the nutrient balance (Liu *et al.*, 2008). However, the Na^+/K^+ ratio reportedly increased under salt stress in many halophytic plants (Moghaieb *et al.*, 2004; Yang *et al.*, 2007). The Ca^{2+} and Mg^{2+} concentrations in leaves of New Zealand spinach decreased under salt treatment; however, both elements in leaves of water spinach were not changed by salinity. The increase of Na^+ accumulation in New Zealand spinach was associated with reduced K^+, Mg^{2+} and Ca^{2+}, indicating a restriction in the uptake of these nutrients, as noted in other halophytic plants (Maggio *et al*., 2000; Debez *et al*., 2004; Aleman *et al*. 2009).

Nitrogen is an essential nutrient element in the biosynthesis of nitrogenous organic solutes in plants. Salt stress reduces N uptake in many plants, and this is attributed to antagonism between NO_3^- and Cl^- (Gouia *et al.*, 1994; Parida & Das, 2004). In the present study, the N uptake of New Zealand spinach was increased at 50 mM NaCl treatment, and was unchanged at 100 and 200mM NaCl treatments relative to the control (Fig. 3). On the other hand, N uptake of water spinach was markedly decreased in the presence of salt stress. Also, in New Zealand spinach, the N partitioning

rate from the roots and stems to the leaves was 60 % to 70%; however that of water spinach was 30% to 50% (data not shown). Nitrogen is the mineral element in plants required in the largest amounts and is a constituent of many plant cell components, including amino and nucleic acids. Therefore, nitrogen deficiency rapidly inhibits plant growth (Hu & Schmidhalter, 2005). The results of the present study suggested that New Zealand spinach showed more active N uptake under saline conditions.

In response to salt stress, the P_o of both species was decreased (Table 4). The reduction of the P_o was lower in New Zealand spinach compared to water spinach at 50 and 100 mM NaCl treatment. The P_o of water spinach was 0 μmol CO_2 m^{-2} S^{-1}, but that of New Zealand spinach was 8.7 μmol CO_2 m^{-2} S^{-1} at 200 mM NaCl treatment. Along with the decreasing P_o , the *gs* and C_i were decreased with increasing salinity. In both species, g_s reduction was marked compared to that of the C_i. These parameters suggest that stomatal closure limited the leaf photosynthetic capacity under saline conditions. On the other hand, the T_r of both species decreased with increasing salinity, and the T_r reduction was high in water spinach relative to that of New Zealand spinach. New Zealand spinach still maintained transpiration under marked saline conditions. Based on the results obtained, it is assumed that New Zealand spinach maintained open stomata under saline conditions, which

increased transpiration rate compared with water spinach. Consequently, water transpiration through the stomata stimulated the translocation of water through the xylem from the roots. This water flow appeared to be regulated mainly by stomatal opening, and might promote the translocation of Na^+ and N from the roots to shoots. Similar result found on *Suaeda salsa* (Fang *et al.,* 2005) and rice (Moradi *et al.,* 2007) under salt stress.

The decreases of soluble sugars concentration in response to salinity stress may depend on net CO_2 assimilation, however, this does not rule out a significant role of soluble sugars in salt tolerance (Ashraf & Harris 2004; Bartels & Sunkar 2005). Data presented in Table 5 shows that the soluble sugars concentration decreased ($P<0.05$) under salt stress, especially, under high NaCl levels (100 and 200 mM) in New Zealand spinach (leaf and stem), but the salt stress had no effect in water spinach (leaf and stem). However, the sugar concentration in stem tissues was more than the leaves of both species, because stems tissue is basically non-photosynthetic, the accumulation of carbohydrates and the composition of those carbohydrates reflected translocation and subsequent metabolism (Kerepesi & Galiba, 2000).

In conclusion, the growth of New Zealand spinach was promoted under saline conditions but that of water spinach was markedly decreased,

indicating that New Zealand spinach is halophytic. The main strategy of salt tolerance in New Zealand spinach seems to be the increase in osmotic adjustment (a crucial role in osmo-regulation of this plant under salt stress), through the accumulation of Na^+ in leaves, and the maintaining of a higher capacity for water uptake and water supply to the leaves.

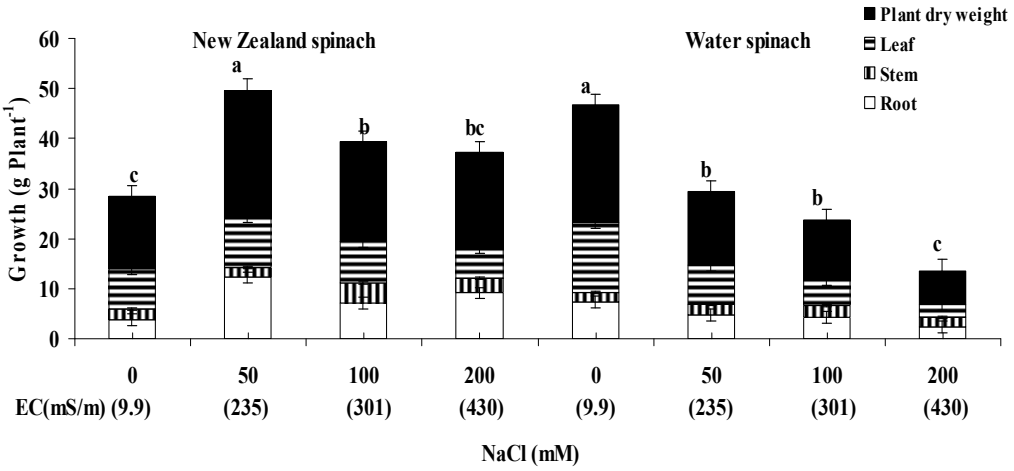

Fig. 1. Effect of salinity on plant dry weight, leaf, stem and root of New Zealand spinach and water spinach after 14 days of salinity treatment. Values represent means ± S.E. Bars with different letters significantly differed at *P<0.05*.

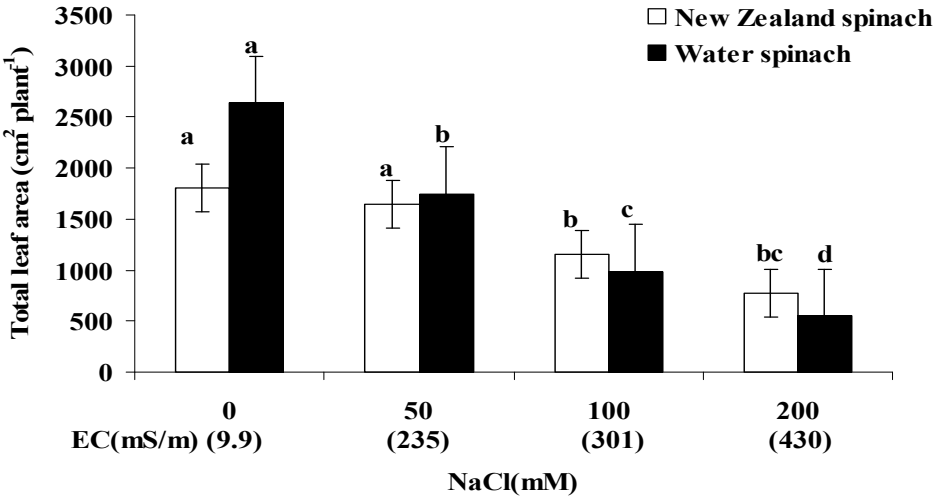

Fig. 2. Effect of salinity on leaf area of New Zealand spinach and water spinach after 14 days of salinity treatment. Values represent means ± S.E. Bars with different letters significantly differed at *P<0.05*.

Table 1. Effect of salinity on leaf water potential (Ψ□), osmotic potential (Ψ_π), turgor, osmotic adjustment (OA) and water content (WC) of New Zealand spinach and water spinach after 14 days of salinity treatments.

		NaCl(mM)			
		0	50	100	200
	EC (mS/m)	9.9	235	301	430
New Zealand spinach	Ψ□ (−MPa)	0.65±0.01^{a}	1.35±0.06^{b}	2.2±0.15^{c}	2.5±0.06^{b}
	Ψ_π (−MPa)	1.01±0.03^{d}	1.87±0.06^{c}	2.76±0.03^{a}	2.61±0.003^{b}
	Turgor	0.36±0.04^{ab}	0.52±0.06^{a}	0.56±0.06^{a}	0.11±0.06^{b}
	OA		0.86	1.75	1.6
	WC	83.81± 1.53^{a}	73.34±1.05^{b}	73.65±0.96^{b}	63.69±2.02^{c}
Water spinach	Ψ□ (−MPa)	0.23±0.02^{b}	0.3±0.03^{b}	1.6±0.06^{a}	1.7±0.06^{a}
	Ψ_π (−MPa)	0.94±0.01^{c}	0.84±0.02^{c}	1.88±0.01^{b}	1.99±0.06^{a}
	Turgor	0.71±0.02^{a}	0.54±0.03^{a}	0.28±0.06^{b}	0.29±0.11^{b}
	OA		0.1	0.94	1.05
	WC	59.58±1.23^{ab}	62.63±1.21^{a}	56.04±1.33^{b}	46.89±0.88^{c}

Values represent means ± S.E. The same letter on each line indicates no significant difference ($P<0.05$).

Table 2. Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations in leaf, stem and root of New Zealand spinach and water spinach after 14 days of salinity treatment. Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations are expressed as (mg g-¹ DW).

		NaCl mM			
		C	50	100	200
EC (mS/m)		9.9	235	301	430
Na^+					
New Zealand spinach					
	Root	1.51±0.07^{d}	4.20±0.19^{c}	6.07±0.30^{b}	12.96±0.78^{a}
	Stem	11.71±0.49^{b}	28.72±1.65^{a}	34.24±4.72^{a}	33.61±1.03^{a}
	Leaf	29.69±1.62^{d}	69.84±0.69^{c}	76.42±1.55^{b}	94.95±2.24^{a}
Water spinach	Root	1.37±0.82^{d}	15.78±1.30^{b}	10.74±1.31^{c}	24.99±1.49^{a}
	Stem	3.69±0.29^{c}	3.61±0.01^{c}	51.98±2.60^{b}	63.52±0.17^{a}
	Leaf	9.49±0.14^{c}	22.50±0.07^{b}	62.77±0.21^{a}	64.31±2.23^{a}
K^+					
New Zealand spinach					
	Root	7.01±0.48^{a}	1.96±0.05^{b}	2.15±0.07^{b}	2.34±0.19^{b}
	Stem	38.53±1.16^{a}	28.57±0.81^{b}	24.52±1.55^{c}	18.29±1.01^{d}
	Leaf	83.41±4.88^{a}	44.55±1.68^{b}	33.35±1.39^{c}	26.90±0.67^{c}
Water spinach	Root	8.01±2.02^{a}	9.97±0.36^{a}	6.17±0.98^{a}	8.13±0.55^{a}
	Stem	49.22±2.34^{a}	42.29±2.44^{ab}	35.61±2.89^{bc}	31.81±2.30^{c}
	Leaf	66.54±6.21^{a}	35.95±10.58^{b}	31.18±7.66^{b}	30.62±3.01^{b}
Ca^{2+}					
New Zealand spinach					
	Root	3.59±2.29^{a}	5.88±2.09^{a}	4.73±2.70^{a}	2.37±0.71^{a}
	Stem	11.20±1.37^{a}	12.81±1.89^{a}	7.63±0.82^{a}	7.21±2.39^{a}
	Leaf	10.91±1.40^{a}	4.93±0.36^{b}	4.39±0.51^{b}	3.15±0.94^{b}
Water spinach	Root	5.63±4.03^{a}	6.69±1.19^{a}	3.77±1.23^{a}	3.76±1.49^{a}
	Stem	19.40±2.73^{a}	22.67±3.04^{a}	20.34±0.54^{a}	22.99±1.89^{a}
	Leaf	11.31±1.14^{a}	14.90±2.31^{a}	14.27±1.04^{a}	16.87±1.69^{a}
Mg^{2+}					
New Zealand spinach					
	Root	4.26±0.27^{a}	2.24±0.54^{b}	2.23±0.91^{b}	2.50±0.37^{ab}
	Stem	4.86±0.42^{a}	3.05±0.52^{b}	3.32±0.42^{b}	3.08±0.26^{b}
	Leaf	6.95±0.62^{a}	2.48±0.18^{b}	3.23±0.13^{b}	2.90±0.28^{b}
Water spinach	Root	3.18±0.76^{a}	3.09±0.88^{a}	5.58±1.39^{a}	2.87±0.16^{a}
	Stem	3.26±0.13^{a}	3.02±0.36^{a}	2.73±0.30^{a}	3.20±0.67^{a}
	Leaf	0.19±0.03^{a}	0.30±0.05^{a}	0.25±0.05^{a}	0.26±0.01^{a}

Values represent means ± S.E. The same letter on each line indicates no significant difference ($P<0.05$).

Table 3. Effect of salinity on sodium/potassium ratio (Na/K) and sodium/ calisum ratio (Na/Ca) in leaf, stem and root of New Zealand spinach and water spinach after 14 days of salinity treatments.

		Na/K				Na/Ca			
		NaCl (mM)							
		0	50	100	200	0	50	100	200
	EC (mS/m)	9.9	235	301	430	9.9	235	301	430
New Zealand spinach	Leaf	0.35	1.57	2.29	3.53	2.72	14.17	17.41	30.14
	Stem	0.31	1.01	1.41	1.84	1.05	2.24	4.49	4.66
	Root	0.22	2.14	2.82	5.54	0.42	0.71	1.28	5.47
Water spinach	Leaf	0.14	0.63	2.01	2.11	0.84	1.51	4.41	3.81
	Stem	0.07	0.09	1.46	2.11	0.19	0.16	2.56	2.76
	Root	0.17	1.58	1.74	3.07	0.24	2.36	2.85	6.65

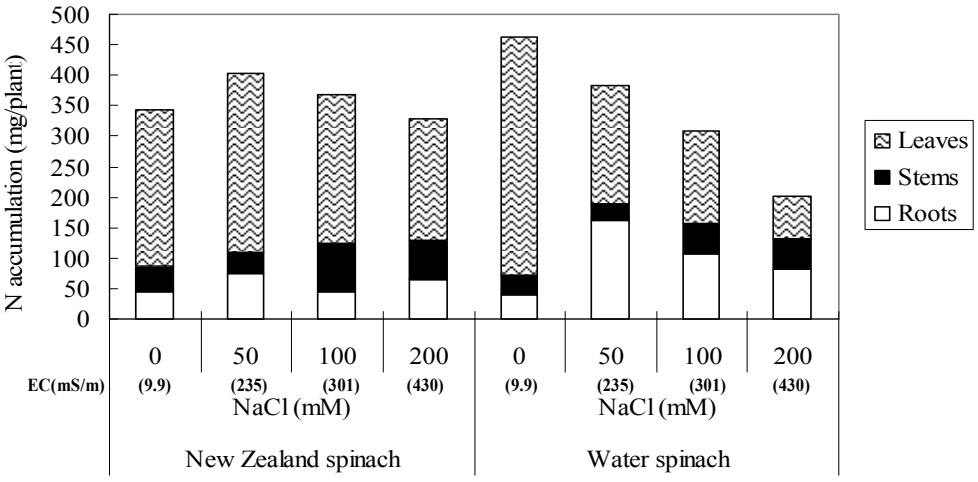

Fig. 3. Effect of salinity on nitrogen accumulation in the leaves, stems and roots in New Zealand spinach and water spinach after 14 days of salinity treatment. Values represent means ± S.E.

Table 4. Effect of salinity on photosynthetic rate(P_o), stomatal conductance(g_s), intercellular CO_2 concentration(C_i) and transpiration rate (T_r), in leaves of New Zealand spinach and water spinach after 14 days of salinity treatment.

	NaCl (mM)			
	0	50	100	200
EC (mS/m)	9.9	235	301	430
New Zealand spinach				
P_o [μ mol CO_2/ m^2/S]	22.5±0.8^a	16.9±1.7^{ab}	15.7±1.4^b	8.7±2.7^c
g_s [mol H_2O /m^2 /S]	1.3±0.2^a	0.4±0.1^b	0.2±0.003^b	0.1±0.03^b
C_i [μ mol CO_2]	2.9±1.5^a	2.5±0.1^b	2.3±0.3^c	2.1±0.9^d
T_r [mol H_2O /m^2 /S]	6.6±0.3^a	2.6±0.2^b	1.8±0.1^c	1.1±0.3^c
Water spinach				
P_o [μ mol CO_2/ m^2/S]	21.0±0.2^a	11.9±0.7^b	10.7±0.2^b	0
g_s [mol H_2O /m^2 /S]	0.6±0.1^a	0.1±0.02^b	0.08±0.04^b	0
C_i [μ mol CO_2]	2.8±2.1^a	1.9±1.4^c	1.7±2.4^d	2.2±1.2^b
T_r [mol H_2O /m^2 /S]	3.3±0.2^a	1.4±0.2^b	0.9±0.4^b	0

Values represent means ± S.E. The same letter on each line indicates no significant difference ($P<0.05$).

Table 5. Effect of salinity on soluble sugar concentration (mg/g DW) in leaves of New Zealand spinach and water spinach after 14 days of salinity treatment.

		NaCl (mM)			
		0	**50**	**100**	**200**
EC (mS/m)		**9.9**	**235**	**301**	**430**
New Zealand spinach					
	Leaf	57.71±2.91^b	69.99±0.89^a	24.19±0.57^d	46.41±1.57^c
	Stem	127.56±8.79^a	126.02±10.29^a	57.50±10.93^b	80.94±2.59^b
Water spinach					
	Leaf	73.62±6.99^a	44.72±2.99^b	53.86±5.81^{ab}	52.94±8.32^{ab}
	Stem	89.48±2.39^{ab}	97.76±3.74^a	82.77±8.10^{ab}	74.17±2.45^b

Values represent means ± S.E. The same letter on each line indicates no significant difference (P<0.05).

Chapter 3

Comparative physiological responses of New Zealand spinach and chard to salinity

3.1. Introduction

In chapter 2, I reported the presence of a large accumulation of Na^+ in New Zealand spinach and growth stimulation at 50 mM NaCl, at which levels the growth of water spinach was severely inhibited leading to death. Such characteristics are commonly observed in most halophyte (Koyro *et al*., 2006; Moghaieb *et al*., 2004; Kurban *et al*., 1999).

Chard (*Beta vulgaris*) is a glycophytic member of the Chenopodiaceae and is distributed in over the world, and is used as a green vegetable. Chard showed a high osmotic adjustment and accumulation of proline and inorganic ions under salt stress, and this plant was more tolerant to salinity than other glycophytic vegetable crops (Ghoulam *et al*., 2002). But there were only a few studies on the contributory role of proline and inorganic ions to the osmotic adjustment in chard under salt stress.

The objectives of this chapter were to study the effect of salinity stress on the accumulation of Na^+, K^+ and proline, osmotic adjustment, water relations, lipid peroxidation and photosynthetic rate in New Zealand spinach in comparison to chard, to assess two species for their salt tolerance and to

give more information on the significance of ions and proline accumulation under salt stress, in order to understand the adaptation mechanism of New Zealand spinach to salinity.

3.2. Materials and Methods

Plant materials and experimental methods

New Zealand spinach and chard were used in this experiment, and salinity treatments, measurement of growth, leaf water relations, photosynthesis, concentrations of Na^+, K^+ and N were measurement by methods according to the chapter 2, from April to June 2009, except the measurements of the osmotic potential at full turgor ($\psi_{\pi(100)}$), relative water content (RWC), Ca^{2+} and Mg^{2+} concentrations, proline and lipid peroxidation contents as below.

Measurements of osmotic potential at full turgor ($\psi_{(\pi 100)}$) and relative water content (RWC).

The osmotic potential at full turgor ($\psi_{\pi(100)}$) was calculated by adjusting for the relative water content (RWC), as described by Wilson *et al.* (1979). RWC was measured as described by Saneoka *et al.* (1995).

Measurement of Ca^{2+} and Mg^{2+} concentrations

The Ca^{2+} and Mg^{2+} concentrations were determined using Atomic absorption spectrophotometer (170-30, Hitachi, Japan).

Measurement of lipid peroxidation

The level of lipid peroxdation in the leaf blade was expressed as malondialdehyde(MDA) content, measured by the method reported by Buege and Aust (1978). One hundred milligrams of frozen leaf blade was homogenized with 3 ml of 10mM HEPES, PH 7.0, at 4°C. The homogenate was transferred to a medium containing trichloroacetic acid (0.918 M), 2-thiobarbituric acid (25.7mM), butylated hydroxytoluene(1.8mM), ethanol (0.343N) and HCl (0.25N). The mixture was heated at 95°C for 30 min and then quickly cooled on ice. After centrifuging at 3000 ×g for 10 min, the absorbance of the supernatant was read at 535nm. The value of non-specific absorption at 600nm was subtracted from the reading at 535 nm. The content of MDA was calculated using the extinction coefficient of 1.56×10^{-5} cm^{-1}.

Measurement of proline

Dried ground samples used for proline determination were transferred to vials subjected to methanol extraction, and stored in the dark at 4ºC. Proline was determined spectrophotometrically following the ninhydrin method

described by Bates *et al.* (1973), using L-proline as a standard and then determined using spectrophotometer (U-3310, Hitachi, Ltd. Tokyo, Japan).

Statistical analysis

Data (n=3) were examined by one-way ANOVA analysis of variance. Multiple comparisons of means of data between different salinity treatments within the plants were performed using Duncan's test at the 0.05 significance level (all tests were performed with SPSS Version 16.0 for Windows).

3.3. Results

Plant growth

The shoot dry weight of New Zealand spinach was increased 15% at low salt level (50mM NaCl) and then decreased at 100 and 200mM NaCl treatment compared to the control plants (Fig. 4A). On the other hand, that of chard was marked decreased by salinity, and the reduction of the shoot dry weight at 50, 100 and 200mM NaCl treatment compared to control plants were 33%, 49% and 58%, respectively. The changes of relative growth rate were similar to the dry weight (Fig. 4B). Increasing concentration of NaCl treatments significantly reduced ($P<0.05$) the leaf

area of both species, but the reduction level of chard was higher than that of New Zealand spinach (Fig.5).

Photosynthesis

Photosynthetic rate (P_o), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) decreased with increasing NaCl concentration (Table 6). The P_o was higher in New Zealand spinach compared with chard at each salt treatment. C_i decreased similarly to P_o. The g_s rate was strongly affected by salinity compared to C_i, and its reduction was higher in chard compared with New Zealand spinach. Transpiration rate (T_r) also decreased by salinity; however the T_r in New Zealand spinach was still higher than that of chard at 100 and 200mM NaCl treatment.

Ions accumulation

The Na^+ concentration in both species increased ($P<0.05$) with increasing NaCl concentration; however there was no difference in Na^+ concentration between the two species (Table 7). On the other hand, the K^+ concentration was slightly decreased by NaCl treatment in New Zealand spinach. In contrast, it was severely decreased ($P<0.05$) in chard. Concentrations of Ca^{2+} and Mg^{2+} decreased in all part tissues of both species after 14 days of

salinity treatment (Table 7). The data show that Na^+/ K^+ and Na^+/Ca^{2+} ratios increase with increasing salt stress in both species (Table 8).

Salinity increased ($P<0.05$) total N in leaves in both species after 14 days of salt treatment (Table 9).

Leaf water status

The relative water content(RWC) in both species were decreased with increasing NaCl concentration, but the reduction of RWC was less in New Zealand spinach than chard (Table 10). Leaf water potential (ψ_l) in both species was also decreased by salt stress; the value of ψ_1 in New Zealand spinach was significantly higher than in chard at all salt stress treatments. The leaf turgor was increased by salinity; the increase of turgor was higher in New Zealand spinach than in chard. Osmotic potential at full turgor ($\psi_{\pi(100)}$) decreased by salt stress in both species, but the decrease of $\psi_{\pi(100)}$ was higher in New Zealand spinach than in chard. The values of osmotic adjustment which were calculated as the difference in values of $\psi_{\pi(100)}$ between non-salinized and salinized plants were a 1.25, 1.48 and 1.58-fold higher in New Zealand spinach than those in chard at 50, 100, and 200mM NaCl treatment, respectively.

Proline content

Proline content in both species increased with increasing NaCl concentration (Fig. 6A). The proline content in New Zealand spinach was 34, 48 and 14–fold higher than that in chard at 50, 100 and 200 mM NaCl treatment, respectively.

Malondialdehyde (MDA) content

The MDA content also significantly increased with increasing NaCl concentration in both species (Fig. 6B). The MDA content in chard was 1.8, 1.8 and 1.6-fold higher than that in New Zealand spinach at 50, 100 and 200 mM NaCl treatment, respectively.

3.4. Discussion

The plant dry weight of chard significantly decreased with increasing salt stress; however, the growth of New Zealand spinach slightly increased at 50 mM NaCl treatment, and was unchanged at 100 mM treatment and then decreased at 200 mM NaCl treatment (Fig. 4A,B). The relative growth rate (RGR) of chard also remarkably decreased with increased salinity, whereas RGR of New Zealand spinach was also increased at low level of salt stress (50 mM NaCl treatment). The plant growth in non-halophytic mesophytes species was generally reduced with increasing salinity. The growth response

to salinity in this study is consistent with previous observations on the halophytes *Salicornia europaea* and *Suaeda maritina* (Moghaieb *et al.*, 2004) and *Plantago coronopus* (Koyro, 2006) in which the growth of New Zealand spinach was improved at low level of salinity. These results of growth responses indicated that New Zealand spinach is a halophyte, and so the salt tolerance of this plant was higher than that of chard.

The photosynthetic rate of both species was decreased with increasing NaCl concentration (Table 6). The reduction of the photosynthetic rate was lower in New Zealand spinach compared to chard. The value of photosynthetic rate in New Zealand spinach was higher than that of chard at each NaCl treatment. A similar tendency was observed for the stomatal conductance and intercellular CO_2 concentration, although the impairment of the former by NaCl was more remarkable than that of the later. This finding suggested that stomatal closure limited the leaf photosynthetic capacity under saline conditions. On the other hand, the transpiration rate of both species decreased with increasing salinity, and the transpiration rate in New Zealand spinach was higher compared to chard at all of the treatments. New Zealand spinach still maintained transpiration under marked saline conditions. Based on the results obtained, it is assumed that New Zealand spinach maintained open stomata under saline conditions, which increased

transpiration rate compared with chard. Consequently, water transpiration through the stomata stimulated the translocation of water through the xylem from the roots. This water flow appeared to be regulated mainly by stomatal opening, and might promote the movement of water from the roots to shoots (Moghaieb et al., 2006).

The Na^+ content significantly increased by salt treatments, but there was no significant difference in Na^+ concentration between New Zealand spinach and chard (Table 7). In contrast to Na^+ concentration, K^+ concentration in the leaves of both species was decreased with increasing salinity. The K^+ accumulation in New Zealand spinach was higher than that in chard. The Na^+/K^+ ratios in leaves of both species rose with increasing salinity; however the Na^+/K^+ ratio in the leaves of New Zealand spinach was significantly lower than that of chard. The Na^+ competes with K^+ for intercellular influx, since these cations are transported by common proteins, Na^+ can lead to damage of plasma membrane and, subsequently increase K^+ efflux from intercellular stores. K^+ is also an essential cofactor for enzymes, but Na^+ is not and cannot replace K^+ (Grattan & Grieve, 1999). The data showed that New Zealand spinach posses much more selective mechanism for the uptake of K^+ over Na^+ than chard under salt stress conditions, and the accumulation of Na^+ induced Ca^{2+} and Mg^{2+} deficiencies (Table. 6). Ca^{2+}

and Mg^{2+} are known to play a crucial role in maintaining the structural and functional integrity of plant membranes and considerable roles in cell wall stabilization, regulation of ion transport and selectivity and activation of cell wall enzyme, Therefore, the ability of plants to retain Ca^{2+} is associated with their salt tolerance, however, the accumulation of Na^{+} induced Ca^{2+} and Mg^{2+} deficiencies in plants (Ashraf *et al.*, 2004; Netondo *et al.*, 2004). Similar results were found in *Beta vulgaris* (Koyro *et al.,* 2006) and *Atriplex nummularia* (Silveira *et al.*, 2009).

N is the mineral element that plants require in the largest amounts and is a constituent of many plant cell components, including amino and nucleic acids, therefore (Hu & Schmidhalter, 2005). Salinity increased concentration of N in leaves, stems and root of both species after 14 days of salinity treatment, especially, in leaves under high NaCl levels (200 mM) (Table 9). Similar results were previously reported in the *Tetragonia trigyna* (Watkins *et al.*, 1988).

The leaf water potential (ψ_l) and osmotic potential (ψ_π) of both species was decreased with increasing salinity (Table 10). However, New Zealand spinach exhibited a higher ψ_l and ψ_π than chard under each treatment. The value of osmotic adjustment which was calculated as the difference in $\psi_{\pi(100)}$ between non-salinized and salinized New Zealand

spinach was 0.71, 0.92, and 1.35 at 50, 100, and 200mM NaCl treatment, respectively, and this value in chard was 0.57, 0.62 and 0.91, at 50, 100, and 200mM NaCl treatment, respectively. The salt-tolerant species New Zealand spinach displayed a significantly higher osmotic adjustment than sensitive species chard under salt stress conditions. The turgor was higher in New Zealand spinach than chard. The great increase of turgor in leaves of New Zealand spinach may be responsible for the promotion of growth under low salt stress condition, since a positive turgor is required for cell elongation, stomatal opening and photosynthesis (Parida & Das, 2005).

Both species showed an increased in proline content under salt stress condition (Fig. 6A). There were significantly differences in proline content, with New Zealand spinach showing much higher levels than chard under salt treatment. It is well known that compatible solutes such as proline which accumulates in many plants under salt stress conditions, and acts as a compatible solute, an osmoprotectant, and a protective agent for cytosolic enzymes and cellular organelles (Bohnert & Shen, 1998; Yancy, 2005). Accumulation of proline under salt stress in many plants has been correlated with stress tolerance (Tani & Sasakawa, 2006; Ashraf & Foolad, 2007). Petrusa & Winicove (1997) reported that proline content was rapidly

increased in salt-tolerant alfalfa under salt stress conditions; however in the salt-sensitive plants the response was slow.

The findings data showed that the degree of accumulation of MDA significantly increased in both species subjected to salinity stress (Fig. 6B), but MDA accumulation was greater in chard than in New Zealand spinach. These results indicated that salinity produced less toxic reactive oxygen species in New Zealand spinach compared to chard. The low values of MDA content obtained with New Zealand spinach might account for lower lipid peroxidation levels and less affected membrane permeability. Low lipid peroxidation may be one of the reasons for the observed tolerance of New Zealand spinach exposed to high levels of salinity, Similar results found of cottan (Meloni *et al.*, 2001); azolla (Masood *et al.*, 2006); maize (Neto *et al.,* 2006); sugar and wild beet (Bor *et al.,* 2003); liquorice (Pan *et al.,* 2006); *Agrostis palustris* (Saneoka *et al.*,2004) and mangrove (Parida *et al.*,2004).

Proline is an important component of plant antioxidant systems (Ashraf & Foolad, 2007). Molinari *et al*. (2007) indicated that a proline overproducing transgenic sugarcane had lowered active-oxygen species contents and increased tolerance to drought. Sumithra *et al* (2006) also reported that salinity-tolerant cultivars of *Vigna radiate* showed more accumulated proline and a lower level of lipid peroxidation than salt-sensitive cultivars.

In conclusion, the growth of New Zealand spinach was promoted under saline conditions but that of chard was markedly decreased, indicating that New Zealand spinach is halophytic. The main strategy of salt tolerance in New Zealand spinach seems to be the capacity of osmotic adjustment through the greater accumulation of Na^+ and proline, and the possession of a higher antioxidant system under salt stress conditions.

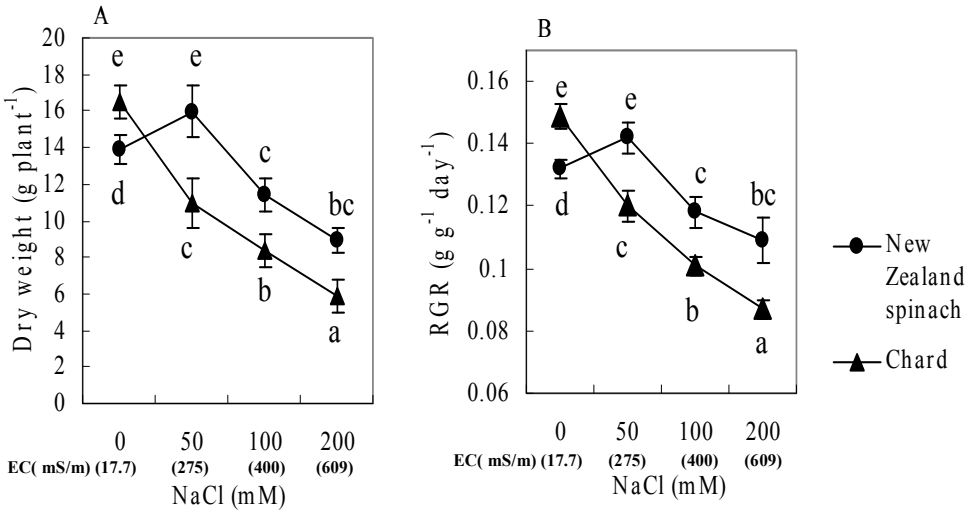

Fig. 4. Effect of salinity on the dry weight of shoots (A) and relative growth rate(RGR)(B) of New Zealand spinach and chard after 14 days of salinity treatment. Values represent means ± S.E. Bars with different letters significantly differed at *P<0.05*.

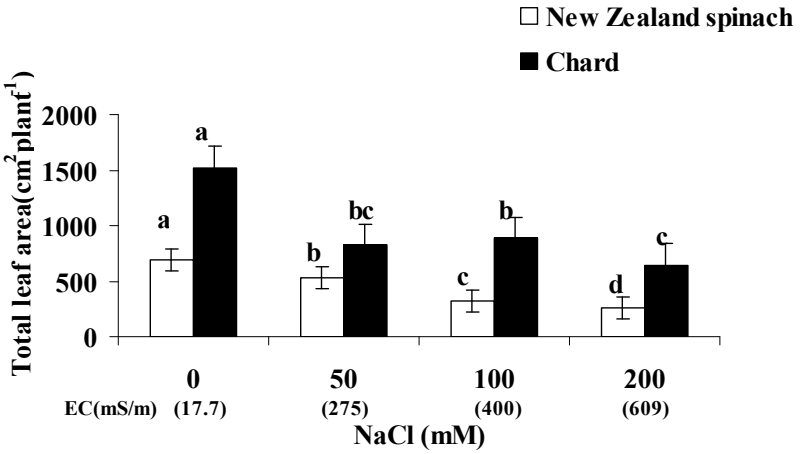

Fig. 5. Effect of salinity on leaf area of New Zealand spinach and chard after 14 days of salinity treatment. Values represent means ± S.E. Bars with different letters significantly differed at *P<0.05*.

Table 6. Effect of salinity on photosynthetic rate(P_o), stomatal conductance(g_s), intercellular CO_2 concentration(C_i) and transpiration rate(T_r) of New Zealand spinach and chard after 14 days of salinity treatment.

	NaCl Treatment(mM)			
	0	50	100	200
EC (mS/m)	17.7	275	400	609
New Zealand spinach				
P_o [μ mol CO_2/ m^2/S]	30.65±0.78[a]	15.27±2.58[b]	16.47±2.22[b]	6.41±1.61[c]
g_s [mol H_2O /m^2 /S]	0.41±0.01[a]	0.09±0.09[b]	0.15±0.01[b]	0.02±0.01[b]
C_i [μ mol CO_2]	372.00±4.61[a]	246.00±1.15[c]	207.67±8.45[d]	277.41±0.21[b]
T_r [mol H_2O /m^2 /S]	5.29±0.15[a]	1.62±0.26[b]	1.20±0.21[b]	0.23±0.11[c]
Chard				
P_o [μ mol CO_2/ m^2/S]	16.80±1.08[a]	12.42±1.85[ab]	8.96±1.89[b]	7.28±1.03[b]
gs [mol H_2O /m^2 /S]	0.42±0.23[a]	0.07±0.02[a]	0.04±0.01[a]	0.04±0.01[a]
C_i [μ mol CO_2]	264.67±6.17[a]	196.57±4.91[b]	181.50±0.29[b]	155.50±4.91[c]
T_r [mol H_2O /m^2 /S]	1.38±0.26[a]	0.26±0.08[b]	0.25±0.02[b]	0.19±0.01[b]

Values represent means ± S.E. The same letter on each line indicates no significant difference($P<0.05$).

Table 7. Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations in leaf, stem and root of New Zealand spinach and chard after 14 days of salinity treatment, concentrations of Na^+, K^+, $Ca^{2+}$and Mg^{2+} are expressed as (mg g^{-1}DW).

			NaCl Treatment (mM)			
			0	50	100	200
	EC (mS/m)		17.7	275	400	609
New Zealand spinach						
	Na^+	Leaf	18.80±2.98^{d}	109.38±4.81^{c}	131.51±3.59^{b}	146.25±3.26^{a}
		Stem	12.58±1.48^{d}	35.08±0.39^{c}	53.44±1.62^{b}	70.87±2.74^{a}
		Root	6.09±0.78^{c}	19.82±1.49^{a}	16.27±1.01^{b}	16.58±0.59^{ab}
	K^+	Leaf	113.42±9.24^{a}	96.29±4.44^{a}	99.49±7.16^{a}	91.80±4.56^{a}
		Stem	85.27±4.26^{a}	61.22±6.13^{b}	58.72±2.34^{b}	61.48±4.8^{b}
		Root	35.71±2.17^{a}	23.90±1.31^{b}	17.79±1.25^{c}	12.63±1.19^{d}
	Ca^{2+}	Leaf	11.29±0.21^{a}	10.28±0.32^{a}	10.78±0.43^{a}	10.60±0.84^{a}
		Stem	18.13±0.76^{a}	14.16±0.33^{b}	13.28±0.73^{b}	13.91±0.91^{b}
		Root	12.42±1.73^{b}	15.62±0.10^{a}	12.44±0.21^{b}	12.17±0.08^{b}
	Mg^{2+}	Leaf	12.44±0.55^{a}	9.33±0.45^{b}	9.36±0.76^{b}	8.72±0.63^{b}
		Stem	6.94±0.31^{a}	5.81±0.33^{a}	6.46±0.57^{a}	6.69±0.28^{a}
		Root	18.25±0.88^{a}	12.73±0.62^{b}	10.42±0.74^{c}	9.40±0.48^{c}
Chard						
	Na^+	Leaf	12.85±1.44^{d}	123.48±3.69^{c}	132.58±1.29^{b}	156.29±2.94^{a}
		Stem	15.19±2.58^{c}	121.73±7.73^{b}	152.02±1.76^{a}	150.11±2.64^{a}
		Root	5.13±0.33^{c}	36.47±2.45^{a}	39.87±0.59^{a}	25.12±0.61^{b}
	K^+	Leaf	101.23±2.97^{a}	81.44±8.04^{b}	80.43±3.38^{b}	59.19±1.53^{c}
		Stem	83.18±1.04^{a}	79.88±0.49^{a}	67.05±2.69^{b}	59.66±1.21^{c}
		Root	32.99±4.94^{a}	22.03±3.86^{a}	25.91±4.81^{a}	7.73±1.03^{b}
	Ca^{2+}	Leaf	9.94±1.67^{a}	9.35±0.65^{a}	9.32±0.45^{a}	10.83±0.02^{a}
		Stem	12.44±1.57^{a}	8.17±0.40^{b}	8.26±0.73^{b}	7.41±0.01^{b}
		Root	7.62±0.11^{c}	8.42±0.01^{b}	8.95±0.01^{a}	5.16±0.01^{d}
	Mg^{2+}	Leaf	9.84±2.15^{ab}	9.18±1.08^{b}	11.31±0.67^{ab}	14.26±1.02^{a}
		Stem	8.43±1.03^{a}	4.82±0.45^{b}	5.25±0.85^{b}	5.26±0.28^{b}
		Root	10.81±0.31^{a}	7.90±0.99^{bc}	10.03±0.40^{ab}	7.12±0.77^{c}

Values represent means ± S.E. The same letter on each line indicates no significant difference($P<0.05$).

Table 8. Effect of salinity on sodium/potassium ratio (Na/K) and sodium/ calcium ratio (Na/Ca) in leaf, stem and root of New Zealand spinach and chard after 14 days of salinity treatment.

		NaCl Treatment(mM)			
		0	50	100	200
	EC (mS/m)	17.7	275	400	609
New Zealand spinach					
Na/K	Leaf	0.17	1.14	1.32	1.59
	Stem	0.15	0.57	0.91	1.15
	Root	0.17	0.83	0.91	1.31
Na/Ca	Leaf	1.67	10.64	12.2	13.8
	Stem	0.69	2.48	4.02	5.09
	Root	0.49	1.27	1.31	1.36
Chard					
Na/K	Leaf	0.13	1.52	1.65	2.64
	Stem	0.18	1.52	2.27	2.52
	Root	0.16	1.66	1.54	3.25
Na/Ca	Leaf	1.29	13.21	14.23	14.43
	Stem	1.22	14.9	18.4	20.26
	Root	0.67	4.33	4.45	4.87

Table 9. Effect of salinity on concentation of nitrogin (mmol mg DW^{-1}) in New Zealand spinach and chard after 14 days at salinity treatment.

	NaCl Treatment(mM)			
	0	50	100	200
EC (mS/m)	17.7	275	400	609
New Zealand spinach				
Leaf	1.33 ± 0.12^{b}	1.48 ± 0.06^{b}	1.69 ± 0.14^{ab}	2.68 ± 0.62^{a}
Stem	0.97 ± 0.02^{b}	0.94 ± 0.05^{b}	$1.11\pm0.04a^{b}$	1.39 ± 0.22^{a}
Root	0.87 ± 0.07^{a}	0.79 ± 0.01^{a}	0.83 ± 0.09^{a}	0.98 ± 0.05^{a}
Chard				
Leaf	3.19 ± 0.16^{bc}	3.00 ± 0.16^{c}	3.56 ± 0.08^{ab}	3.68 ± 0.11^{a}
Stem	1.51 ± 0.20^{c}	1.89 ± 0.09^{bc}	2.45 ± 0.29^{a}	2.87 ± 0.16^{a}
Root	1.58 ± 0.09^{a}	1.92 ± 0.24^{a}	2.98 ± 0.94^{a}	1.74 ± 0.24^{a}

Values represent means ± S.E. The same letter on each line indicates no significant difference($P<0.05$).

Table 10. Effect of salinity on leaf water potential□ Ψ□), osmotic potential ($\Psi_{\pi(100)}$) at full turgor, turgor and osmotic adjustment (OA) in New Zealand spinach and chard after 14 days of salinity treatment.

	NaCl Treatment (mM)			
	0	50	100	200
EC (mS/m)	17.7	275	400	609
New Zealand spinach				
Ψ□ (-MPa)	0.51±0.01^{c}	0.86±0.06^{b}	1.06±0.10^{ab}	1.23±0.10^{a}
$\Psi_{\pi(100)}$ (-MPa)	0.82±0.02^{d}	1.53±0.09^{c}	1.74±0.06^{b}	2.17±0.03^{a}
Turgor (MPa)	0.46±0.02^{c}	1.07±0.10^{b}	1.33±0.10^{b}	2.05±0.10^{a}
OA	□	0.71	0.92	1.35
Chard				
Ψ□ (-MPa)	0.31±0.01^{d}	1.82±0.1^{c}	2.12±0.05^{b}	2.56±0.01^{a}
$\Psi_{\pi(100)}$ (-MPa)	0.89±0.42^{c}	1.46±0.09^{b}	1.51±0.07^{b}	1.79±0.06^{a}
Turgor (MPa)	0.76±0.05^{a}	0.16±0.02^{b}	0.32±0.07^{b}	0.63±0.05^{a}
OA	□	0.57	0.62	0.91

Values represent means ± S.E. The same letter on each line indicates no significant difference(*P<0.05*).

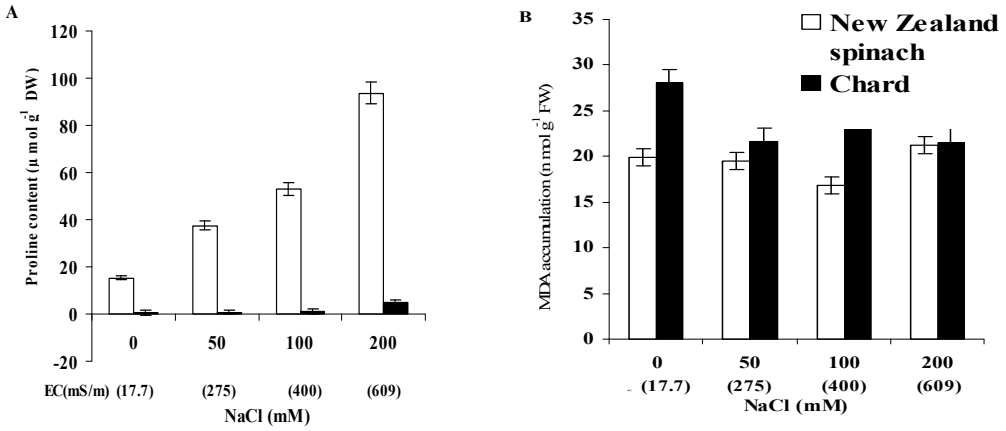

Fig. 6. Proline and MDA contents in the leaves of New Zealand spinach and chard after 14 days of salinity treatment. Values represent means ± S.E. Bars with different letters significantly differed at *P<0.05*.

Chapter 4
General discussion

1. Comparative physiological responses of New Zealand spinach and water spinach to salinity

In the present study, two species were examined under NaCl stress. Plant growth, leaf area and plant dry weight in both species was significantly inhibited by salt stress. However, the dry weight of New Zealand spinach increased under a low NaCl (50mM) level and then decreased at higher salinity stress, this result agrees with previous data reported on *Salicornia europan* and *Suaeda maritina* (Moghaieb *et al.*, 2004) and *Alhagi pseudoalhagi* (Kurban *et al.,* 1999), in which salt treatment at low levels improves plant growth in halophyte. This might be caused by the osmotic adjustment through accumulating Na^+ in plant tissues to lower internal water potential and absorb more water from soils (Liu *et al.*, 2008).

In this study, the leaf water potential and osmotic potential of New Zealand spinach were lower than those of water spinach under salt stress. These results suggest that New Zealand spinach could absorb more water from the saline soil compared to water spinach. In fact, in the present study, the water content of New Zealand spinach was 74%, 73% and 64% at 50, 100 and 200mM NaCl treatments, respectively; however, in water spinach,

the water content was markedly decreased by salt treatment, being 63%, 56% and 44% at 50, 100 and 200mM NaCl treatment, respectively. Therefore, osmotic adjustment increased with increasing NaCl levels, and was greater in New Zealand spinach and lowest in water spinach plants, related to the accumulation of Na^+ ion. The present data agree with previous data reported on *Salicornia europaea* and *Suaeda maritime* under salinity stress (Moghaieb *et al.*, 2004) and wheat (Morgan, 1995).

In the present study, the Na^+ concentration in leaves of New Zealand spinach was higher than that of water spinach at all salt treatments. In New Zealand spinach, 80% of Na^+ taken up by plant was distributed to the leaves. Whereby halophytic plants accumulate and compartmentalize large amounts of Na^+ in vacuoles to lower the osmotic potential (Cheeseman, 1988; Munns, 2002; Ashraf, 2004; Song *et al*., 2009), and absorb more water from soil (Liu *et al.,* 2008).

Na^+ competes with K^+ for intracellular influx, since these cations are transported by common proteins, Na^+ can lead to damage of plasma membrane and, thus, subsequently increases K^+ efflux from intercellular stores. However, concentration of K^+ in leaves and stem was higher than in roots, and decreased with an increase of salinity, and K^+ accumulation induced by salinity was higher in leaves and stems than in roots of both

species, this indicates the high selectivity of K^+ to employ the physiological process. The data showed that New Zealand spinach posses a much more selective mechanism for the uptake of K^+ over Na^+, than water spinach under salinity.

The Ca^{2+} and Mg^{2+} concentrations in leaves of New Zealand spinach decreased under salt treatment; however, both elements in leaves of water spinach were not changed by salinity. The increase of Na^+ accumulation in New Zealand spinach was associated with reduced K^+, Mg^{2+} and Ca^{2+}, indicating a restriction in the uptake of these nutrients, as noted in other halophytic plants (Maggio *et al*., 2000; Debez *et al*., 2004).

Na^+/K^+ ratio and Na^+/Ca^{2+} ratio in leaves, stems and roots increased with increasing NaCl levels in both species, but Na^+/K^+ ratio was higher in roots of New Zealand spinach than in leaves and stems, indicating that root had the ability to select K^+ to keep the nutrient balance (Liu *et al.*, 2008).

Salinity reduced total N content in leaves and stems, but it decreased that in root tissues of New Zealand spinach. But the N content in all plant part of water spinach was not altered by salinity stress. The data shows that the levels of N content in tissues of New Zealand spinach higher than that of water spinach. Similar results were previously reported in the *Tetragonia trigyna* (Watkins *et al.*, 1988).

Salinity reduces net photosynthetic rate (P_o), transpiration rate (T_r), and stomatal conductance (g_s) and intercellular CO_2 concentration (C_i), in both species, salt induced reduction of photosynthesis can be caused by stomatal limitation with stomatal clousure, and disturbance of photosynthetic activity at high tissue salt concentration, excess salt in the photosynthetic tissues can cause shrinkage of thylakoids and stacking of adjacent membranes in grana, and ionic imbalance can cause the reduction of K^+ in chloroplasts and disintegration of the second photosystem PSII (Ashraf, 2004; Parida & Das, 2005), however, Salinity tolerance is related to the maintenance of net photosynthetic rate and stomatal conductance (Ashraf, 2004). But both the P_o and T_r in New Zealand spinach were maintained at a higher level than that in water spinach.

Data presented that the soluble sugar contents decreased ($P<0.05$) under salt stress, especially, under high levels (200 mM NaCl) of New Zealand spinach (leaf and stem), this result agrees with pervious data reported on kidney bean and mung bean under salt stress (Saffan, 2008), the soluble sugar contents of water spinach (leaf and stem) not effected by salt stress, this result agrees with pervious data reported on *Schinopsis quebracho* (Meloni *et al.,* 2008).

These results indicated that the New Zealand spinach was high tolerant (halophytes) and more tolerant to salinity than water spinach. The significant changes of leaf area, plant dry weight, gas exchange parameters, leaf water potential and accumulation of Na^+ ion and N accumulation in leaves of both species, help clarify the physiological mechanisms, and then, compared the salt tolerance between New Zealand spinach and water spinach under salinity stress.

2. **Comparative physiological responses of New Zealand spinach and chard to salinity**

In the present study, two species were examined under NaCl stress. Plant growth, leaf area and dry plant weight in both species was significantly inhibited by salt stress, except the dry weight of New Zealand spinach increased under a low (50mM) NaCl, then decreased with increasing salinity, beside of the dry weight of chard was decreased ($P<0.05$) under all salinity levels increased after 14 days of salt stress. This result agrees with previous data reported on *Salicornia europaea* and *Suaeda maritime* (Moghaieb *et al.*, 2004) and *Alhagi pseudoalhagi* (Kurban *et al.*, 1999), in which salt treatment at low levels improves plant growth in halophyte. The growth stimulation at moderate salinities was found to be related to the high

uptake of ions, involved in the cell expansion and turgor maintenance (Amor *et al.*, 2005).

The leaf water potential and osmotic potentials of New Zealand spinach were lower than those of chard under salt stress. From the results of these parameters, New Zealand spinach could be absorbing more water from the saline soil compared to chard. In fact, in the present study, the water contents of New Zealand spinach was 84.2%, 83.5% and 80.1% at 50, 100 and 200mM NaCl treatments after 14 days of salt stress, respectively; however, in chard, the water content was markedly decreased by salt treatment, being 84%, 80.4% and 72.8% at 50, 100 and 200mM NaCl treatments after 14 day of salt stress, respectively. Similar result was found on *Atriplex halimus* under salt stress (Martinez e*t al.,* 2004).

In this study the salt treatment decreased all the gas exchange properties, especially, the photosynthetic rate, and subsequently the whole plant biomass suggest that the impairment of plant growth by salt stress mainly results from the suppression of photosynthetic activity, the salt treatment caused substantial reduction of CO_2 diffusion, reduced stomatal conductance and intercellular CO_2, the resulting low CO_2 concentration in the chloroplast might have reduced photosynthesis rate. This result agrees with previous

data reported on rice (Moradi & Ismail, 2007), mangrove (Parida *et al.,* 2004) and *Suaeda salsa* (Fang *et al.*, 2005) under salt stress.

In the present study, the Na^+ concentration of leaves of New Zealand spinach was higher than that of chard at all salt treatments. In New Zealand spinach, 93% of Na^+ uptake up was distributed to the leaves. The marked decline in the osmotic potential of New Zealand spinach was mainly due to a large accumulation of Na^+ ions in the leaves, as observed in many reports, whereby halophytic plants accumulate and compartmentalize large amounts of Na^+ in vacuoles to lower the osmotic potential (Cheeseman, 1988; Munns, 2002; Ashraf, 2004; Song *et al*., 2009), and absorb more water from soil (Liu *et al.,* 2008).

Na^+ competes with K^+ for intracellular influx, since these cations are transported by common proteins, Na^+ can lead to damage of plasma membrane and, thus, subsequently increase K^+ efflux from intercellular stores. In this study, concentration of K^+ in leaves and stem was higher than in roots, and decreased with an increase of salinity, and K^+ accumulation induced by salinity was higher in shoots than in root of both species, this indicates the high selectivity of K^+ to employ the physiological process. The data showed that chard plants possesses a much more selective mechanism for the uptake of K^+ over Na^+, than New Zealand spinach under salinity.

Similarity result founded on *Beta vulgaris* (Koyro *et al.*, 2006), sugar beet (Ghoulam *et al.*, 2002), *Crithmum maritimum* (Amor *et al.,* 2005).

The Ca^{2+} and Mg^{2+} concentrations in leaves of New Zealand spinach decreased on salt treatment; however, both elements in leaves of chard were not changed by salinity. The increase of Na^{+} accumulation in New Zealand spinach was associated with reduced K^{+}, Mg^{2+} and Ca^{2+} concentrations indicating a restriction in the uptake of these nutrients, as noted in other halophytic plants (Maggio *et al*., 2000; Debez *et al*., 2004).

The Na^{+}/K^{+} ratio in leaves, stems and roots increased with increasing NaCl levels in both species, but Na^{+}/K^{+} ratio was higher in roots of chard than in leaves and stems, indicating that root had the ability to select K^{+} to keep the nutrient balance (Liu *et al.*, 2008). However, the Na^{+}/K^{+} ratio in plant tissue of chard was higher than that of New Zealand spinach under salinity.

Salinity increased concentration of N in leaves, stems and root of both species, after 14 days of salt stress, especially, in leaves under high NaCl levels (200mM). The data show that the levels of N concentration in tissues of chard were higher than that of New Zealand spinach. Similar results were previously reported in the *Tetragonia trigyna* (Watkins *et al.,* 1988).

Proline is compatible solutes that accumulate in response to osmotic stress, and the accumulation of these osmolytes represents an important adaptive response to salt and drought stress (Rhodes *et al.*, 1993). The present data indicated that both species accumulated ($P<0.05$) proline after 14 days of salt stress, and chard plants accumulate higher levels of proline in their leaves under salt conditions than New Zealand spinach. Similar result founded on *Acacia auriculiformis* and *Acacia mangium* under salt stress (Nguyen *et al.*, 2004).

Lipid peroxidation was evaluated by the determination of MDA content in leaf tissues. The finding in the present study showed that the level of MDA accumulation significantly increased in both species subjected to salinity stress, but MDA accumulation was greater in Chard than in New Zealand spinach. The data shows that the accumulated level of New Zealand spinach was higher than that of chard. The low values of MDA content obtained with chard might account for lower lipid peroxidation levels and less affected membrane permeability, Similar results were found in cottan (Meloni *et al.,* 2003); azolla (Masood *et al.*, 2006); maize (Neto *et al.,*2006); sugar beet (Bor *et al.,*2003); liquorice (Pan *et al.*, 2006); *Agrostis palustris* (Saneoka *et al.,*2004) and mangrove (Parida *et al.,*2004).

Osmotic adjustment increased with increasing salinity, and was greater in New Zealand spinach than that in chard. Osmotic adjustment seemed to be related to the accumulation of Na^+, MDA and proline.

These results indicated that the New Zealand spinach was more tolerant to salinity than chard. The significant changes of plant dry weight, gas exchange parameters, leaf water potential and accumulation of Na^+ ions, MDA, proline in leaves of both species, help clarify the physiological mechanisms, and then, compared the salt tolerance between New Zealand spinach and chard under salinity stress.

3. **Salinity tolerance of New Zealand spinach compared with water spinach and chard**

The growth of New Zealand spinach increased at 50 mM NaCl salt stress, then decreased slightly, whereas that of water spinach and chard were reduced significantly with increasing salinity. Leaf water potential(ψ_l) and osmotic potential(ψ_π) become more negative with an increase in salinity, The reduction level of (ψ_l) and (ψ_π), were higher in New Zealand spinach than water spinach and chard, and New Zealand spinach accumulated more Na^+ ions in the leaves, and show more active N uptake than water spinach. Whereas the accumulation of Na^+ was lower in New Zealand spinach than chard, but the K^+ concentration of New Zealand spinach was higher than in

chard. And the accumulation of proline was higher than in chard. The lipid peroxidation (as measured Malondialdehyde MDA) content of chard was significantly higher than in New Zealand spinach. Salt stress reduce photosynthetic rate(P_o) and transpiration rate(T_r) of all species, but both the P_o and T_r in New Zealand spinach were maintained at a higher level than that in water spinach and chard. This result indicated that New Zealand spinach is halophytic and more salinity tolerant than water spinach and chard.

Chapter 5
Summary

Salinity had drawn extensive attention throughout the world because over 6% of the earth's land area (about 800 million hectare) is affected by salinity. About 75% of the area of central and southern parts of Iraq is saline soil and salinity is seriously limiting crop production. The ability of vegetation to survive under higher salinity conditions is important for the distribution of plants and agriculture.

New Zealand spinach (Tetragonia tetragonioides L.) is widely cultivated throughout the world for use as a vegetable, ground cover and medicinal plant; however the mechanism of salt tolerance has not been clarified. The objective of this study was to compare the nutrio-physiological responses to salinity among New Zealand spinach, water spinach and chard, and to discuss the salinity tolerance of New Zealand spinach to salinity.

Experiment 1. Comparative nutrio-physiological responses to salinity in New Zealand spinach and water spinach

The seeds of New Zealand spinach and water spinach (Ipomoea aquatica) were germinated in seedbeds with a soil mixture for two weeks, then at six weeks after transplanting, the plants were subjected to three levels of salinity

treatment through irrigation with nutrient solutions containing 0, 50, 100 and 200mM NaCl twice (at 10:00 a.m. and 15:00 p.m.) every day until water drained from the bottom of the pots for 14 days.

The growth of water spinach was markedly and gradually reduced with increasing salinity, whereas that of New Zealand spinach was increased with elevating salinity, indicating that New Zealand spinach is halophytic. The leaf water potential and the osmotic potential was also gradually decreased with increasing salinity; the reduction of the leaf water potential and the osmotic potential were higher in New Zealand spinach than water spinach. New Zealand spinach accumulated more Na^+ ions in the leaves compared with water spinach. The photosynthetic rate and the transpiration rate of both species decreased with increasing salinity, but both the photosynthetic rate and the transpiration rate in New Zealand spinach were maintained at a higher level than in water spinach.

Experiment 2. Comparative nutrio-physiological responses to salinity in New Zealand spinach and chard

New Zealand spinach and chard (Beta vulgaris) were exposed to salt stress by daily irrigation with 0, 50, 100 and 200mM NaCl solution for 14 days same as Experiment 1.

The growth of chard was markedly inhibited by NaCl treatment, whereas, in New Zealand spinach, plant growth increased at a low NaCl concentration (50mM) and slightly decreased with increasing salinity. The leaf water potential and the osmotic adjustment in New Zealand spinach was higher than that in chard under salt stress. The proline content of New Zealand spinach rose with increasing salinity, and the accumulation of proline was higher than in chard in most of the salt stress treatments. Salt stress induced Na^+ accumulation in the leaves of both species, but the accumulation was lower in New Zealand spinach than in chard. The K^+ content of both species decreased with increasing salinity, but the K^+ content of New Zealand spinach was higher than that of chard. The lipid peroxidation level measured as malondialdehyde (MDA content) in chard was significantly higher than that in New Zealand spinach at a higher salinity. The photosynthetic rate was decreased by salt stress, but that in New Zealand spinach was maintained at a higher level compared with chard.

Conclusion

The growth of New Zealand spinach was promoted by the uptake of moderate amount of salt, but water spinach and chard was sever impairment of growth by salinity, indicating that New Zealand spinach is halophytes.

The main strategy of salt tolerance in New Zealand spinach seems to be an increased capacity of osmotic adjustment through the accumulation of Na^+ ions, proline and a more active antioxidant system under salt stress conditions. These results suggested that New Zealand spinach is more tolerant to salinity than water spinach and chard.

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