Salt Mass Balance Study and Plant Physiological Responses for an Enhanced Salt Phytoremediation System

by

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Author’s declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Salinity is one of the most severe environmental factors that limits global crop yield. Enhanced phytoremediation using plant growth promoting rhizobacteria (PGPR) has proven to be an effective and environmentally responsible approach to remove salt from the surface soil and reclaim salt-impacted soil for crop production.

PGPR enhanced phytoremediation systems (PEPS) were applied to two research sites, Cannington Manor North (CMN) and Cannington Manor South (CMS) in southern Saskatchewan. The sites were impacted by brine leakage during upstream oil and gas production. A salt mass balance study was performed based on data collected from these two sites. Both sites were planted in June. Soil samples were taken in June 2009 (beginning of the season), August (midseason) and October (end of the season). Soil salinity changes throughout the season were monitored by measuring soil electrical conductivity (EC). The average surface soil EC_e decreased from 3.7 dS/m to 3.1 dS/m at CMN, and from 10.2 dS/m to 9.2 dS/m at CMS in 2009 season. Plant samples that were collected in August and October were analyzed for sodium and chloride concentrations. These values were then converted into predicted EC_e changes for the soil to compare with the actual changes in soil EC_e. Plant uptake of NaCl was calculated to account for 25.2% and 28.1% of the decrease in surface soil EC_e at CMN and CMS, respectively. However, plant samples were washed prior to salt content analysis. A considerable amount of salt could have been lost during the washing process. Several plant samples from other salt-impacted sites in Saskatchewan and Alberta were selected to examine salt loss due to tissue washing. The salt ions lost by washing were determined to be 44.4% for Na^+ and 63.8% for Cl^-.

After the adjustment of plant NaCl uptake data by the loss due to washing, plant accumulation of NaCl accounted for 59.9% of the decrease in surface soil EC_e at CMN and 56.1% at CMS. When plant uptake of K^+ and Ca^{2+} were also taken into consideration by a simulation study, the decrease in surface soil EC_e that was caused by plant uptake of salt ions accounted for 107.5% at CMN and 117.5% at CMS. This indicated that plants can have a significant role in the remediation of salt-impacted soil.

The effects of PGPR (*Pseudomonas spp.* UW4 and *Pseudomonas corrugata* CMH3) treatment on selected physiological indicators, such as proline, superoxide dismutase (SOD), membrane leakage and photosynthesis, were examined on annual ryegrass (*Lolium multiflorum*). Plants were grown under three saline conditions: non-saline topsoil, non-saline topsoil spiked with NaCl to 10 dS/m, and high saline soil collected from a salt-impacted site diluted with non-saline topsoil to reach 10 dS/m. The shoot fresh weight of plants grown in spiked salt soil decreased by 74% and in diluted salt soil by 44%, respectively, compared to control soil. Both types of salt soil increased SOD activities by approximately 50%, proline concentrations by 20 to 25 fold, and membrane leakage levels by 1.6 to 2.8 fold. Significant impairment of photosynthetic performances, as indicated by the decreases in the chlorophyll fluorescence parameters F_v/F_m, yield and qP, and a parallel increase in qN, was also observed using Pulse Amplitude Modulation (PAM) fluorometry for plants in diluted impacted soil. PGPR moderately increased fresh weight and SOD activity. Both UW4 and CMH3 significantly increased proline concentration and lowered membrane leakage relative to untreated plants. Therefore, PGPR improve plant performance under salt stress by elevating proline levels, which can act as a quencher of destructive reactive oxygen species. PGPR treatment also restored all the

chlorophyll fluorescence parameters nearly to the non-stressed level, indicating protection of photosynthetic tissues of PGPR treated plants under salt stress.

Overall, PEPS was successfully applied to the salt-impacted sites. Plant uptake of salt played a major role in the decrease of surface soil EC_e. PGPR's role in enhancing plant performance under salt stress was suggested by the elevated proline concentrations, the decreased membrane leakage levels and the restored photosynthetic activity.

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AL	Actinic light used in PAM fluorometry
Alameda	The Alameda site
ASC	Ascorbate
APX	Ascorbate peroxidases
CAT	Catalase
CMN	The Cannington Manor North site
CMS	The Cannington Manor South site
dd H2O	De-ionized and distilled water
DW	Dry weight
EC	Electrical-conductivity
$EC_{1:2}$	Electrical-conductivity of a soil extract with 1 part of soil to 2 parts of water (w/v)
EC_e	Electrical-conductivity of a saturated soil paste extract
Fd	Ferredoxin
FNR	Ferredoxin $NADP^+$-reductase
FR	Far-red light used in PAM fluorometry
GB	Glycinebetaine
GSH	Glutathione
HKT	High-affinity K^+ transporters
IAA	Indole-3-acetic acid

IS	Ion strength
LHCI	Light-harvesting chlorophyll protein complex I
ML	Modulated measuring light used in PAM fluorometry
NHX1	Tonoplast Na^+/H^+ antiporters
NSCCs	Non selective cation channels
OD	Optical density
P5CS	Pyrroline-5-carboxylate synthetase
P5CR	Pyrroline-5-carboxylate reductase
PAM	Pulse amplitude modulated
PEAMT	Phosphoethanolamine: N-methyltransferase
PGPR	Plant growth-promoting rhizobacteria
POX	Peroxidase
ProDH	Proline dehydrogenase
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
RuBisCo	Ribulose-1,5-bisphosphate carboxylase oxygenase
SAM	S-adenosyl-methionine
SOD	Superoxide dismutase
SOS1	Salt overly sensitive plasmas membrane Na^+/H^+ antiporter
SAR	Sodium adsorption ratio
SP	Saturating pulse used in PAM fluorometry
TSB	Tryptic soy broth

Chlorophyll Fluorescence Nomenclature

F Actual fluorescence intensity at any given time

F' Fluorescence at any light level and induction state

F_o Minimal fluorescence in dark-adapted tissue; fluorescence intensity with all PSII reaction centers open while the photosynthetic membrane is in the non-energized state (qP = 1 and qN = 0)

F_m Maximal fluorescence in dark-adapted tissue; fluorescence intensity with all PSII reaction centers closed (qP = 0), all non-photochemical quenching processes are at a minimum (qN = 0)

F_v Variable fluorescence in dark-adapted tissue; maximum variable fluorescence in the state when all non-photochemical processes are at a minimum; F_m-F_o

F_s Fluorescence in steady states; a period within which the fluorescence intensity does not change while the external circumstances remain constant

F_v/F_m Excitation transfer efficiency in dark-adapted tissue; $(F_m-F_o)/F_m$

F_o' Minimal fluorescence in light-adapted tissue (quick application of Far-Red PSI light); fluorescence intensity with all PSII reaction centers open in any light adapted state

F_m' Maximal fluorescence in light-adapted tissue; fluorescence intensity with all PSII reaction centers closed in any light adapted state

F_v' Variable fluorescence in light-adapted tissue; maximum variable fluorescence in any light adapted state; $F_m' - F_o'$

F_v'/F_m' Excitation transfer efficiency in light-adapted tissue; $(F_m' - F_o')/F_m'$

qP Photochemical quenching; $(F_m' –F)/(F_m' - F_o')$

qN	Non-photochemical quenching; $1-(F_m'-F_o')/(F_m-F_o)$
Yield	Effective quantum yield of PSII; $(F_m'-F_s)/F_m'$

Chapter 1 – Introduction

About 7% of the world's total land is affected by excessive salt (Munns, 2002). NaCl is the most soluble and widely distributed salt found in soil (Munns & Tester, 2008). Natural salt-impacted soils arise from the weathering of rocks, the deposition of oceanic salts and other processes (Läuchli & Luttge, 2002). However, secondary salinization commonly occurs as an outcome of agricultural practices. For example, poor irrigation practices and management can result in salt-impacted soil (Läuchli & Luttge, 2002). Over 30% of the total agricultural production comes from irrigated land, the productivity of which is about twice that of the rain-fed land (Flowers & Yeo, 1995). Irrigated salinization threatens the sustainability of high agricultural productivity. Other anthropogenic activities that can lead to salt contamination include oil and gas production, road salt application for snow removal and the transportation of saline material for industrial use (Howat, 2000; Läuchli & Luttge, 2002).

Many salt-contaminated sites in Canada are associated with the accidental release of brine that may occur during oil and gas exploration and transport (Howat, 2000). Salt in brine can easily move with water over the surface or down through the soil, and thereby degrade soil properties and impair vegetative growth (Howat, 2000). Salinity has been one of the key environmental problems limiting the crop yield in the world (Flowers & Yeo, 1995). The control of salinization and the improvement of crop tolerance to salt stress are of critical importance to satisfy increasing demand for food, as the world's population increases (Läuchli & Luttge, 2002).

One way to improve crop performance under salinity stress is to develop salt-tolerant cultivars (Läuchli & Luttge, 2002). This can be accomplished by either exploiting natural genetic variations or introducing novel genes that can affect the degree of salt tolerance (Flowers, 2004). However, the transgenic approach has only been performed on a few model plants, such as *Arabidopsis*, tobacco and rice (Yamaguchi & Blumwald, 2005). The transformation of many

crop species is technically challenging (Yamaguchi & Blumwald, 2005). In addition, in most of the cases, the assessment of salt tolerance in transgenic plants is carried out under greenhouse conditions, which might have limited correlation to the tolerance in the field (Yamaguchi & Blumwald, 2005).

Another way to manage increased salt in soils and enhance crop productivity is to reclaim saline soil (Läuchli & Luttge, 2002). Salt-impacted soils are difficult to remediate because Na^+ and Cl^- ions are highly mobile in soils and not volatile. While excavation and landfilling is sometimes used to remove hot spots where Na^+ and Cl^- concentrations are high, it is not a practical option for site-wide remediation (Alberta Environment, 2001). Leaching the soil with water, followed by discharge through appropriate drainage system has been widely used to remove excess soluble salt from soil (Cardon et al., 2007). However, leaching can only be used when abundant sources of high quality water are available. Adding large quantity of water to the root zone might result in nutrient loss from the surface soil (Cardon et al., 2007). Additionally, downward movement of a large amount of salt may compromise groundwater quality, especially for those sites where the water table is shallow (Alberta Environment, 2001). Remediation of salt-impacted sites should protect groundwater quality and minimize negative impacts to land use and soil productivity (Alberta Environment, 2001). An alternative to leaching salt from the root zone is phytoremediation (Chang, 2008; Gerhardt et al., 2009).

1.1 Soil salinity measurement

The term soil salinity refers to the soluble and readily dissolvable inorganic solutes (essentially Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^-, NO_3^- and CO_3^{2-}) in the soil or in an aqueous extract of a soil sample (Rhoades et al., 1999). Electrical conductivity (EC) is an easily measured

index of the total concentration of ionized solutes in an aqueous sample (US Salinity Laboratory Staff, 1954). Soil salinity has been assessed in terms of laboratory measurements of the electrical conductivity of the extract of a saturated soil-paste sample (EC_e) (Rhoades et al., 1999). In the International System of Units (SI), electrical conductivity is reported as siemens (reciprocal of ohm) per metre (S/m), or as decisiemens per metre (dS/m). One dS/m is equivalent to one milliohm/cm (Rhoades et al., 1999).

EC of a 1:2 soil-water mixture ($EC_{1:2}$) can be measured with an electrode to give an indication of soil salt content (Sonnevel & Vandenen, 1971). $EC_{1:2}$ are easier to use than that of the saturation paste. It can be converted to EC_e by multiplying a K value (Equation 1.1). K value is dependent on soil properties and ranges from 2 to 4 (Sonnevel & Vandenen, 1971).

$$EC_{\mathrm{e}} = \mathrm{K} \times EC_{1:2} \quad (1.1)$$

When the concentration of free salt ions increases in soils, the EC_e will increase proportionally (Läuchli & Luttge, 2002). The equivalent concentrations of NaCl in the soil and in water solutions of different EC_e values are listed in Table 1.1. Soil with an EC_e higher than 4 dS/m is considered as saline soil, which can negatively affect the growth and yield of most plant species (Läuchli & Luttge, 2002). A general guideline of soil quality at different salinity levels is listed in Table 1.2.

Table 1.1: The equivalent concentrations of NaCl in the reference soil and in the water solution of a range of EC_e values at 23 ºC.

EC_e (dS/m)	NaCl (g/kg reference soil)[1]	NaCl (mM)[2]
2.0	0.5	12.9
4.0	1.4	32.7
6.0	2.3	52.6
8.0	3.2	72.4
10.0	4.1	92.2
15.0	6.3	141.8
20.0	8.4	191.4
25.0	10.6	241.0
30.0	12.8	290.6

1. The data are based on the measurement of EC_e in the NaCl spiked un-impacted topsoil (reference soil). The EC_e of the topsoil is 0.5 dS/m.
2. The data are retrieved from Wu, 2009.

Table 1.2: Soil quality guidelines for unrestricted land use (Adapted from Alberta Environment, 2001).

	Rating categories			
	Good	Fair	Poor	Unsuitable
Topsoil EC_e (dS/m)	<2	2 to 4	4 to 8	>8
Subsoil EC_e (dS/m)	<3	3 to 5	5 to 10	>10

1.2 Salt tolerance level of plants

Halophytes are plants that are natural to highly saline soil like maritime marshes and salt deserts, which can have soil EC_e of approximately 60 dS/m (Flowers et al., 1986). Only 2% of the world's plant species are halophytes, including saltbush (*Atriplex* spp) (*Halophyte DataBase, salt-tolerant plants and their uses.*). Most crops are glycophytes, which are defined as non-halophytes whose growth is severely inhibited by NaCl (Flowers et al., 1986). Production of many crops is impacted when soil EC_e is higher than 4 dS/m, which is approximately 1.4 g NaCl

per kg of soil (Läuchli & Luttge, 2002). The salt-tolerance list (Table 1.3) is arranged according to major crop divisions (U.S. Department of Agriculture, 1954). EC_e in each column represents the salinity level at which a 50% decrease in yield may be expected as compared to yield on nonsaline soil under comparable growing conditions (U.S. Department of Agriculture, 1954).

Table 1.3: Relative salt tolerance of various crop plants (Adapted from U.S. Department of Agriculture, 1954).

Tolerance level	EC_e	Vegetable Crops	EC_e	Forage Crops	EC_e	Field Crops
High	10-12 dS/m	Asparagus Spinach	12-18 dS/m	Saltgrass Western wheatgrass	10-16 dS/m	Barley Sugar beet cotton
Moderate	4-10 dS/m	Tomato Broccoli Cauliflower Lettuce Pea Cucumber	4-12 dS/m	Perennial ryegrass Alfalfa Tall fescue Annual ryegrass	6-10 dS/m	Rye Wheat Oat Rice Corn Sunflower
Low	3-4 dS/m	Radish Celery Green beans	2-4 dS/m	Meadow foxtail Red clover Burnet	< 4 dS/m	Field beans

1.3 Phytoremediation of salt-impacted soil and salt mass balance in soil

Phytoremediation is the use of plants and their associated microbes to exclude, isolate, immobilize or degrade contaminants in soil (Pilon-Smits, 2005). Compared to the traditional physical and chemical methods, phytoremediation is considered a cost-effective and environmentally responsible approach (Gerhardt et al., 2009). The technique has been successfully applied to clean up soils impacted with petroleum, metals, and other persistent contaminants (Gerhardt et al., 2009; Pilon-Smits, 2005). Phytoremediation of salt is based on the ability of plants to accumulate salt ions into aboveground biomass, which can then be removed

from the site (Gerhardt et al., 2009).

Salt decreases in surface soil have been observed using phytoremediation (Chang, 2008; Wu, 2009). Several factors can affect salt movement in soil during phytoremediation. Rainfall infiltration and percolation are forces that lead to downward migration of salt, while evapotranspiration of water pulls salt up into plant tissue (Läuchli & Luttge, 2002). Salt that has been leached down might rise to the surface again by capillary action when the water table rises within two meters of the soil surface (Goebel, 2003). In contrast, phytoremediation of salt is irreversible as long as the plant material is mowed and removed from the site (Gerhardt et al., 2009). With the salt content decreasing year by year, the site can be permanently remediated, provided no further salt contamination occurs. Therefore, plant uptake of salt is preferable during phytoremediation over leaching of salt below the root zone. A study of salt mass balance and the assessment of phytoremediation efficacy can help illustrate how salt migrates in the soil profile and how plants reduce the surface soil salinity. The surface EC_e decrease that is caused by plant uptake of NaCl and the decrease caused by natural leaching beyond the root zone can be calculated to estimate their respective contributions to the decrease in salt from the surface soil.

Studies have shown salt uptake is proportional to plant biomass production (Chang, 2008). Therefore, better plant growth will likely result in higher remediation efficiency. However, salt-impacted soil has a negative effect on biomass production (Chang, 2008).

1.4 Plant responses to salt stress

Exposure of plant roots to salt will lead to a series of physiochemical changes that might develop into acute or chronic stress if the salt is not removed (Greenway & Munns, 1980; Munns, 2002). High concentration of salt built up in the plant root zone can decrease water potential in

aboveground tissues, and induce similar responses in plants that are exposed to drought conditions (Munns, 2002). These include immediate decreases in leaf and root elongation rates, followed by decreased leaf emergence, final leaf size and seed production after days or weeks (Table 1.4) (Munns, 2002). As plants start to take up salt ions, ion-specific stress becomes apparent (Munns, 2002; Munns & Tester, 2008). Symptoms such as chlorosis of leaf tips and abscission of old leaves can be seen after days or weeks (Munns, 2002). If salt is not removed, ion accumulation might threaten young leaves where active photosynthesis takes place and result in the plant death before seed maturation (Ashraf, 2004; Munns, 2002). Oxidative stresses induced by water deficiency and ion toxicity can cause further tissue damage (Abogadallah, 2010). The sequential responses of plants exposed to salt stress are summarized in Table 1.4. Plants can activate defense mechanisms to partially neutralize the negative impact of these stresses and acclimate to saline environment (Ashraf, 2009; Zhu, 2001).

Table 1.4: Plant responses to salinity at different time scales (adapted from Munns, 2002).

Time	Osmotic stress effects	Ion-specific effects
Minutes to hours	Reduced leaf and root elongation rate, then partial recovery	
Days	Reduced rate of leaf emergence	Injury visible in older leaves
Weeks	Reduced final leaf size	Death of older leaves
Months	Reduced seed production	Younger leaves die; plants may die before seeds mature

1.4.1 Osmotic stress

Salt-induced osmotic stress is not only caused by NaCl. KCl and mannitol can cause similar effects. A decreased ability of plants to take up water from the soil is one of the initial consequences of elevated salt soil levels (Munns, 2002). Plants can develop a positive osmotic potential when a high concentration of salt ions is present around the roots causing water to flow

from the roots to the soil (Munns, 2002). Salt induced osmotic stress can also lead to stomatal closure to restrict the water loss by transpiration, and might thereby cause a decrease in CO_2 diffusion rate and photosynthetic fixation of CO_2 (Flexas et al., 2004).

One way to counteract the osmotic stress is to continuously pump sodium and chloride ions to the aboveground tissues (Tester & Davenport, 2003). This strategy has been effectively employed by various halophytes, and known to be one of the key features that distinguish halophytes from glycophytes (Tester & Davenport, 2003; Zhu, 2001). Halophytes can tolerate higher concentrations of salt ions in leaf cells than glycophytes, and therefore use readily available salt ions as osmolytes (Tester & Davenport, 2003). In contrast, the low tolerance to ion toxicity of glycophytes prevents them from taking up a large quantity of salt ions for osmotic potential balancing (Tester & Davenport, 2003). Another mechanism that is widely used by both halophytes and glycophytes is to biosynthesize a series of organic compounds, called compatible osmolytes (Ashraf & Harris, 2004; Hare et al., 1998). Compatible osmolytes are usually of low molecular weight, highly water soluble, and non-toxic at high cellular concentrations (Ashraf & Foolad, 2007). These solutes include soluble sugars, organic acids, polyols and nitrogen containing compounds such as amino acids, amides, and quaternary ammonium compounds (QACs) (Ashraf & Harris, 2004). They can counteract the negative effects of high osmotic pressure in plant tissues, and they can also serve as osmoprotectant, which play a role in the detoxification of reactive oxygen species, stabilization of enzymes and proteins, and the protection of membrane integrity (Hare et al., 1998). A positive correlation between the accumulation of osmolytes and salt stress tolerance in plants has been found (Delauney & Verma, 1993; Lutts et al., 1999; Nanjo et al., 1999).

As the most commonly found QACs in plants that are subjected to salt stress, glycinebetaine

(GB) (Figure 1.1 A) accumulates in many crops (Ashraf & Foolad, 2007; T. Chen & Murata, 2011). GB is synthesized from choline by a two-step oxidation reaction: choline is oxidized to betaine aldehyde by choline monooxygnase (CMO), which is then converted to GB by NAD^+-dependent betaine aldehyde dehydrogenase (BADH) (Chen & Murata, 2011). Mainly synthesized in chloroplasts under osmotic stress, GB is known to play a vital role in protecting thylakoid membranes, thereby maintaining photosynthetic efficiency under salt stress (Ashraf & Foolad, 2007; Papageorgiou & Murata, 1995). GB might also protect the CO_2-fixing enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo), under salt stress, and thereby sustain the fixation of CO_2 (Makela et al., 2000; Papageorgiou & Murata, 1995). Furthermore, GB activates the expression of genes for ROS-scavenging enzymes, which decrease the levels of ROS in cells, resulting in the alleviation of salinity effects on the photosynthetic machinery (Banu et al., 2009; Hoque, Okuma et al., 2007). In addition to the osmoprotectant role in chloroplast, GB is shown to be present in the cytoplasm and act as an osmolyte (Ashraf & Foolad, 2007). GB in the cytosol has also been correlated with the ion homeostasis (Ashraf & Foolad, 2007). However, little is known about the transport process of GB from the chloroplast to the cytosol, and the knowledge of how GB interacts with ion channels and transporters related to plant salt tolerance is scarce (Chen & Murata, 2011).

Transgenic plants over accumulating GB have exhibited enhanced tolerance to salt stress (Chen & Murata, 2002; Huang et al., 2000). Although considerable efforts have been made to increase overall levels of GB in transgenic plants, the expression level in plants that naturally do not produce GB can be limited due to the low availability of substrates like choline and metabolic flux from cytoplasm into chloroplast where GB is synthesized (Huang et al., 2000; McNeil et al., 2001; Nuccio et al., 1998). The key enzyme of choline synthesis is

phosphoethanolamine: N-methyltransferase (PEAMT). The lack of GB accumulation in transgenic lines overexpressing CMO was found to result from the elevated concentration of endogenous phosphocholine, which is a strong inhibitor of PEAMT in vitro, indicating the availability of choline is one of the major limiting factors for the successful development of transgenic GB accumulators (McNeil et al., 2001). The gene sequence of the second enzyme in GB synthesis pathway, BADH, was also used to transform non-GB accumulating plants, resulting in strong tolerance to salt stress (Kumar et al., 2004). Exogenous application of GB and the precursor of GB have also been examined. However, due to the limited number of reports on the effectiveness of different concentrations of GB in different plant species, the results of GB treatment vary from full alleviation of the adverse effects to no effect on the growth and biomass (Harinasut et al., 1996; Heuer, 2003).

Proline (Figure 1.1 B) is also widely synthesized by plants in the cytosol in response to environmental stresses (Hare et al., 1999). In plants, the precursor for proline biosynthesis is glutamic acid. Two enzymes, pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR), play major roles in proline biosynthetic pathway (Delauney & Verma, 1993). Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants (Hare & Cress, 1997; Mattioli et al., 2009; Nayyar & Walia, 2003). For example, a study showed that in the apical region of maize roots growing at a water potential of −1.6 MPa, proline accounted for up to 50% of the osmotic adjustment (Voetberg & Sharp, 1991). However, the role of proline in osmoregulation has been questioned. Previous studies also found that more than 90% of the osmotic adjustment in salt stressed tomato and quinoa was achieved by plant uptake of salt ions rather than proline accumulation (Hariadi et

al., 2011; Shalata & Tal, 1998) and elevated level of proline seemed to be a symptom of injury rather than an indicator of salt tolerance (Lutts et al., 1999). Despite of the controversial role of proline as an osmolyte, overproduction of proline by either inducing the synthesis pathway enzyme P5CS or suppressing the proline dehydrogenase (ProDH), the enzyme that is responsible for proline degradation, resulted in increased salinity tolerance in transgenic plants (Kishor et al., 1995; Nanjo et al., 1999; Yoshiba et al., 1997). The protective role of proline other than the osmotic adjustment has been proposed to result from its ability to stabilize the cell membranes and proteins, scavenge free radicals, and buffer cellular redox potential under stress conditions (Mattioli et al., 2009; Matysik et al., 2002). Furthermore, proline is known to induce expression of salt stress responsive genes, which have proline responsive elements in their promoters (Oono et al., 2003; Satoh et al., 2002). Exogenous application of proline has been shown to confer plants salt stress tolerance through the activation of antioxidant systems (Banu et al., 2009; Hoque et al., 2007). However, plants exhibit concentration dependent responses to the foliar spray. At low concentrations, exogenous application can provide a stress preventing or recovering effect; however, high concentration may exacerbate the deleterious effects of salt stress (Hare et al., 2002).

Figure 1.1: Chemical structures of glycinebetaine (A) and proline (B)

1.4.2 Ion-specific stress

Compared to the immediate onset of osmotic stress, ion-specific stress develops over time, as plants gradually accumulate sodium and chloride ions to toxic concentrations in tissues (Munns, 2002). For most species, Na^+ appears to reach a toxic concentration before Cl^- does. In some woody species, such as citrus and grapevine, chloride is more toxic than sodium (GuetaDahan et al., 1997; Munns & Tester, 2008). Therefore, most current studies on ion-specific stress on plants focus on sodium transport.

At the soil-root interface, the electrochemical gradient favors the passive flux of Na^+ from water in the soil into plant roots (Kronzucker & Britto, 2010). The main import sites for Na^+ are non selective cation channels (NSCCs) and high-affinity K^+ transporters (HKT) in root epidermal cell membranes (Figure 1.2) (Demidchik et al., 2002; Rodriguez-Navarro & Rubio, 2006; Russell, 2000). Before being loaded into the xylem and transported to shoot tissues in the transpiration stream, a majority of Na^+ is likely to be pumped back to the soil solution via SOS1 (salt overly sensitive Na^+/H^+ antiporter) located in the plasma membrane (Figure 1.2), or sequestered into the root cell vacuoles, mediated by NHX1 (tonoplast Na^+/H^+ antiporters) (Figure 1.2) (Mansour et al., 2003; Tester & Davenport, 2003). It has been suggested that the exclusion of Na^+ from roots is a key mechanism to minimize salt damage to plants growing in saline soil (Munns & Tester, 2008; Tester & Davenport, 2003). By comparing Na^+ concentrations outside plant roots and in xylem, it has been estimated that at 200 mM external Na^+ (20 dS/m), about 97% of all Na^+ presented to the root surface must be excluded. This is the case for both glycophytes and halophytes (Munns et al., 1999). However, most Na^+ that is delivered to the shoot remains in the shoot, because the retrieval of Na^+ from the shoot to the root through the phloem is negligible in most plants (Tester & Davenport, 2003). Therefore, Na^+

transport is largely unidirectional and result in the progressive accumulation of Na^+ in leaf blade, which is the main site of Na^+ toxicity for most plants (Tester & Davenport, 2003). The first visible sign of ion toxicity is the chlorosis of old leaves; starting at the tips and margins and working back through the leaf (Tester & Davenport, 2003). Old leaves can act as a sink for Na^+, and thereby avoid ion toxicity in young leaves, where active photosynthesis occurs. Na^+ toxicity for the whole plant can be lowered when old leaves abscise (Kronzucker & Britto, 2010; Rodriguez-Navarro & Rubio, 2006).

The ion K^+ serves as an essential nutrient for plants (Taiz & Zeiger, 2006). High concentration of Na^+ in the cytoplasm competes with K^+ for binding sites essential for enzyme functions and cause toxicity (Bhandal & Malik, 1988). When cytoplasmic Na^+ reaches 100 mM, enzyme activity starts to be inhibited (Maser et al., 2002). In addition, K^+ is a crucial ion responsible for the binding of tRNA to ribosomes in protein synthesis processes, which would be disrupted by elevated Na^+ level or K^+ deficiency (Blaha et al., 2000). Certain halophytes require Na^+ for growth and osmotic adjustment, but the majority of plants cannot fulfill physiological functions when K^+ is replaced by Na^+ (Kronzucker & Britto, 2010). However, due to physiochemical similarities between K^+ and Na^+, the acquisition of K^+ is limited when high concentrations of Na^+ compete with K^+ for uptake (Maathuis & Amtmann, 1999; Zhu, 2003). Therefore, the maintenance of a high cytosolic K^+/Na^+ ratio is considered to be one of the most important determinants of plant salt tolerance (Maathuis & Amtmann, 1999).

Na^+/H^+ antiporters are membrane proteins that move Na^+ against its electrochemical potential coupled with the motive force generated by a proton gradient, which is provided either by H^+-pyrophosphatase or H^+-ATPase (Figure 1.2) (Taiz & Zeiger, 2006). Na^+/H^+ antiporters play an essential role in maintaining proper K^+/Na^+ ratios in the cytoplasm (Craig Plett & Moller,

2010; Maathuis & Amtmann, 1999). The most well studied plasma membrane Na^+/H^+ antiporters is SOS1 in *Arabidopsis thaliana* (AtSOS1) (Shi et al., 2000). Salt stress induces a Ca^{2+} signal that activates the SOS3/SOS2 protein kinase complex, which then phosphorylates SOS1. Consequently, Na^+/H^+ antiporter is activated. SOS1 can mediate efflux of Na^+ from the epidermal cells at the root tip, and the retrieval of Na^+ from xylem (Qiu et al., 2002). The Atsos1 mutant was shown to overaccumulate Na^+ in both root and shoot tissue (Shi et al., 2000; Davenport et al., 2007), whereas plants that constitutively overexpress AtSOS1 have decreased Na^+ in the shoot and the xylem (Shi et al., 2003). SOS2 also activates tonoplast Na^+/H^+ antiporter NHX1, which is responsible for the compartmentalization of Na^+ into vacuoles (Craig Plett & Moller, 2010). Studies have indicated that the overexpression of NHX1 can improve the salt tolerance of Arabidopsis (Apse et al., 1999), tomato (Zhang & Blumwald, 2001), and wheat (Xue et al., 2004).

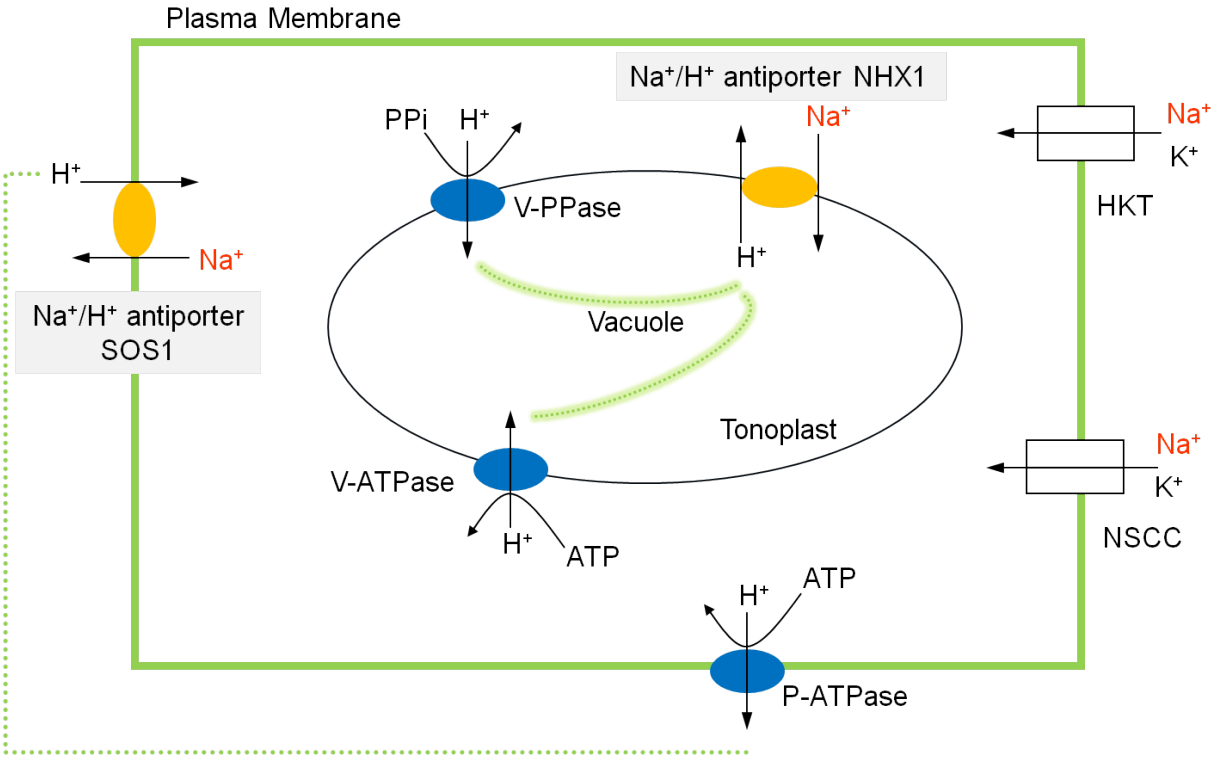


Figure 1.2: Schematic diagram showing the ion transporters, channels and pumps which have been characterized as being involved in Na^+ transport in plants (Adapted from Mansour et al., 2003). The various proteins are localized on the putative membrane. The influx of Na^+ into cells is mediated by non selective cation channels (NSCCs) and HKT transporters. The efflux of Na^+ from cells is mediated by the Na^+/H^+ antiporter (SOS1). Vacuolar partition of Na^+ is mediated by a vacuolar Na^+/H^+ antiporter (NHX1) and the electrochemical potential is provided by the vacuolar H^+-pyrophosphatase (V-PPase) and the vacuolar H^+-ATPase (V-ATPase).

1.4.3 Oxidative stress

In plants, reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$) hydroxyl group (HO·) and hydrogen peroxide (H_2O_2), are continuously generated as byproducts of cellular metabolism in various compartments including the chloroplasts, mitochondria, and peroxisomes (Apel & Hirt, 2004). Under non-stressed conditions, the concentrations of ROS in plant cells are low: normally a production rate of 240 μM s^{-1} for superoxide (O_2^-) and a steady-state level of 0.5 μM H_2O_2 in chloroplasts (Polle, 2001). However, ROS production can be

increased to a rate of 240-720 μM s^{-1} for O_2^- and a steady-state level of 5-15 μM H_2O_2 by abiotic and biotic stresses that disrupt the cellular homeostasis of production and scavenging of ROS (Polle, 2001). This rapid increase of ROS in cells is called an "oxidative burst", which imposes oxidative stress on plants (Apel & Hirt, 2004).

Chloroplasts are a major site for ROS production in stressed plants (Miller et al., 2010). Three major physiological systems within a chloroplast that are affected by salt stress can yield ROS: (1) Ion toxicity can cause damage to the enzymes of the electron transport systems. As a result, the light energy captured by the light harvesting complex (LHC) excites a triplet (ground-state) oxygen to singlet oxygen (Krieger-Liszkay, 2005), which is a representative ROS, rather than being used in electron excitation. (2) The restricted supply of CO_2 due to stomatal closure under salt stress results in underperformance of the Calvin cycle which fixes carbon and regenerates $NADP^+$. With insufficient amounts of $NADP^+$ as an electron acceptor, molecular oxygen is reduced to superoxide by ferredoxin in photosystem I (PSI) (Hsu & Kao, 2003). (3) Under non-stressed condition, approximately 10% of the electrons leak out from the transport chain. Stress has been shown to induce an increase of electron leakage from the photosystem II (PSII) reaction center, and facilitate electron transfer to molecular oxygen, thereby produce more superoxide and hydrogen peroxide (Hasegawa et al., 2000). In addition to abiotic strategies to produce ROS as mentioned above, several enzymes have been shown to be triggered by salt stress and contribute to the generation of ROS (Apel & Hirt, 2004). The cell membrane-bound NADPH oxidases catalyze the production of superoxide by the one-electron reduction of oxygen using NADPH as the electron donor (Apel & Hirt, 2004). The $\cdot O_2^-$ generated by this enzyme serves as a starting material for the production of a large variety of reactive oxidants (Apel & Hirt, 2004). The apoplastic diamine oxidase is also reported to be responsible for ROS

production under salt stress (Abogadallah, 2010; Hernandez et al., 2001).

When maintained at low concentrations, ROS can participate in cell signaling (Blokhina et al., 2003). For example, H_2O_2 can activate several mitogen-activated protein kinase (MAPK) signaling cascades, which appears to be central for mediating cellular responses to multiple stresses (Apel & Hirt, 2004; Ouaked et al., 2003). However, once overproduced and accumulated in cellular compartments such as chloroplasts, ROS interact with DNA, pigments, proteins, lipids, and other essential cellular components leading to a series of random destructive processes (Apel & Hirt, 2004). These include DNA and protein denaturation, as well as lipid peroxidation resulting in the loss of membrane integrity (Apel & Hirt, 2004; Blokhina et al., 2003). Therefore, equilibrium between the formation and scavenging of ROS needs to be tightly controlled in plant cells.

Plants have evolved both enzymatic and non-enzymatic mechanisms to scavenge excessive ROS (Apel & Hirt, 2004; Miller et al., 2010). A variety of antioxidant enzymes, such as superoxide dismutases (SOD), catalases (CAT) and ascorbate peroxidases (APX) are utilized by plants to remove ROS. SOD can catalyze superoxide to H_2O_2, while CAT and APX subsequently detoxify H_2O_2 to H_2O (Ashraf, 2009). Non-enzymatic antioxidants include the low molecular weight cellular buffers like ascorbate and reduced glutathione (GSH). A high ratio of reduced to oxidized ascorbate and GSH is essential for ROS scavenging in cells (Mittler, 2002; Noctor & Foyer, 1998).

Oxidative damage in plants can be assessed in a number of ways, such as changes in the levels of antioxidant enzymes, lipid peroxidation, and membrane leakage (Campos et al., 2003; Mittler, 2002). The membrane leakage technique is an appealing method because it is simple, rapid, inexpensive, and allows a large number of samples being analyzed at the same time (Bajji

et al., 2002). ROS-induced increased permeability of cell membrane can result in the leakage of electrolytes contained within the membrane into surrounding tissues (Campos et al., 2003). The degree of injury in cell membranes can be estimated by measuring of the conductivity of the leaked contents from plant tissues immersed in water (Bajji et al., 2002; Whitlow et al., 1992).

1.4.4 Salt stress and photosynthesis

Plant photosynthesis is the basis of plant growth and biomass production. Photosynthesis is a physiological process in plants that couples energy of light to form O_2, carbohydrates and adenosine triphosphate (ATP) as energy sources (Taiz & Zeiger, 2006). Photosynthesis is initiated by absorption of light and transfer of photon energy to electrons. The electrons that are excited to a high energy level pass through the electron transport chain in the thylakoid membrane, and are finally transferred to the electron acceptor $NADP^+$ to form NADPH (Taiz & Zeiger, 2006). The proton gradient generated during the electron transport is coupled to phosphorylation of adenosine diphosphate (ADP) and produce ATP (Taiz & Zeiger, 2006). With the energy and the reducing power generated by the light reactions, carbon fixation is achieved in the Calvin cycle (Taiz & Zeiger, 2006).

The photosynthetic electron transport chain consists of three protein complexes: PSII, the cytochrome b_6f complex, and PSI (Taiz & Zeiger, 2006). When a pair of chlorophyll molecules (P680) in PSII absorbs photon, the resulting high-energy electrons are transferred to a pheophytin molecule, and travel to two plastoquinone molecules (Taiz & Zeiger, 2006). Notably, once plastoquinone has accepted an electron, it is not able to accept another until it has passed the first onto a subsequent electron carrier (Maxwell & Johnson, 2000). After receiving two electrons, the plastoquinone molecules are reduced to QH_2, take up two protons from the stroma,

and leave the PSII. The charge separation occurs, as the P680 molecule gains positive charge and becomes a strong oxidant that can strip electrons from water molecules, which are thereby split to release O_2 (Taiz & Zeiger, 2006). The QH_2 diffuses through the membrane to the cytochrome b_6f complex (cyt b_6f) and then to the next mobile component plastocyanin, which migrates to Photosystem I (PSI) containing the light-harvesting center I (LHCI), the reaction center P700 and a number of electron acceptors (Taiz & Zeiger, 2006). The electrons are excited again and then transferred to $NADP^+$ via ferredoxin (Fd) and ferredoxin $NADP^+$-reductase (FNR) to produce NADPH. The formation of NADPH initiates the proton flow back into the thylakoid space via ATP synthase/hydrolase complex ATPases, which synthesize ATP (Taiz & Zeiger, 2006). The NADPH and ATP produced by the light dependent reactions are used in the Calvin cycle, which converts CO_2 and water into organic compounds (sugars) (Taiz & Zeiger, 2006). The key enzyme of the cycle is Ribulose-1,5-bisphosphate carboxylase oxygenase, known as RuBisCo, which catalyzes carboxylation of rubulose-1,5-bisphosphate with carbon dioxide (Taiz & Zeiger, 2006).

Photosynthesis is impaired in salt stressed plants for several reasons. First, stomatal closure induced by osmotic stress restricts the availability of CO_2 for carboxylation reactions (Brugnoli and Bjorkman, 1992). It has been shown that CO_2 diffusion in the liquid phase from the mesophyll wall to the site of CO_2 reduction in chloroplast is also inhibited, which results in decreased CO_2 supply for photosynthesis (Flexas et al., 2004). Second, excessive salt in the photosynthetic tissues can cause swelling of thylakoids and decrease the number of grana stacks (Bruns & Hechtbuchholz, 1990; Mitsuya et al., 2000). The distortion of chloroplast membrane structure lowers overall photosynthetic rates (Bruns & Hechtbuchholz, 1990; Mitsuya et al., 2000). Thirdly, decreased leaf expansion resulting in a buildup of unused photosynthates in growing tissues may generate feedback signals to downregulate photosynthesis (Parida & Das,

2005). Therefore, measurement of photosynthesis can be used as an indicator of plants under salt stress.

Photosynthetic performance of plants can be evaluated indirectly through the measurement of chlorophyll fluorescence (Maxwell & Johnson, 2000). The light energy captured by chlorophyll in a leaf can be consumed in three ways: re-emission as light (chlorophyll α fluorescence), energy to drive photosynthesis, and heat dissipation. These three processes compete with each other. An increase in the efficiency of one will result in a decrease in the yield of the other two (Maxwell & Johnson, 2000). The variations of chlorophyll fluorescence originate mainly in the biophysical condition of PSII (Walz, 1993). For this study, chlorophyll α fluorescence was measured using pulse amplitude modulation fluorometry (PAM), which consists of a main control unit including various light sources, detectors and electronics hardware, special fiber optics and a data acquisition software (Walz, 1993). PAM provides fast assessment of the overall photosynthesis. Comparison of several chlorophyll α fluorescence parameters (e.g. F_v/F_m, yield, qP, and qN) can be used to assess the efficiency of photochemistry in plants under different conditions (Maxwell & Johnson, 2000; Walz, 1993).

The light sources available in PAM include the measuring light (< 0.4 μmol photon $m^{-2}s^{-1}$), actinic light (used to drive photosynthesis, approximately 300 μmol photon $m^{-2}s^{-1}$), saturation pulse (approximately 2000 μmol photon $m^{-2}s^{-1}$) and far-red light (used for stimulating PSI, 730 nm) (Walz, 1993). The biophysical condition of the PSII reaction center is related to the maximum and minimum fluorescence (F_m and F_o). Maximum fluorescence occurs when the PSII reaction centers are all closed (Maxwell & Johnson, 2000). Because no photochemistry takes place, the light energy is converted to fluorescence and heat (Maxwell & Johnson, 2000). The measurement of F_m and F_o is achieved by the adaptation of the leaf tissue in the dark. Following

dark adaptation, the minimum fluorescence value (F_o) of the sample can be measured by switching on the modulated measuring light, which is set low enough to prevent activation of the photosystem. Then a very strong pulse of white light (saturation pulse) is applied (Walz, 1993). PSII reaction centers are quickly exhausted by excited electrons that cannot be immediately transported away, and fully closed (Maxwell & Johnson, 2000). As photosynthesis is temporarily inhibited, chlorophyll fluorescence increases. The sample is exposed to enough light so all the closed reaction centers are full of photons, allowing the measurement of the maximum amount of fluorescence (F_m). The difference between F_m and F_o is the variable fluorescence (F_v). F_v/F_m provides a measure of PSII photochemical efficiency (Equation 1.2).

$$\text{Maximum quantum yield} = (F_m - F_o)/F_m = F_v/F_m \quad (1.2)$$

The parameter F_v/F_m is a measure of maximum quantum yield of PSII, or the potential quantum efficiency if all PSII centers are open (Maxwell & Johnson, 2000). The measurement of F_v/F_m provides information on the probability that a trapped photon will end up in the reaction center and cause a photochemical event. Any change in the state of PSII will cause a decrease in F_v/F_m (Maxwell and Johnson 2000). The optimal value of F_v/F_m varies between 0.79 to 0.83 for most plant species (Brugnoli & Lauteri, 1991); F_v/F_m is usually lower than the optimal value when plants are under stress.

After reaching to maximum value, the fluorescence level starts to decrease in a few minutes. This phenomenon is termed fluorescence quenching (Maxwell & Johnson, 2000). Two competing processes are distinguished. First, as the rate at which electrons are transported away from PSII increases, stomata are open, and the enzymes involved in carbon fixation are gradually activated, the fluorescence emission is quenched by photochemical energy conversion at the PSII centers, which is referred to as photochemical quenching (qP) (Maxwell & Johnson, 2000). On

the other hand, energy dissipation in the form of heat is also increased, which is termed non-photochemical quenching (qN) (Maxwell & Johnson, 2000). Typically, changes in these two processes will be complete within about 15-20 min and reach an approximate steady-state (Maxwell & Johnson, 2000). To quantify qP and qN, and the overall quantum yield of photochemical energy conversion, measurements in the steady illuminated state are most informative (Walz, 1993). An actinic light is applied and at appropriate intervals, further saturating flashes are applied. The maximum fluorescence in the light F_m' can be measured (Walz, 1993). A decrease in F_m' is usually linked with non-photochemistry (Walz, 1993). The steady-state value of fluorescence immediately prior to the flash is sampled and termed F_s (Walz, 1993). After a flash, actinic light is removed simultaneously with a switching-on of a far-red light, which allows the measurement of F_o' (Walz, 1993). The photochemical quenching parameters always relate to F_m' and F_s. The effective quantum yield (Yield, Equation 1.3) measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry, and gives an indication of the overall rate of electron transport (Maxwell & Johnson, 2000).

$$\text{Yield} = (F_m' - F_s)/\, F_m' = \Delta F\, /F_m' \quad (1.3)$$

The parameter qP (Equation 1.4), which is an indication of the proportion of PSII reaction centers that are open in illuminated state and equals the approximate oxidation of PSII (Maxwell & Johnson, 2000; Schreiber et al., 1986), is calculated as:

$$qP = (F_m' - F_s)/\,(F_m' - F_o') \quad (1.4)$$

The term qN (Equation 1.5) measures the non-photochemical quenching of fluorescence, which is related to the dissipation of energy as heat and indicates the extent of photoinhibition (Maxwell & Johnson, 2000; Schreiber et al., 1986).

$$qN = 1 - (F_m' - F_o)/(F_m - F_o) \quad (1.5)$$

1.5 Plant growth promoting rhizobacteria (PGPR) in plant salt stress alleviation

Studies have shown that the inoculating plants with PGPR can confer salt tolerance (Kohler et al., 2009; Nadeem et al., 2010). Several mechanisms have been proposed to explain how PGPR enhances the tolerance of plants to various abiotic stresses. These include the secretion of phytohormones (e.g. auxins and cytokinins), and the lowering of plant stress ethylene levels by the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al., 2007).

This study focuses on ACC-deaminase producing *Pseudomonads* and their roles in conferring salinity tolerance to selected plant species that have been applied in the phytoremediation of salt-impacted sites. Plants grown under high salinity usually produce higher than usual amounts of stress hormones such as ethylene (Glick et al., 2007). It has been shown that PGPR with ACC-deaminase activity act as a sink for ACC, which is the precursor to ethylene, and thereby lower plant stress ethylene conferring tolerance to plants (Figure 1.3) (Cheng et al., 2007; S. Mayak et al., 2004a; S. Mayak et al., 2004b). Soil-borne *Pseudomonads* have excellent root-colonization ability, exude useful enzymes, and have proven to be efficient bio-fertilizers (Glick, 1995; Vessey, 2003). ACC-deaminase producing *Pseudomonad* PGPR strains have been shown to effectively lower stress ethylene levels and help plants withstand salinity stress. For example, *Pseudomonas putida* UW3 and *Pseudomonas spp.* UW4 isolated from the University of Waterloo campus, and *Pseudomonas corrugata* CMH3 isolated from high salt-impacted soil from a farm in Saskatchewan were effective at conferring salt tolerance (Chang, 2008; Cheng et al., 2007; Mayak et al., 2004a; Mayak et al., 2004b). These physiological advantages show PGPR's protective role in plant acclimation to salt stress, and the

potential to enhance phytoremediation rates of salt-impacted soil. However, PGPR-induced proline accumulation, antioxidant enzyme activities and membrane integrity has not been extensively examined for plants that have been subjected to salt stress (Kohler et al., 2009; Nadeem et al., 2007; Paul & Nair, 2008). This proposal hypothesizes that PGPR can positively adjust these physiological indicators and help plant acclimate to saline environment.

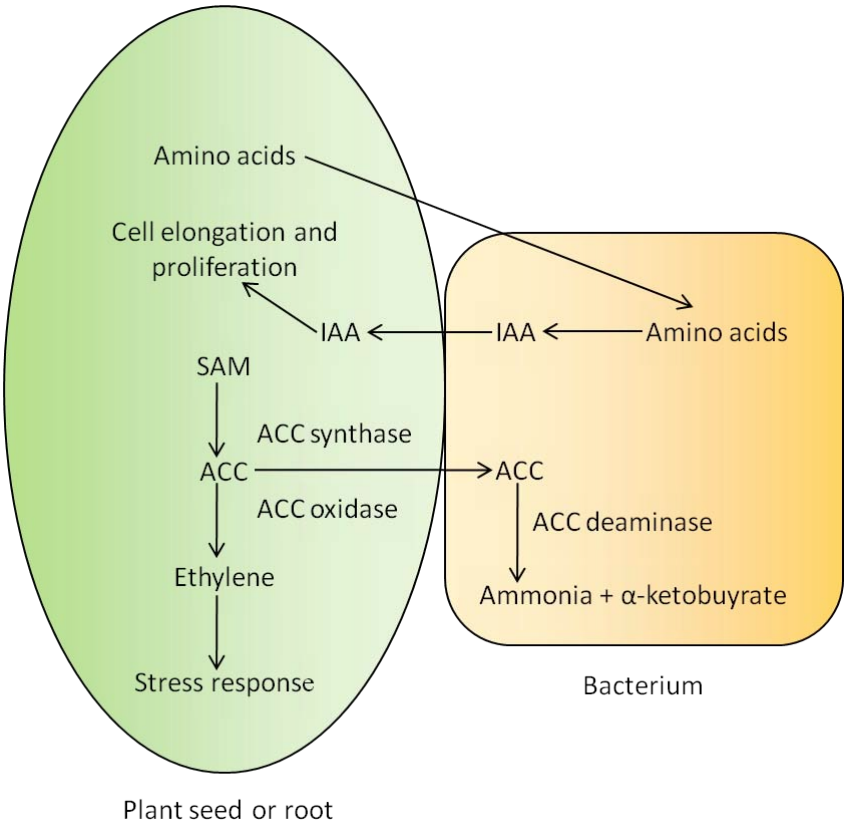


Figure 1.3: Schematic diagram of PGPR with ACC deaminase activity lower ethylene level in plant seed or root (Glick et al., 1998). ACC produced by plant can be transferred to the bacterium, where ACC deaminase of the bacterium degrades ACC to ammonia and α-ketobutyrate. Decreased level of ethylene can improve plant growth and thereby confer plant acclimation to stress. Some PGPR are also capable of producing phytohormones, IAA, which further stimulates plant growth by conferring plant cell proliferation as well as root elongation.

1.6 Protection from salt stress

To achieve better plant growth in saline soils, physiological processes can be directly manipulated. Successful examples include the exogenous application of proline, overexpression of Na^+/H^+ antiporters, and the up-regulation of antioxidant enzymes (Ashraf, 2009; Hoque, Okuma et al., 2007; Shi et al., 2000). Indirect methods, such as inoculation with plant growth promoting rhizobacteria (PGPR), can also be applied to neutralize negative physiological responses (Kohler et al., 2009; S. Mayak et al., 2004a).

1.7 Objectives

From the above, there are various important questions that need to be answered to improve phytoremediation of salt-impacted soil. First, it is not clear what happens to the salt during phytoremediation. Salt levels have been shown to decline in surface soil has been observed; however, how the decreased amount of salt accumulates in between plant tissues and in the soil horizons remains unknown. Second, it is unclear how tolerant the commonly used species PGPR *Pseudomonas corrugata* CMH3 is to salt. Third, a series of physiological changes, such as proline accumulation, membrane leakage and photosynthesis activity, behind plant acclimation to salt stress with PGPR treatment are not well understood,

Based on previous work that frequently observed the enhanced biomass accumulation of plants in association with PGPR under salt stress and unsolved questions mentioned above, this study will focus first on salt mass balance in the soil profile and plant tissues. Secondly, the salt tolerance ability of the PGPR strain applied to the seeds will be examined. Thirdly, the PGPR enhancement of plant physiological performances and biomass production will be studied. Thus, the hypotheses of this study are:

(1) Plant uptake of NaCl plays a major part and contributes more than leaching in the surface soil salinity decrease during phytoremediation of salt-impacted soil.

(2) Both *Pseudomonas spp.* UW4 and *Pseudomonas corrugata* CMH3 can grow well in high saline media.

(3) PGPR can enhance proline accumulation, SOD activity, membrane integrity and photosynthesis in salt stressed plants.

In order to test the hypotheses, the objectives of this study are to

(1) Perform a salt mass balance study by calculating the amount of salt accumulated in plant tissue and in different soil layers to evaluate the fate of salt during phytoremediation.

(2) Assess the salt tolerance of a commonly used PGPR *Pseudomonas corrugata* CMH3 isolated from a salt-impacted site (EC_e of 35 dS/m $\approx$ 2% NaCl solution) in comparison to *Pseudomonas spp.* UW4, isolated from campus soil, by examining their growth in saline media;

(3) Evaluate the effect of salinity and PGPR treatment on plant physiological indicators by measuring the changes in proline contents, membrane leakage and SOD levels, and photosynthesis.

Chapter 2 – Material and methods

2.1 Salt mass balance studies

Three salt-impacted sites in Saskatchewan were used for a salt mass balance study. Soil salinities and plant salt uptake were monitored during phytoremediation. Salt mass balance calculations were performed based on data collected in the 2009 field season.

2.1.1 Salt-impacted sites and sampling maps

The Cannington Manor North (CMN) and Cannington Manor South (CMS) sites are located near Carlyle, Saskatchewan, Canada. Salt contamination was caused by the leakage of brine water storage tanks resulting in the spread of brine water over a large area (Figure 2.1).

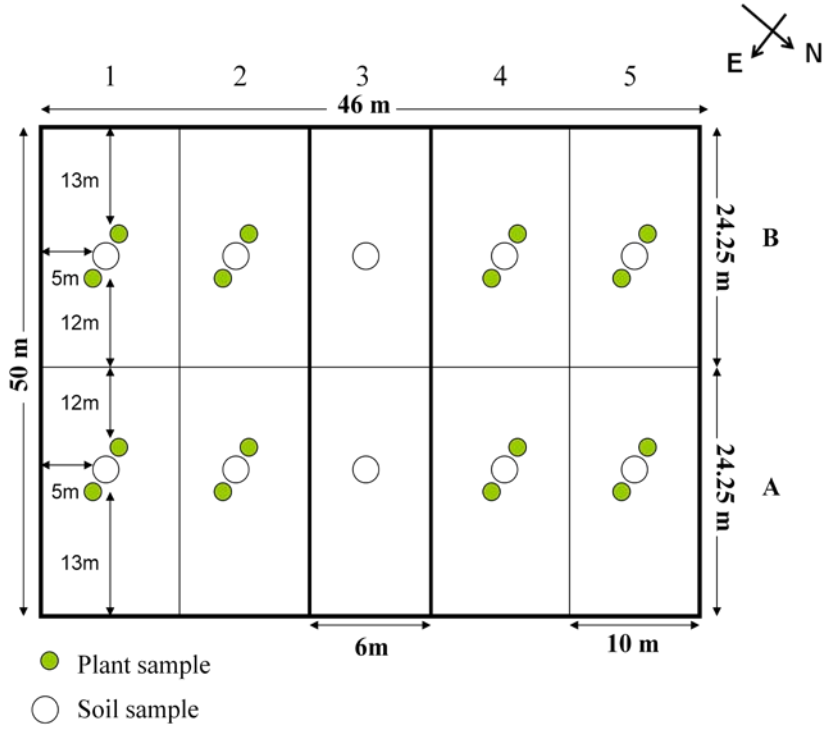


Figure 2.1: The dimensions and sampling map of the Cannington Manor North (CMN) site. The rectangular shaped site is divided into 5 sections (1, 2, 3, 4, 5), and each is sub-divided into 2 sub-sections (A and B). The middle strip (plots 3A and 3B) was left unplanted to serve as a control area (blank). Plant samples (green dots) are taken in August and October; soil samples (white dots) are taken in June and October in three depths (0-25 cm, 25-50 cm and 50-75 cm), and August in one depth (0-25 cm).

The CMS site, which is 400 m south of the CMN site, has a low area on the east portion of the site where frequent flooding occures in the spring. It was heavily impacted by a brine water spill and the resulting soil salinity levels were much higher than CMN (Figure 2.2). Gypsum ($CaSO_4$) and manure were applied to both CMN and CMS sites before planting in May 2007.

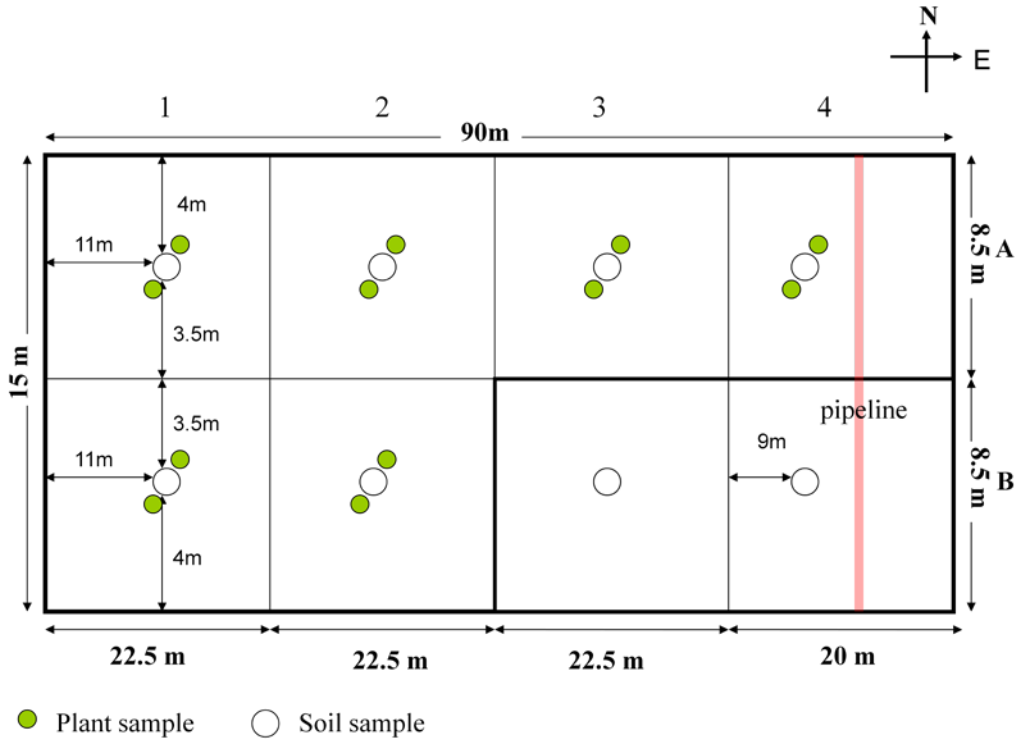


Figure 2.2: The dimensions and sampling map of the Cannington Manor South (CMS) site in the 2009 growing season. The site is divided into 4 sections (1, 2, 3, and 4), and each is further sub-divided into 2 sub-sections (A and B). The southeast corner (plots 3B and 4B) of the site is set up as the unplanted control area (Blank). The vertical line across section 4 indicates the buried pipeline, which requires sampling point shift to avoid touching the pipeline.

The Alameda site (AL) is located near Alameda, Saskatchewan. The source of salt contamination was from leakage of one of the lines that attaches to the brine tanks (Figure 2.3). A 4-inch layer of manure was mixed into the surface soil of this site in May 2007. The average

EC_e, 31 dS/m (ranging from 19 to 52 dS/m), was the highest of the three sites before planting in 2009.

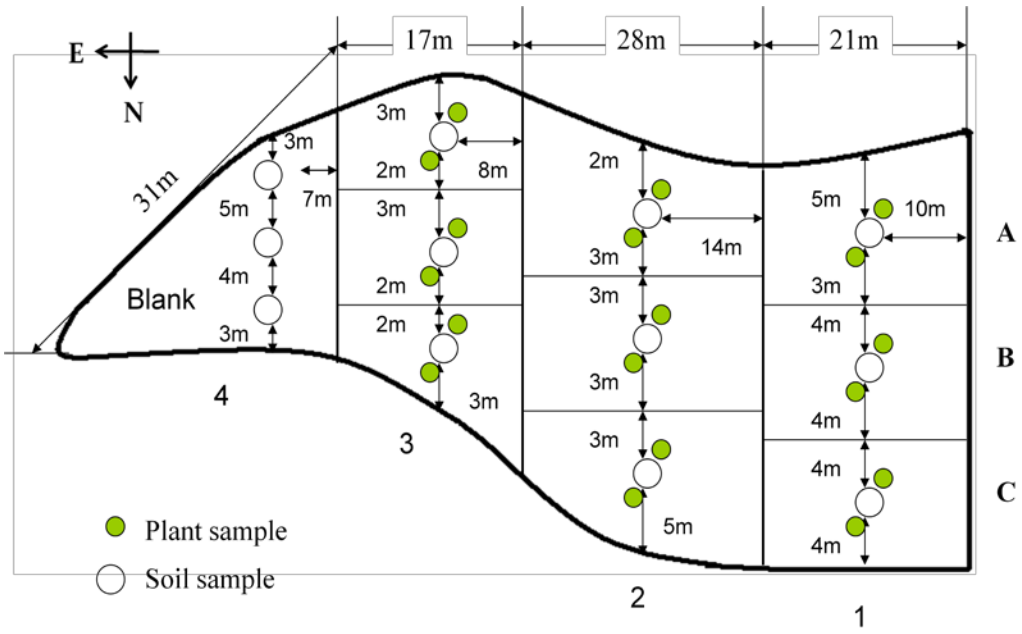


Figure 2.3: The dimensions and sampling map of the Alameda site in the 2009 growing season. The irregularly-shaped site is divided into 4 sections, each of which is further sub-divided into 3 sections (A, B, and C). Plot 4 is set up as a blank with no seeds being planted. Soil samples are taken from each plot only from the surface layer (0-25cm) due to the unknown pipeline positions underneath.

2.1.2 Seed treatment with PGPR

Seed treatment with PGPR was performed as previously published methods (Gerhardt et al., 2009). The bacterial culture of CMH3 grown in TSB was transferred aseptically into a sterile 50 mL Falcon tube, centrifuged at 2000 r.p.m. for 20 minutes. The cell pellets were washed and resuspended with 50 mL of ddH_2O (de-ionized and distilled water) to remove secondary metabolites, and centrifuged again at 2000 r.p.m. for 20 minutes. The final bacterial pellet was resuspended in sterile ddH_2O to an absorbance of 2.0 at 600nm.

Methylcellulose (Sigma, Oakville, Canada) was also added to facilitate adhesion of the

bacterial cells to the seed surface. To prepare the polymer, 15.0 g of methylcellulose powder (Sigma, Oakville, Canada) was dissolved in 1.0 L of ddH_2O and stirred for one hour. The solution was then autoclaved for at 121º C and 15 psi for 30 minutes, after which a gelatinous solid formed. Upon cooling, the gel liquefied (reverse-gelatinisation) into a slurry form. This polymer was added to the bacterial suspension at a rate of 200 mL per liter of bacterial suspension. A commercial non-toxic blue colorant (Color Coat Blue, Becker Underwood, Saskatchewan) was added into the bacterial-polymer slurry at a ratio of 17.5 mL to 1 L of slurry. The presence of colorant was necessary to meet safety regulations requiring all treated seeds to be visibly colored to avoid animal consumption.

An aliquot of 10 mL of the blue bacterial-polymer slurry were applied to 2.5 L equivalent of seeds of oats, and 20 mL to 2.5 L grasses using a seed treater (HEGE 11, Wintersteiger Inc., Austria) and the machine ran for two minutes. The dried seeds were immediately transferred into bags ready for use in both field remediation and greenhouse studies.

2.1.3 Soil sampling and EC determination

Soil samples were taken from the field sites at the beginning of the planting season (June), midseason (August), and end of season (October). Using an auger to obtain soil samples, a composite sample made up of 3 soil cores was taken from each plot at 3 depths (0-25cm and 25-50cm, 50-75cm). Approximately 500 mL of soil was placed in plastic bags and stored in coolers until they were tested for soil salinity in the laboratory.

Soil samples were air dried to remove moisture, ground, and then sieved through a 2 mm particle size sieve. Soil salinity ($EC_{1:2}$) was measured by mixing an aliquot of 15 g of soil with 30 mL double-deionized H_2O (ddH_2O) in a 50 mL plastic culture tube (Chang, 2008). The

mixture was shaken at 80 r.p.m. for 30 minutes before centrifugation at 2000 r.p.m. for 10 minutes. The $EC_{1:2}$ of the supernatant was measured with a conductivity meter (Oakton Instruments, Vernon Hills, IL, US). EC_e of the samples were determined using Equation 1.1 (Wu, 2009).

Samples with a range of $EC_{1:2}$ values from each site were chosen for EC_e measurement and K value determination. An aliquot of 50 g soil was mixed with sufficient ddH_2O in a 100 mL jar to reach saturation. The characteristics required for saturation include: (1) a shiny appearance of the soil paste; (2) the paste flowed slightly when dispersion was made in the surface; and (3) soil paste slided cleanly from an aluminum spatula. The sample was allowed to settle for at least 4 hours and checked again to ensure the saturation criteria were met. If free water was observed on the surface, a small amount of soil would be added and the paste remixed. If the soil was stiffened or dried, ddH_2O would be added and the paste remixed. This was repeated until all saturation criteria were met. The mixture was transferred to a 50 mL Falcon sterile culture tube and centrifuged at 2000 r.p.m. for 10 minutes. The electrical conductivity of the supernatant was then measured with the bench Conductivity Meter (Oakton Instruments, Vernon Hills, IL, US) (Wu, 2009).

2.1.4 Plant sampling and tissue salt level determination

In this project, four plant species, oats (*Avena sativa*), tall fescue (*Festuca arundinacea*), tall wheatgrass (*Agropyron elongatum*) and annual ryegrass (*Lolium multiflorum*) were used. They were selected because they are moderately salt-tolerant species and were native to Saskatchewan. The PGPR, *Pseudomonas corrugata* CMH3 (CMH3), which was isolated from CMS site, was used in seed treatment prior to planting. The mixture of oat and grass seeds treated with CMH3

was planted at the beginning of the season (June 2009) and sampled at midseason and the end of the season.

Table 2.1: A summary of seed planting information in three salt-impacted sites in Saskatchewan in 2009.

Salt-impacted sites	CMH3-treated seeds (lbs)			
	Oat	Tall fescue	Tall wheatgrass	Annual ryegrass
Cannington Manor North (CMN)	125	50	——	60
Cannington Manor South (CMS)	100	50	50	——
Alameda	100	50	50	——

All of the plant species in table 2.1 are common to southeast Saskatchewan, and are known to be moderately to highly salt tolerant (Miyamoto et al., 2004; USDA-ARS, 2009). At CMN, where the initial EC_e (3.7 dS/m) was the lowest of the three research sites, annual ryegrass was planted instead of tall wheatgrasses (Table 2.1). Compared to tall wheatgrass, annual ryegrass is less tolerant to high salinity, but usually yields more biomass at moderate salt levels (Greenberg, unpublished data). However, in high salt-impacted sites, such as CMS and Alameda where plant establishment was severely inhibited and germination rates became the principal determinant for biomass production, tall wheatgrass was a more favorable candidate for phytoremediation.

Plants were sampled by harvesting the above ground biomass within a 50 cm × 50 cm square frame. Two plant samples were taken from each plot, with one exactly on top of the soil sampling point, and the other within a 1 m distance of the soil sampling point. Plant samples were kept in coolers until analysis could be performed at the University of Waterloo for analysis. All samples were washed three times with deionized water and air dried for seven days prior to dry weight measurement. Dry plant tissues were sent to an accredited lab ALS Environmental

Inc. (Waterloo, ON) for the analyses of sodium and chloride contents. Considerable amount of salt loss might be resulted from washing. The assessment of salt loss due to sample washing was stated in the result section 3.2. Sodium concentrations were determined using the method USEPA 6020 method, which stipulated that plant tissues should be completely digested in nitric acid and analyzed by ICP-MS (Inductively Coupled Plasma Mass Spectroscopy). Measurement of soluble chloride was performed using IC (Ion Chromatography) according to APHA method 4110B. Salt mass in plant tissues were determined by salt ion concentrations (mg/kg plant dry weight) times plant dry biomass.

2.1.5 Salt balance and remediation efficiency

In the arid regions of Saskatchewan, salt could migrate vertically in the soil profile. For the planted area, assuming that salt runoff from the site was minimal, soil salinity changes would be equal to the difference between the EC_e at the beginning of the season and EC_e at the end of the season. An EC_e decrease in the surface soil could be due to leaching of salt by precipitation and plant extraction. Only plant uptake will permanently remove salt from the surface soil. The remediation efficacy was therefore calculated by the percentage of plant derived change of EC_e out of the total change of surface soil EC_e.

The plant derived decrease of EC_e can be estimated from the theoretical ion strength (*IS*) in plant tissues.

$$IS = \sum_{i=1}^{n} c_i z_i^2 \quad (2.1)$$

In Equation 2.1, c_i represents molar concentration of a specific ion (mmol/L); z represents the magnitude of the charge of the ion. Due to z being squared, divalent ions like calcium would contribute more to ionic strength than monovalent ions such as sodium and chloride. The ionic

strength (*IS*) was expressed in terms of mol/L and was the sum of the contributions of all ions.

The ions accumulated in above ground plant tissues were analyzed by ALS Laboratories Inc., which returned ppm values (mg ion per kg plant tissue). Using the total biomass production, the mass of salt ions taken up by plants in the entire field was calculated, and then converted to the number of mmol of ions.

These ions had been stored in field's rooting zone, defined as the top 25 cm of the entire field, before being taken up by plants. The volume of water required to measure $EC_{1:2}$ of these ions was calculated by soil mass in top 25 cm of the site times 2, according the established method of $EC_{1:2}$ measurements (see 2.1.3).

The total mass of the soil in one plot could be calculated by soil bulk density (ρ) times plot area (A) times sampling depth (h):

$$1\ m^2 = 10\ 000\ cm^2;\ 1\ g = 0.001\ kg; \mathrm{h} = 25\ \mathrm{cm}$$

$$kg\ soil = \rho Ah = \rho\ (g/cm^2\ ) \times A\ (m^2) \times h\ (cm) = 250\rho A\ (kg\ soil) \quad (2.2)$$

Therefore, the amount of water needed for the measurement of $EC_{1:2}$ was $500\rho A$ (L). The molar concentrations of ions (c_i), such as sodium, calcium, potassium and chloride, could then be calculated respectively by the Equation 2.3.

$$c_i = \frac{mmol\ \text{of ion in plant}}{500\rho A} \quad (2.3)$$

The theoretical ionic strength in the plant tissues could then be calculated by Equation 2.1.

According to Alva et al, 1991, electrical conductivity could be related to ionic strength by the Equation 2.4. In this equation, the ionic strength (*IS*) was expressed in terms of mmol/L.

$$IS = 0.013 \times EC_{1:2} \quad (2.4)$$

The decrease in $EC_{1:2}$ as a result of plant uptake could then be calculated by the rearranged

Equation 2.4.

$$EC_{1:2\ plant} = \frac{IS}{0.013} \quad (2.5)$$

This value was then multiplied by the previously determined K value of each site to find the equivalent change in $EC_{e\ plant}$. Remediation efficacy of field sties was therefore calculated by dividing the calculated $EC_{e\ plant}$ (Table 3.2) by the measured change in surface soil EC_e (Equation 2.6).

$$\text{Remediation efficacy} = \frac{EC_{e\ plant}}{Soil\ EC_e\ decrease} \quad (2.6)$$

2.2 PGPR salt tolerance

The PGPR *Pseudomonas corrugata* CMH3 and *Pseudomonas spp.* UW4 were cultured in Tryptic Soy Broth (TSB) spiked with 0% and 2% sodium chloride (w/v) (EC_e=34.9 dS/m), higher than the EC_e value of the site where CMH3 was isolated (Chang, 2008). All greenhouse and laboratory experiments performed with CMH3 and UW4 in this project were at salinities lower than this level. The bacterial cultures were grown at room temperature (23±1 ºC) on a rotary shaker (170 r.p.m.) for 28 hours. Optical density readings at 600 nm (OD_{600}) were taken every hour to assess the growth of bacteria under salt stress (Wu, 2009).

2.3 Physiochemical indicators under salt stress

Topsoil was generously donated by the Parkview Cemetery & Crematorium in Waterloo (EC_e=0.5 dS/m). Diluted salt-impacted soil was prepared by mixing the salt-impacted soil from the CMS site with topsoil to reach an EC_e of 10 dS/m. Spiked salt soil was prepared by mechanically mixing NaCl powder into topsoil to an EC_e of 10 dS/m. Pots containing un-impacted soil were set up as positive control (Table 2.2).

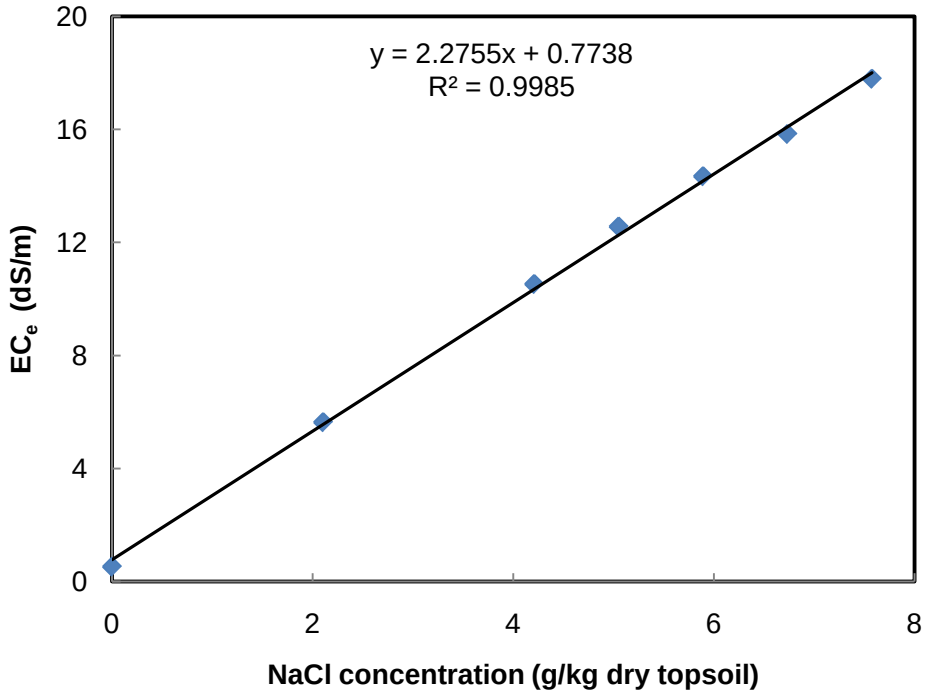


Figure 2.4: The relationship between the concentrations of NaCl solutions added into the salt treated pots and soil EC.

PGPR-treated or untreated annual ryegrass seeds were planted in pots without holes at the bottom to ensure that salt was not leached out by watering. Seeds were evenly spread out to reach a seeding rate of 0.0362 g/cm^2, and a thin layer of sieved soil was applied to cover the seeds. All pots were randomized in trays and placed in the greenhouse. The day time temperature ranged from 25 to 35 ºC and the night time temperature ranged from 18 to 27 ºC. Plants were irrigated once or twice before germination and irrigated daily to the soil field capacity after germination. Lighting source was natural sun light with no supplemental lighting.

Plants were sampled 9, 12, 15, and 18 days after germination. Fresh leaves were collected and chopped to approximately 1 cm segments for further physiological assays (Table 2.2).

Table 2.2: A summary of greenhouse trial design for physiological assays.

Factors	Treatments
Plant species	Annual ryegrass (*Lolium multiflorum*)
Salinity	Topsoil (0.5 dS/m)
	NaCl spiked soil (10 dS/m)
	Diluted salt-impacted soil (10 dS/m)
PGPR	-PGPR
	Pseudomonas putida UW4
	Pseudomonas corrugata CMH3
Time points	9, 12, 15, 18 days after the germination of control plants
Replicates	3 replicates for each trial. Repeat the trial three times (November, December and January)

2.3.1 Determination of free proline

Free proline content was determined following the method proposed by Bates et al. (1973). Proline quantification will be based on the formation of a brick red coloured proline–ninhydrin complex in an acidic medium. A plant sample (0.2 g) was homogenized in 1 mL of sulfosalicylic acid (3%) using a mortar and pestle, the homogenate was centrifuged for 5 minutes, and the supernatant was used to quantify proline. An aliquot of 100 µl extract was mixed with 100 µl of 3% sulfosalicylic acid, 200 µl glacial acetic acid, and 200 µl acidic ninhydrin, and heated at 100 ºC for 1 hour. Toluene (1 mL) was added to the reaction mixture. The tube with the reaction mixture was vortexed for 20 seconds and allowed to sit on bench for 5 minutes to allow the separation of the organic and water phases. The chromophore containing toluene was isolated (organic phase), and absorbance at 520 nm was read using a spectrophotometer, and this was compared to a toluene blank. The concentration of proline was estimated by referring to a standard curve made using solutions of known concentrations of proline.

2.3.2 Assessment of membrane leakage

The ion leakage measurement was modified from previously published procedures (Bajji et al., 2002; Campos et al., 2003). Fresh leaf tissues (0.5 g fresh weight) were cut into approximately 1 cm long segments, rinsed with ddH_2O, and blot-dried with Kimwipe. Segments were submerged in 10 mL of ddH_2O in a 50 mL centrifuge tube, which was placed into a vacuum dessiccator. The samples were subjected to a vacuum at a rate of 100 L/min for 2 hours using a vacuum pump (Savant, VP 100, New York, USA). An EC value of the solution was then measured at room temperature of 23±1 °C using an electrical-conductivity meter (Oakton Instruments, IL).

2.3.3 Superoxide dismutase assay

The assay for the estimation of SOD activity was based on the formation of a blue coloured formazone by nitro-blue tetrazolium and O_2^- radical, which absorbed light at the 560 nm wavelength. The presence of the enzyme, SOD, decreased the generation of $O_2^{\cdot -}$ radical and consequently resulted in a decreased absorbance (Dhindsa et al., 1981). Three millilitres of the reaction mixture containing 13.33 mM methionine, 75 μM nitroblue tetrazolium chloride, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, 0.05 mL enzyme extract. The reaction was started by adding 2 μM riboflavin and placing the test tubes under three 15 W fluorescent light tubes for 15 minutes. A complete reaction mixture without enzymes, which gave the maximum color, served as the control. The reaction was stopped by switching off the light and placing the test tubes in the dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme which reduced the absorbance reading to 50% in comparison with test

tubes lacking enzyme (Dhindsa et al., 1981).

2.3.4 PAM measurement

Chlorophyll *a* fluorescence was measured with pulse amplitude modulated (PAM) fluorometer (PAM-2100, Heinz Walz GmbH, Eichenring, Germany). Annual ryegrass was grown under two salinity levels, diluted salt-impacted soil (10 dS/m) and un-impacted topsoil (0.5 dS/m) for 18 days (sowing method and greenhouse conditions were described in Section 2.5).

Whole plants were dark adapted for 30 min prior to PAM analyses to ensure all PSII reaction centers were open. PAM measurements were made on attached leaves with the aid of a 0.8 cm diameter fiber optic cable. The minimal fluorescence in dark-adapted tissue, F_o, was adjusted to 0.400 ± 0.040 by changing the fluence rate of the measuring light (gain) (Abogadallah, 2010; Babu et al., 2001). The maximum fluorescence in dark-adapted tissue, F_m, was measured by a single non-modulated saturating 0.6 second light pulse (2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of Photosynthetically Active Radiation). After 30 seconds, fluorescence in steady state, F_s, was measured using the non-modulated 640-700nm actinic radiation (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 14 minutes after the fluorescence reached steady state (Lees, 2006; Ueckermann, 2008). A single non-modulated saturating 0.6 s light pulse was triggered every minute to measure the maximum fluorescence during steady state photosynthesis, F_m', in the presence of actinic light (Lees, 2006; Ueckermann, 2008). The PAM parameters derived first were F_v/F_m (maximum photosystem II [PSII] activity) followed by photochemical quenching (qP; net energy storage), non-photochemical quenching (qN; heat dissipation), and Yield (PSII activity at steady state). These

parameters were all calculated using PamWin software (PC software PamWin V 2.00, Heinz Walz GmbH, Germany).

2.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc). Data of soil salinity change over the 2009 season were analyzed using one-way ANOVA followed by the post-hoc Tukey test at $P < 0.05$. Study for salt washing loss was analyzed by T-test at $P < 0.05$. Plant fresh weight, physiological parameter change over time for annual ryegrass, such as SOD, proline, membrane leakage and PAM results were analyzed by two-way AVONA followed by Bonferroni post-tests to compare PGPR treatment effect. Assumptions below were met (1) the samples being tested are independent; (2) the samples has a normal distribution with unknown mean μi; (3) all of the samples have the same standard deviation σ.

Chapter 3 – Results

3.1 Salt mass balance investigation

During 2009 planting season, soil and plant samples from three sites were analyzed, and salt mass balance calculations were carried out.

3.1.1 The Cannington Manor North site

The average EC_e of the surface soil was 3.7 dS/m (ranging from 2.2 to 5.8 dS/m) at CMN prior to planting in June 2009. All plots were planted with a mixture of CMH3-treated oats, annual ryegrass and tall fescue, except the blank area, which was sprayed with the herbicide glyphosate twice during growing season to control for weeds. Planted plots had 100% plant coverage by midseason (August 13, 2009) (Figure 3.1).

	1	2	Blank	4	5	
0-25cm	4.6	2.8	5.0	2.2	5.8	
	3.2	1.8	3.1	1.7	2.7	
25-50cm	5.7	4.7	7.3	1.8	8.6	B
	9.1	4.0	7.4	2.0	7.5	
50-75cm	5.1	3.7	6.9	2.1	6.4	
	6.3	3.6	5.8	1.7	8.0	
0-25cm	2.8	2.2	2.8	4.4	4.8	
	3.3	1.4	2.1	7.7	3.2	
25-50cm	3.4	2.6	4.4	7.3	7.0	A
	3.2	4.5	3.5	13.4	9.8	
50-75cm	3.2	2.3	4.1	6.0	5.3	
	3.9	4.3	5.7	9.4	8.0	
0-25cm	3.7	2.5	3.9	3.3	5.3	
	3.2	1.6	2.6	4.7	3.0	
25-50cm	4.6	3.6	5.9	4.6	7.8	
	6.1	4.2	5.4	7.7	8.6	
50-75cm	4.1	3.0	5.5	4.0	5.9	
	5.1	4.0	5.7	5.5	8.0	

Figure 3.1: Soil EC_e in dS/m of CMN site by sampling in three depths in 2009 growing season. The first row for each depth shows EC_e at the beginning of the season (June 4, 2009) prior to planting; the second row shows EC_e at the end of the season (October 14, 2009). Numbers below the map indicate average EC_e for each plot. Green shaded area (plot 1, 2, 4, 5) indicates midseason plant coverage.

At midseason, about 40% of the blank area was covered by weeds, which were removed two weeks later by the second round of glyphosate application (Figure 3.2).

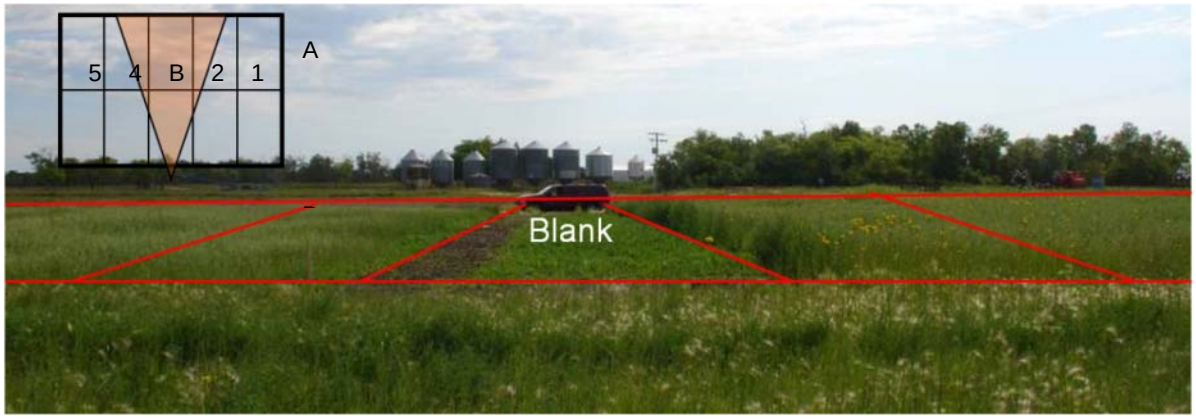


Figure 3.2: An overview of plant growth at midseason (August) in CMN. The plots were lined out and labeled with 1, 2, blank, 4, and 5. The map in the top left corner shows direction from which the image was taken. “B” indicates the blank area.

In the planted area, the soil EC_e increased by midseason when plants were in their active growth period (Figure 3.3). However, a 16% decrease in EC_e was observed at the end of the season relative to the beginning of the season. In the blank area, a negligible change in EC_e was observed at midseason. At the end of the season the EC_e decreased by 33% in the plots without plants relative to the beginning of the season.

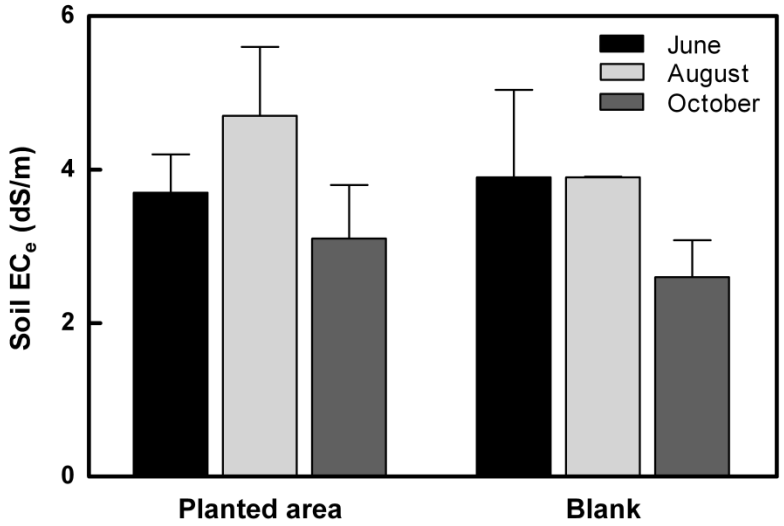


Figure 3.3: CMN 0-25cm soil EC_e seasonal change (June, August, and October) in both planted and blank areas. Error bars indicate the standard error.

In surface soil (0-25 cm), salinity decreased from 3.69 dS/m to 3.13 dS/m; however, at both the 25-50 cm and 50-75 cm depths salinity increased (Figure 3.4), which may be the result of leached solutes from the surface moving down into lower soil horizons or variation among samples.

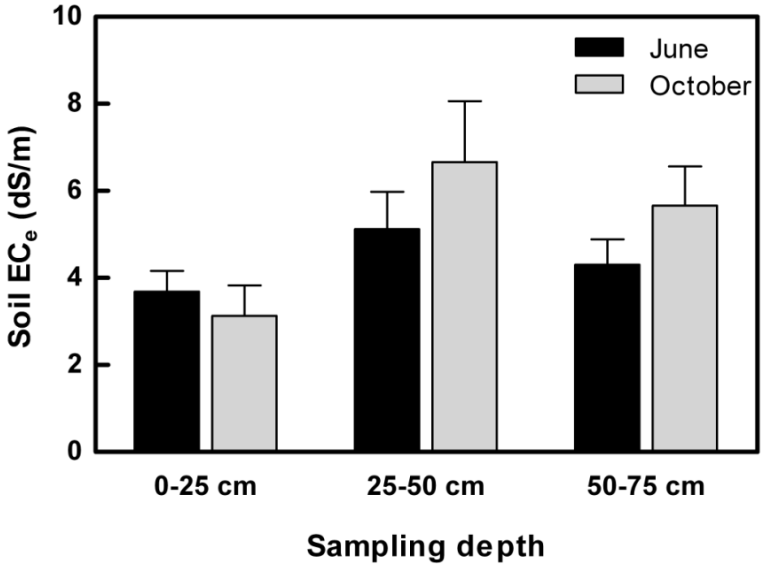


Figure 3.4: CMN soil EC_e changes (June to October) at three sampling depths (0-25 cm, 25-50 cm, 50-75 cm) in the planted area. Error bars indicate the standard error.

The mass of sodium and chloride accumulated in plants in the entire field were calculated using the plant dry weight (Table 3.1). All the water soluble ions in the soil contribute to the conductivity of a soil extract, so all the ions taken up by plants should be considered when the surface soil EC_e decrease caused by plant uptake of NaCl and the remediation efficacy were assessed. However, in the season 2009, only sodium and chloride concentrations in plant tissues were analyzed.

Table 3.1: Plant biomass and salt ion uptake in plant tissues in different plots of CMN.

	Plot	Plant DW[1] (g/m^2)	Plot area (m^2)	Plant DW[2] (kg)	Na^3 (mg/kg)	Na^4 (kg)	Cl^3 (mg/kg)	Cl^4 (kg)	NaCl[5] (kg)
Midseason	1	760	500	380	4405	1.67	23100	8.97	10.64
	2	340	500	170	4070	0.69	21450	3.25	3.94
	4	313	485	152	3955	0.60	14650	2.55	3.15
	5	413	485	200	8810	1.77	30400	7.65	9.42
	Total	——	1970	902	——	4.73	——	22.42	27.15
End season	1	160	500	80	2445	0.20	6025	0.48	0.68
	2	124	500	62	2105	0.13	6540	0.41	0.54
	4	136	485	66	1820	0.12	11080	0.71	0.83
	5	128	485	62	1450	0.09	3565	0.22	0.31
	Total	——	1970	270	——	0.54	——	1.84	2.38
Season 2009	Total	——	1970	1172	——	5.27	——	24.26	29.53

1. DW stands for dry weight
2. Plant DW (kg) of each plot was calculated by Plant DW(g/m2) × Plot area (m2).
3. Raw data of plant Na^+ and Cl^- contents were in mg per kg plant dry weight.
4. Na^+ or Cl^- mass in each plot were calculated by Na^+ or Cl^- (mg/kg plant DW) × kg Plant DW.
5. NaCl uptake for the whole site is calculated by adding up total Na^+ and Cl^- mass.

Soil mass of the surface 25 cm of the entire site was calculated by Equation 2.2:

$$M_{soil} = 250\rho A \text{ (kg)} = 250\times1.01\times1970 \text{ (kg)} = 4.97\times10^5 \text{ (kg)}$$

The volume of water needed to measure $EC_{1:2}$ was calculated by using the soil mass times 2, according to the method of $EC_{1:2}$ measurement:

$$V_{water} = 4.97\times10^5 \text{ (L)}\times2 = 9.95\times10^5 \text{(L)}$$

Table 3.2: Ion strength and $EC_{e\ plant}$ calculation.

Na (mol)	Na^1 (mmol/L)	Cl (mol)	Cl^1 (mmol/L)	IS^2 (mmol/L)	$EC_{1:2\ plant}{}^3$ (dS/m)	$EC_{e\ plant}{}^3$ (dS/m)
229	0.2303	683	0.6869	0.9172	0.0706	0.1411

1. The mass of sodium and chloride were converted to number of mol of ions, which was then divided by V_{water} to obtain molar concentration values of sodium and chloride (c_i) according to Equation 2.3.
2. IS was calculated by Equation 2.1.
3. $EC_{1:2plant}$ was calculated by Equation 2.5, and multiplied by CMN surface soil K value 2.0, to obtain $EC_{e\ plant}$.

Change in soil salinity was calculated by subtracting the EC_e at the beginning of the season from the EC_e at the end of the season:

$$\text{Soil } EC_e \text{ decrease} = 3.69 \text{ dS/m} - 3.13 \text{ dS/m} = 0.56 \text{ dS/m}$$

Remediation efficacy was determined by dividing the calculated $EC_{e\ plant}$ (Table 3.2) by the measured change in surface soil EC_e.

$$\text{Remediation efficacy} = \frac{0.1411 \text{ dS/m}}{0.56 \text{ dS/m}} \times100\% = 25.2\%$$

Therefore, plant uptake accounted for 25.2% of the salinity decrease in the surface soil.

However, salt may have been lost when plants were washed with deionized water. Rinsing plants in the water not only removed the dirt, but also could have washed away the salt that has been secreted to the surface of the leaves. Moreover, because plants were cut during sampling

process, washing would exacerbate the salt loss from the cutting wounds. During the first year (2007) of phytoremediation of these sites, plant samples were not washed before analysis. Plants accumulated much higher amount of salt (mg NaCl/kg plant dry weight) than 2009. Plant uptake was 46.1 g NaCl/kg plant dry weight in CMS site in 2007 (Chang, 2008), whereas it was 36.1 g NaCl/kg plant dry weight in 2009. Therefore, plant salt uptake in CMN and CMS sites might have been largely underestimated.

To estimate the amount of salt that was lost by washing, six plant samples from three salt-impacted sites near Weyburn, Saskatchewan, and three plant samples from one site near Red Earth (RE), Alberta, were selected to test the effect of washing plant material on salt content. Soil from both Weyburn and Red Earth sites was determined to be sandy loam to loam soil, which is the same as both the CMN and CMS sites. Plant samples from where salinity was high (>10 dS/m), moderate (about 8 dS/m) and low (<5 dS/m) were selected. Each of these plant samples was split into two halves. One half was washed the same way as CMN and CMS samples were washed, and the other half was not washed. Both the washed and unwashed samples were sent to ALS for sodium and chloride concentration analysis. Results were listed in Table 3.3.

Table 3.3: Sodium and chloride concentrations of washed and unwashed plant samples from salt-impacted sites in Red Earth (RE) and Weyburn.

Site	Sample ID	Washed Na(mg/kg)	Unwashed Na(mg/kg)	Percent Na loss[3]	Washed Cl (mg/kg)	Unwashed Cl (mg/kg)	Percent Cl loss[3]
Red Earth[1]	1	2370	5170	54.2%	9160	25300	63.8%
	2	913	1280	28.7%	5820	23800	75.5%
	3	555	1270	56.3%	6210	21300	70.8%
Weyburn[2]	1-1	1580	6380	75.2%	7430	14000	46.9%
	1-2	1780	2890	38.4%	5410	11800	54.2%
	1-3	1800	2680	32.8%	3420	11600	70.5%
	2-1	2540	4260	40.4%	13800	40800	66.2%
	2-2	1610	4400	63.4%	12200	37200	67.2%
	3-1	2990	3330	10.2%	8980	21900	59.0%
	Average	1793	3518	44.4%	8048	23078	63.8%

1. Red Earth samples are labeled by the sampling number.
2. Weyburn samples are labeled as site number (1, 2, and 3) followed by the sampling number.
3. Percent Na and Cl loss were calculated by subtracting 100% from $[Na]_{washed}/[Na]_{unwashed}$

Though the Red Earth site is located in Alberta, and Weybern sites are in Saskatchewan, the difference of the average loss of ions in washed plant samples from these two sites was insignificant ($P<0.05$) (See Appendix Figure 1). Therefore, the location of the sites did not significantly affect the percent ion loss during plant tissue washing. This suggested that the average ion loss data obtained from Table 3.3 could be applied to the adjustment of remediation efficacy in Cannington Manor sites.

The sodium and chloride concentrations in washed and unwashed plant samples were averaged, respectively. Both sodium and chloride concentrations in plant tissues were significantly decreased ($P<0.05$) as a result of washing (Figure 3.5). The percentage of Na^+ and Cl^- that was lost due to washing could be determined by subtracting 100% from the concentrations in washed samples divided by the corresponding concentrations in unwashed samples. The percentage of salt loss was then calculated by using 100% minus the percent of salt

remained in plants. The mean Na^+ loss was 44.4%, and Cl^- loss was 63.8% as a result of washing. The data in Table 3.1 and 3.2 were adjusted by compensating for the loss. Results are shown in Table 3.4 and 3.5.

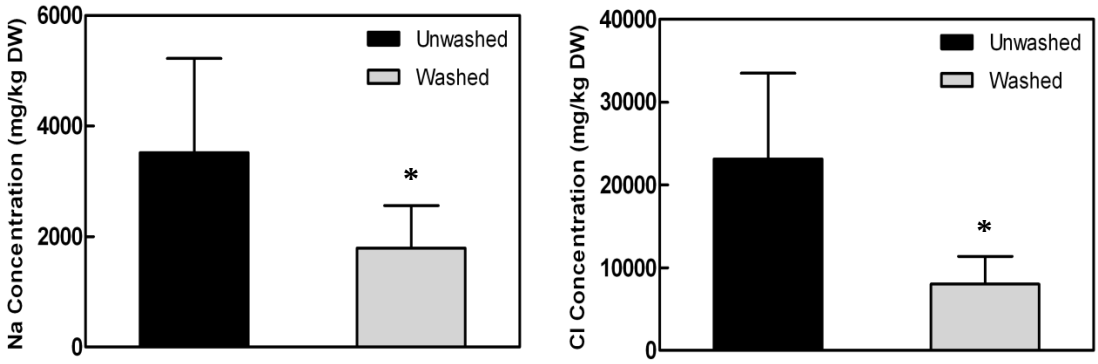


Figure 3.5: A comparison of Na^+ and Cl^- accumulation in washed and unwashed samples from Weyburn and Red Earth salt-impacted sites. Error bar indicates the standard error. Statistical analysis was performed using unpaired Tukey's test. * indicate significant differences observed when comparing washed and unwashed samples at $P<0.05$.

Table 3.4: Plant Na^+ and Cl^- uptake in different plots of CMN after the adjustment for salt loss due to washing.

	Plot	Na (mg/kg)	Na (kg)	Cl (mg/kg)	Cl (kg)	NaCl (kg)
Midseason	1	7923	3.01	23100	24.25	27.26
	2	7320	1.24	21450	10.07	11.32
	4	7113	1.08	14650	6.15	7.23
	5	15845	3.17	30400	16.79	19.96
	Total	——	8.51	——	57.26	65.77
End season	1	4398	0.35	6025	1.33	1.68
	2	3786	0.23	6540	1.12	1.35
	4	3273	0.22	11080	2.02	2.24
	5	2608	0.16	3565	0.61	0.77
	Total	——	0.96	——	5.08	6.05
Season 2009	Total	——	9.47	——	62.34	71.81

The mass of sodium and chloride were converted to the number of mol of ions, which was

then divided by V_{water} to obtain molar concentration values of sodium and chloride (c_i) according to Equation 2.3. IS was calculated using Equation 2.1. $EC_{1:2\ plant}$ was calculated by Equation 2.5, and multiplied by CMN surface soil K-value 2.0, to obtain $EC_{e\ plant}$.

Table 3.5: Computed results of number of mol and molar concentrations of sodium and chloride, IS, $EC_{1:2\ plant}$ and $EC_{e\ plant}$.

Na (mol)	Na (mM)	Cl (mol)	Cl (mM)	IS (mM)	$EC_{1:2\ plant}$ (dS/m)	$EC_{e\ plant}$ (dS/m)
412	0.4139	1756	1.7651	2.1790	0.1676	0.3352

The remediation efficacy was recalculated according to the Equation 2.6:

$$\text{Remediation efficacy} = \frac{0.3352\ \text{dS/m}}{0.56\ \text{dS/m}} \times 100\% = 59.9\%$$

Another problem is that in addition to sodium and chloride, other soluble ions in the soil were also absorbed by plants, such as calcium and potassium. Due to their high solubility in water extraction, Ca^{2+} and K^{+} might also largely contribute to soil EC_e values and plant derived salinity decrease in surface soil. In addition, a large amount of gypsum ($CaSO_4 \cdot 2H_2O$) was applied to both CMN and CMS sites to ameliorate saline soil prior to planting in 2007. Though plant samples were washed in 2008, plant metal scan data showed that the calcium concentration in plant samples ranged from 4620 mg/L to 7970 mg/L. Potassium uptake values were also far beyond 10000 mg/L (Table 3.6). Since only sodium and chloride concentrations in plant tissues were analyzed in 2009, the EC_e decrease caused by plant uptake might still be underestimated even if the data are adjusted for the NaCl loss through washing. Therefore, a simulation was made to study the surface soil EC_e decrease caused by plant uptake of salt ions in the 2009 season.

To further adjust the data, the values of sodium, calcium and potassium concentrations in

plant samples of each plot at CMN in 2008 were obtained from ALS. The Na^+, Ca^{2+} and K^+ concentrations were averaged to obtain the mean uptake values for the whole site. The ratios of $[K^+]/[Na^+]$ and $[Ca^{2+}]/[Na^+]$ in 2008 were calculated and used to estimate Ca^{2+} and K^+ uptake in 2009. After adjusting for loss due to washing, Na^+ uptake was 9550 mg/kg at midseason, and 3516 mg/kg at the end of the season of 2009. The Ca^{2+} and K^+ concentrations were then calculated by Na^+ concentrations times corresponding ratios.

Table 3.6: Plant uptake of Ca^{2+}, K^+ and Na^+ in washed samples from CMN site at midseason 2008 and data adjustment.

Sample ID	Ca (mg/kg)	K (mg/kg)	Na (mg/kg)
CMN #1	4620	19900	10100
CMN #2	5290	19100	8590
CMN #3	7970	30300	3720
CMN #4	4830	21500	3090
Mean	5678	22700	6375
Ratio to Na uptake	0.89	3.56	1.00
Adjusted values midseason	8506	34007	9550
Adjusted values End season	3131	12520	3516

The mass and molar concentrations of Ca^{2+} and K^+ were calculated in the same way as Na^+ and Cl^- were calculated. IS, $EC_{1:2\ plant}$ and $EC_{e\ plant}$ were then calculated by Equation 2.1, 2.5, and $EC_{1:2\ plant}$ times CMN soil K value 2.0, respectively.

Table 3.7: A summary of Ca^{2+}, K^+, Na^+ and Cl^- uptake and plant EC_e calculation after the adjustment for Ca^{2+} and K^+.

Ca (mM)	K (mM)	Na (mM)	Cl (mM)	IS (mM)	$EC_{1:2\ plant}$ (dS/m)	$EC_{e\ plant}$ (dS/m)
0.2139	0.8771	0.4139	1.7651	3.9117	0.3009	0.6018

Remediation efficacy was recalculated according to the Equation 2.6:

$$\text{Remediation efficacy} = \frac{0.6018 \text{ dS/m}}{0.56 \text{ dS/m}} \times 100\% = 107.5\%$$

3.1.2 The Cannington Manor South site

The average EC_e of the surface soil was 3.7 dS/m (ranging from 2.2 to 5.7 dS/m) in CMN and 18.9 dS/m (ranging from 5.6 to 32 dS/m) in CMS prior to planting in June 2009 (Figure 3.6). The site was planted with mixture of CMH3-treated oats, tall wheatgrass and tall fescue; while the blank area remained unplanted and was sprayed twice with glyphosphate to control weeds. By midseason, plots 1 and 2 were 100% covered with vegetation (Figure 3.6 and 3.7); while the plant coverage in plots 3A and 4A was sparse and uneven (Figure 3.8).

	1	2	3	4	
	5.7	14.3	29.9	32.1	
0-25cm	4.4	13.7	21.2	49.0	
	4.8	14.2	20.2	22.2	
25-50cm	3.4	18.7	19.6	21.8	A
	4.0	12.2	23.8	26.8	
50-75cm	2.4	14.1	12.2	21.7	
	6.2	15.0	21.6	26.4	
0-25cm	6.8	11.8	18.6	24.4	
	5.4	14.2	13.6	41.2	
25-50cm	7.6	10.1	8.5	13.4	B
	4.8	12.2	14.8	20.0	
50-75cm	3.0	7.7	7.0	8.4	
	5.9	14.6	27.5		
0-25cm	5.6	12.8	28.3		
	5.1	14.2	24.3		
25-50cm	5.5	14.4	15.8		
	4.4	12.2	21.4		
50-75cm	2.7	10.9	12.3		

Figure 3.6: Soil EC_e in dS/m of the CMS site at three sampling depths in the 2009 growing season. The first row for each depth shows EC_e at the beginning of the season (June 4, 2009) prior to planting; the second row shows sampling at the end of the season (October 14, 2009). Numbers below the map indicate average EC_e for each plot. Green shaded area (plot 1 and 2) indicates midseason plant coverage. Yellow shaded area (plot 3A and 4A) was planted in June, but uneven growth was observed in both midseason

and the end of the season.

Figure 3.7: An overview of plant growth at the low salinity end (plot 1 and 2) at midseason (August) at CMS. The border of the site is indicated by the red line. The map on the top left corner shows the direction from which the image was taken. White patches (3B and 4B) in the map indicate the blank area.

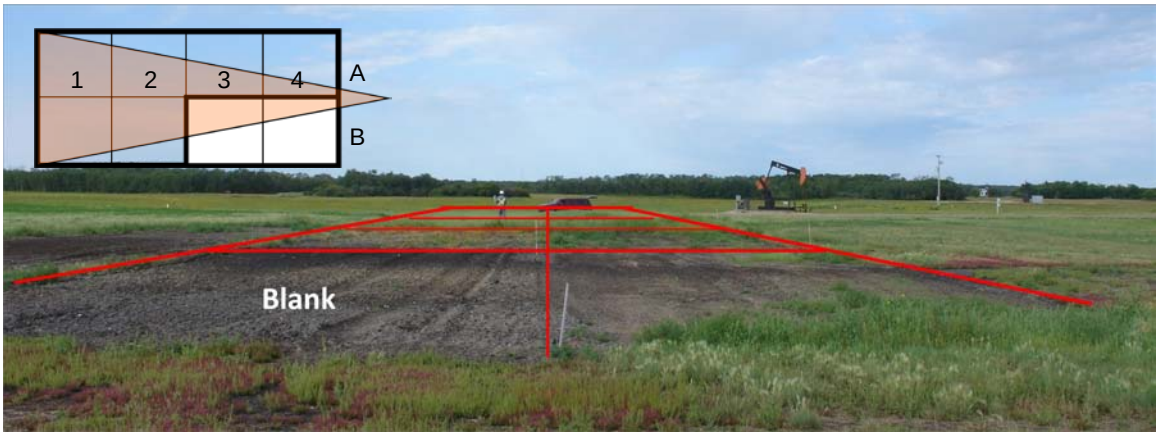


Figure 3.8: An overview of plant growth at midseason (August) at CMS at the high salinity end. Different plots were indicated by red lines. The left down corner was the blank area. The map in the top left corner shows the direction from which the image was taken. White patches (3B and 4B) in the map indicate the blank area.

In the planted area, the trend in seasonal changes of EC_e was the same as the CMN site: an increase at midseason and a decrease at the end of the season (Figure 3.9). The blank area

exhibited the opposite trend, and only small changes in EC_e were observed in October relative to June.

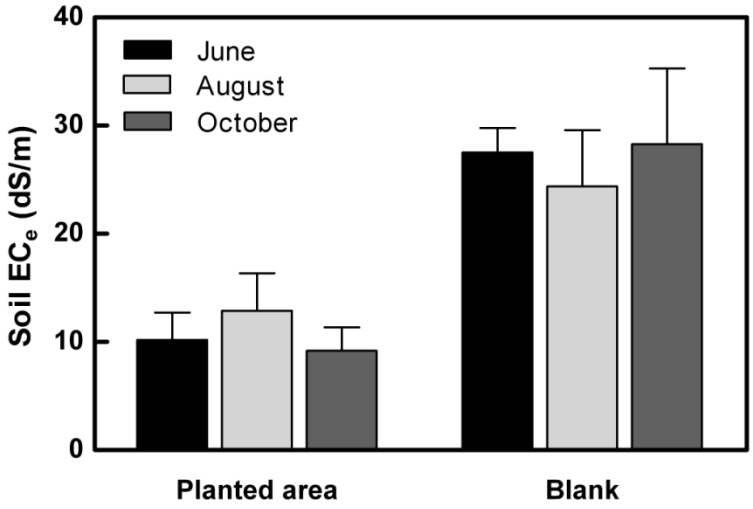


Figure 3.9: CMS 0-25cm soil ECe seasonal change (June, August, and October) in both planted and blank areas. Error bars indicate the standard error.

EC_e in the surface layer (0-25 cm) decreased from 10.22 dS/m to 9.18 dS/m. No apparent change was observed at the 25-50 cm depth, and the 50-75 cm soil salinity exhibited a decline (18%) (Figure 3.10).

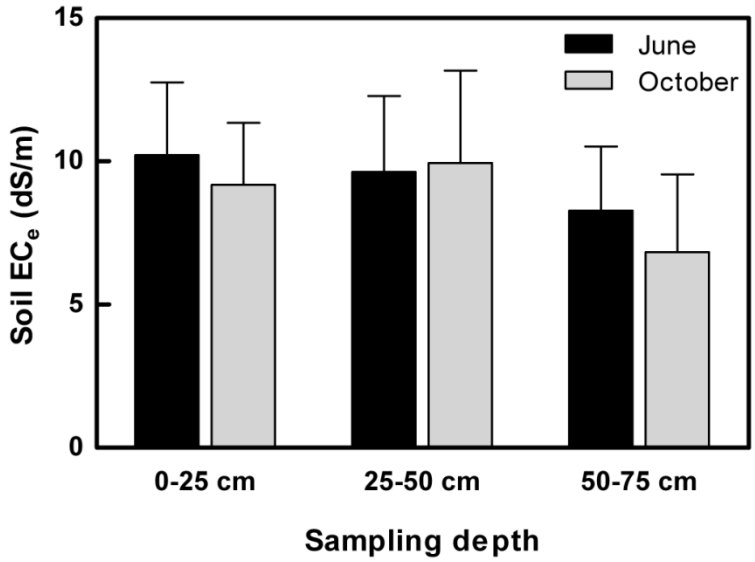


Figure 3.10: CMS soil EC_e seasonal change (June and October) at three sampling depths (0-25 cm, 25-50 cm, 50-75 cm) in the planted area. Error bars indicate the standard error.

Table 3.8: Plant biomass and salt ion uptake in plant tissues in different plots of CMS.

	Plot	Plant DW[1] (g/m^2)	Plot area (m^2)	Plant DW[2] (kg)	Na^3 (mg/kg)	Na^4 (kg)	Cl^3 (mg/kg)	Cl^4 (kg)	$NaCl^5$ (kg)
Midseason	1	600	337.5	202.5	3700	0.75	22150	5.45	6.2
	2	360	337.5	121.5	11655	1.42	32400	6.00	7.42
	Total	——	675.0	324.0	——	2.17	——	11.45	13.62
End season	1	120	337.5	40.5	2870	0.12	6025	0.22	0.34
	2	88	337.5	29.7	1685	0.05	6540	0.23	0.28
	Total	——	675.0	70.2	——	0.17	——	0.45	0.62
Season 2009	Total	——	675.0	394.2	——	2.34	——	11.90	14.24

1. DW stands for dry weight.
2. Plant DW (kg) of each plot was calculated by Plant DW(g/m^2) × Plot area (m^2).
3. Raw data of plant Na^+ and Cl^- contents were in mg per kg plant dry weight.
4. Na^+ or Cl^- mass in each plot were calculated by Na^+ or Cl^- (mg/kg plant DW) × kg Plant DW.
5. NaCl uptake for the whole site is calculated by adding up total Na^+ and Cl^- mass.

Soil mass of the top 25 cm of the entire site was calculated by Equation 2.2:

$$M_{soil} = 250\rho S \text{ (kg)} = 250\times0.75\times675 \text{ (kg)} = 1.27\times10^5 \text{ (kg)}$$

The volume of water required to measure $EC_{1:2}$ was equal to soil mass times 2:

$$V_{water} = 2\times1.27\times10^5 \text{ (L)} = 2.53\times10^5 \text{(L)}$$

Table 3.9: Ion strength and plant-caused EC_e decrease calculation.

Na (mol)	Na^1 (mmol/L)	Cl (mol)	Cl^1 (mmol/L)	IS^2 (mmol/L)	$EC_{1:2\ plant}{}^3$ (dS/m)	$EC_{e\ plant}{}^3$ (dS/m)
102	0.4021	335	1.3249	1.7270	0.1328	0.2922

1. The mass of sodium and chloride were converted to number of mol of ions, which was then divided by V_{water} to obtain molar concentration values of sodium and chloride (c_i) according to Equation 2.3.
2. IS was calculated by Equation 2.1.
3. $EC_{1:2\ plant}$ was calculated by Equation 2.5, and multiplied by CMN surface soil K value 2.0, to obtain $EC_{e\ plant}$.

Soil salinity decrease was calculated by the EC_e at the beginning of the season minus the EC_e at the end of the season:

$$\text{Soil } EC_e \text{ decrease} = 10.22 \text{ dS/m} - 9.18 \text{ dS/m} = 1.04 \text{ dS/m}$$

Remediation efficacy was determined by dividing the calculated $EC_{e\ plant}$ (Table 3.9) by the measured change in surface soil EC_e. Therefore, plant uptake accounted for 28.1% of the salinity decrease in the surface soil.

$$\text{Remediation efficacy} = \frac{0.2922 \text{ dS/m}}{1.04 \text{ dS/m}} \times100\% = 28.1\%$$

The data in Table 3.8 and 3.9 were adjusted by compensating for loss of ions due to washing the tissues in the same way as the adjustment for CMN data. Results are shown in Table 3.10 and 3.11.

Table 3.10: Adjusted plant Na^+ and Cl^- uptake in different plots of CMS. Na^+ or Cl^- mass in each plot were calculated by Na^+ or Cl^- (mg/kg plant DW) × kg Plant DW.

	Plot	Na (mg/kg)	Na (kg)	Cl (mg/kg)	Cl (kg)	NaCl (kg)
Midseason	1	6655	1.35	61180	12.39	
	2	20962	2.55	89492	10.87	
	Total	——	3.89	——	23.26	27.25
End season	1	5162	0.21	16642	0.67	
	2	3031	0.09	18064	0.54	
	Total	——	0.30	——	1.21	1.51
Season 2009	Total	——	4.19	——	24.47	28.76

Table 3.11: Computed results of number of mol and molar concentrations of sodium and chloride, IS, $EC_{1:2\ plant}$ and $EC_{e\ plant}$.

Na (mol)	Na (mM)	Cl (mol)	Cl (mM)	IS (mM)	$EC_{1:2\ plant}$ (dS/m)	$EC_{e\ plant}$ (dS/m)
182	0.7201	689	2.7245	3.4445	0.2650	0.5829

The remediation efficacy was recalculated according to the Equation 2.6:

$$\text{Remediation efficacy} = \frac{0.5829\ \text{dS/m}}{1.04\ \text{dS/m}} \times 100\% = 56.1\%$$

CMS plant metal scan data in 2008 showed that the average calcium concentration in plant samples ranged from 5940 mg/L to 9510 mg/L. Potassium uptake values were also greater than 10000 mg/L (Table 3.11). The data again were adjusted in the same way as they were adjusted for CMN.

The sodium concentration in plants, after the adjustment for washing loss, was 13809 mg/kg at midseason and 4096 mg/kg at the end of the season (Table 3.12). The calcium and potassium levels were then calculated by sodium concentrations times the corresponding ratios.

Table 3.12: Plant uptake of Ca^{2+}, K^+, Na^+ in washed samples from CMS site at midseason 2008 and data adjustment.

Sample ID	Ca (mg/kg)	K (mg/kg)	Na (mg/kg)
CMS #1	9510	20300	8110
CMS #2	5940	20800	7270
CMS #3	9100	17800	4370
Average	8183	19633	6583
Ratio to Na^+ uptake	1.24	2.98	1.00
Adjusted values midseason	17165	41181	13809
Adjusted values End season	5092	12216	4096

The mass and molar concentrations of Ca^{2+} and K^+ were calculated in the same way as Na^+ and Cl^-. IS and $EC_{e\ plant}$ were then calculated by Equation 2.1 and Equation 2.5, respectively.

Table 3.13: A summary of Ca^{2+}, K^+, Na^+ and Cl^- uptake and plant EC_e calculation after the adjustment.

Ca (mM)	K (mM)	Na (mM)	Cl (mM)	IS (mM)	$EC_{1:2\ plant}$ (dS/m)	$EC_{e\ plant}$ (dS/m)
0.5847	1.4389	0.7201	2.7245	7.2223	0.5556	1.2222

Remediation efficacy was recalculated according to the Equation 2.6:

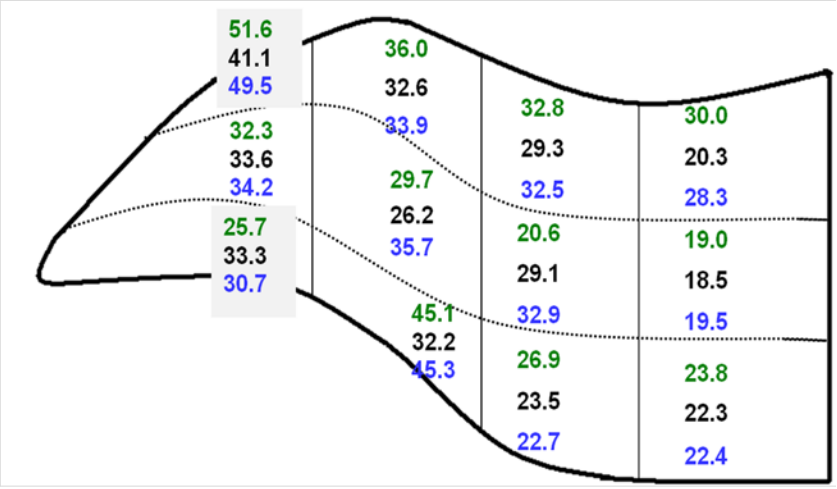
$$\text{Remediation efficacy} = \frac{1.2222\ \text{dS/m}}{1.04\ \text{dS/m}} \times 100\% = 117.5\%$$

3.1.3 The Alameda site

The Alameda site (AL) is located near Alameda, Saskatchewan. The history and source of salt contamination is unknown. A 4-inch layer of manure was mixed into the surface of this site in May 2007.

All plots were planted with a mixture of CMH3-treated oats, tall wheatgrass and tall fescue.

Due to high soil salt concentrations in plots 3A and 3C and the blank (Plot 4), plant establishment was fully inhibited. Furthermore, the blank area did not require glyphosate application to control weed growth. Several days after sowing, a hard frost occurred which may have killed any cereal or grass seeds that had already germinated. However, kochia (*Kochia scoparia*), a competitive salt, drought, and cold tolerant weed species in southeast Saskatchewan, flourished throughout the site. During the midseason visit, kochia dominated the site; while oats and the grasses were sparse (Figure 3.11 and 3.12). By mowing the site after the midseason, the oats and grasses recovered by the end of the season.



Beginning	**36.5**	**36.9**	**26.8**	**24.3**
Middle	**36.0**	**30.3**	**27.3**	**20.4**
End	**38.1**	**38.3**	**29.4**	**23.4**

Figure 3.11: Soil EC_e in dS/m of Alameda site through sampling surface soil in 2009 growing season. The first row for each depth shows EC_e at the beginning of the season (June 3, 2009) prior to planting; the second row shows sampling in the middle of the season (August 14, 2009); the third row shows sampling at the end of the season (October 15, 2009). Numbers below the map indicate average EC_e for each plot.

Figure 3.12: An overview of plant growth at midseason (August 13, 2009) in Alameda. The field was covered with weeds (mostly Kochia) and sparsely with with oats and grasses planted at the beginning of the season. The sketch map on the top left corner shows the direction from which the image was taken.

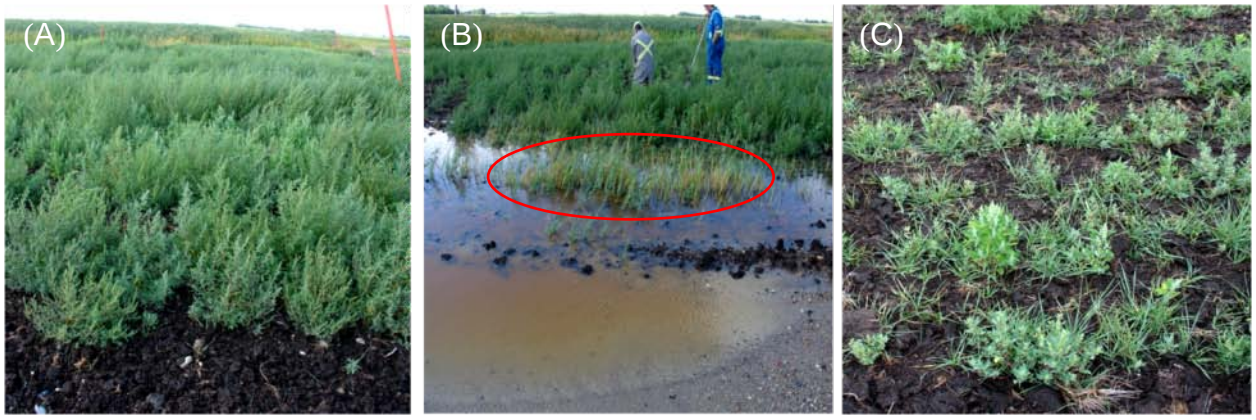


Figure 3.13: A comparison of weeds, oats and grasses growth at midseason in Alameda site. The flourishing kochia across the field (A), the growth of oats in a puddle on the edge of the site (B) (red circled), where the salinity might have been lowered down by the rain water, and the growth of grasses (C), which were strongly inhibited by the high salinity are shown

The uneven plant coverage and the general low production of plant biomass made it impossible to estimate the plant contribution to the lowered salinity level at midseason. As indicated by Figure 3.14, only small EC_e changes were observed at the end of the season

compared to the initial EC_e values.

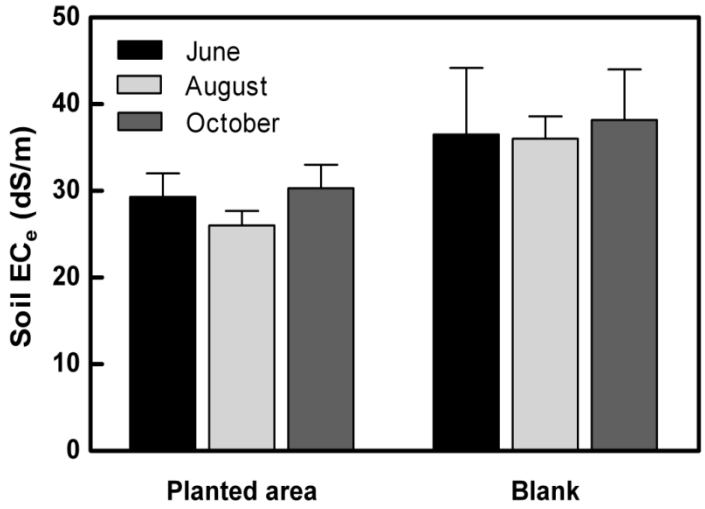


Figure 3.14: Alameda 0-25cm soil EC_e seasonal change (June, August, and October) in both planted and blank area. Error bars indicate the standard error.

3.2 Measurement of PGPR growth in saline media

Pseudomonas corrugata CMH3 was isolated from the CMS site where EC_e was 35 dS/m (approximately 2% NaCl solution) (Wu, 2009). Bacterial growth was monitored in TSB media with no added salt or spiked with 2% NaCl over 28 hours. The growth of *Psudomonas putida* UW4, which was isolated from the University of Waterloo campus, was also examined under the same condition.

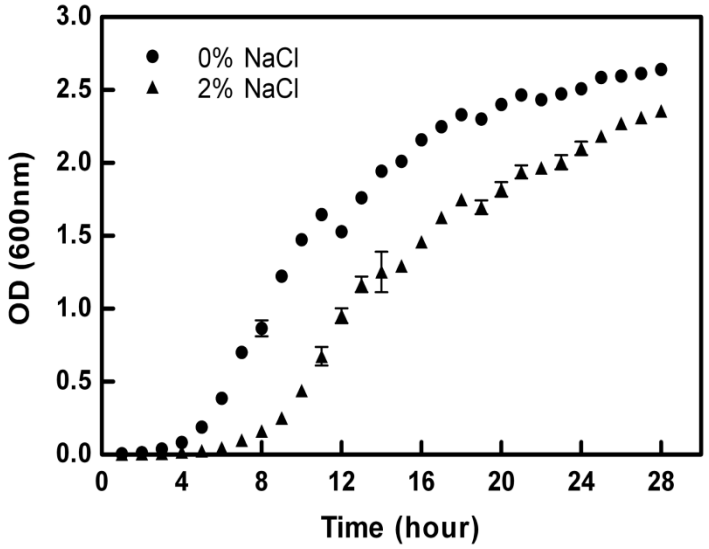
For UW4, the log phase of the growth curve with 2% of salt started at the seventh hour, and did not reach its stationary phase within the twenty eight hour testing period; whereas exponential growth of the control (0%) culture started at approximately the fourth hour, and reached its stationary phase at about twenty hours. UW4 growing in both salt-stressed and non-stressed media reached OD_{600} 2.0 within 24 hours (Figure 3.15).

For CMH3, the log phase of the growth curve with no salt started at about the sixth hour,

while the one with 2% salt started at about the ninth hour. Though the exponential phase was delayed by approximately 3 hours in the salt treatment, no more than 20% inhibition of growth (1 - OD value of growth in 2% NaCl TSB ÷ OD value of growth in control TSB) could be observed in the stationary phase (after 21 hours) (Figure 3.15). It was expected that CMH3 performance in the salt-impacted sites and greenhouse studies would not be significantly affected by the high salinity.

The growth of CMH3 was slower than UW4 in TSB media. At the room temperature in non-saline media, it took more than 20 hours for CMH3 to reach OD_{600} 2, while it took only 17 to 20 hours for UW4.

(A)



(B)

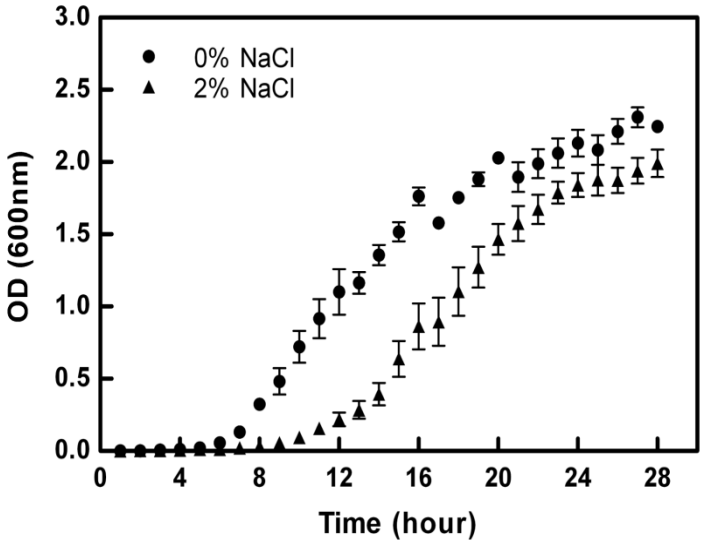


Figure 3.15: Growth curve of UW4 (A) and CMH3 (B) in TSB without salt and with 2% NaCl over 28 hours at 23±2ºC. Samples were performed in triplicates (N= 3). Error bars indicate the standard errors.

3.3 Greenhouse studies to examine PGPR effect on plant physiological indicators

To assess the PGPR effect on the physiological performance of plants under salt stress, annual ryegrass was grown in un-impacted soil (0.5 dS/m), NaCl spiked topsoil (10 dS/m), and CMS saline soil diluted with un-impacted soil (10 dS/m).

3.3.1 Plant growth and fresh weight measurement

The plants in un-impacted control soil were healthy and light green in color, and showed no inhibition on germination; whereas plant germination and growth in both types of salt-impacted soil were inhibited. Plants in NaCl spiked soil had a slower growth, lower germination rates and plant density than those in diluted CMS salt soil, even though the salinity level of both types of soil were 10 dS/m. In addition, plants growing in salt soil generally had a darker green color than those growing in control soil (Figure 3.16). The color difference became more apparent as time elapsed.

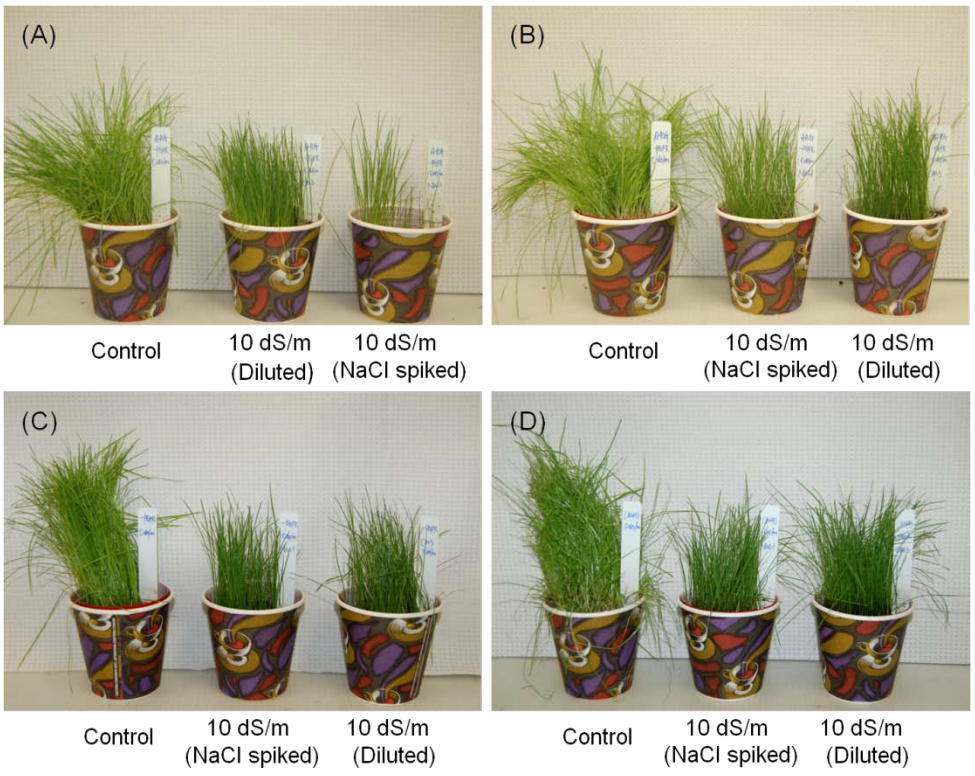


Figure 3.16: Annual ryegrass (*Lolium multiflorum*) growth in topsoil (0.5 dS/m, labeled as "Control"), CMS soil diluted with topsoil to 10 dS/m (labeled as "Diluted"), and topsoil spiked with NaCl to 10 dS/m (labeled as "NaCl spiked") at 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of the plants grown in control soil.

Under salt stress, plants inoculated with UW4 and CMH3 were higher in biomass production compared to un-inoculated plants throughout the experimental period. There is a positive effect of PGPR on plant growth in NaCl spiked soil and diluted CMS salt-impacted soil (Figure 3.17 and 3.18).

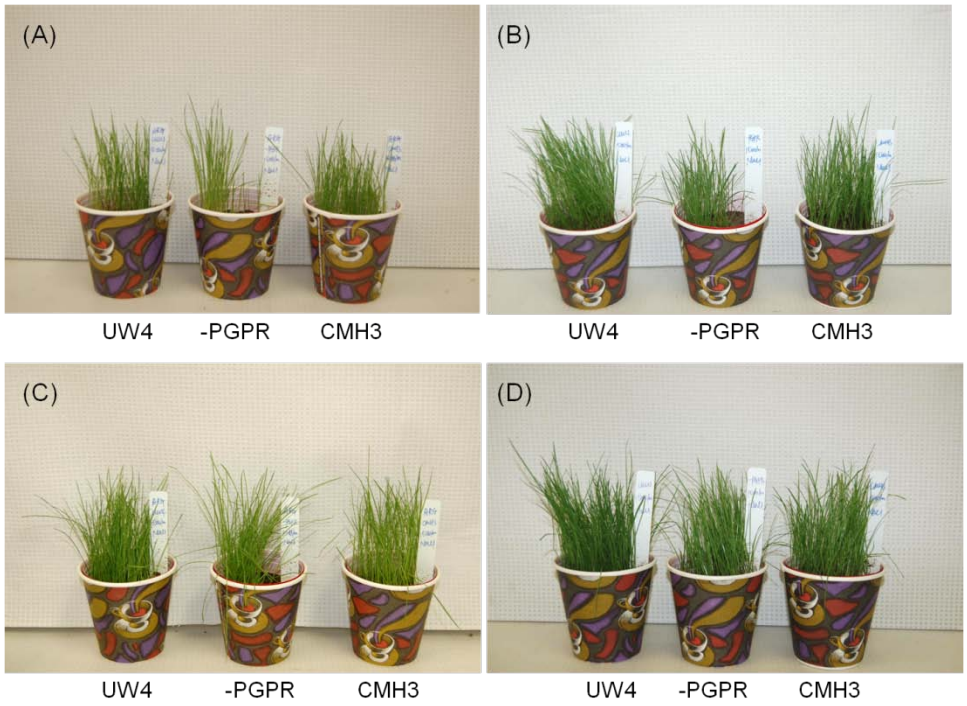


Figure 3.17: A comparison of annual ryegrass (*Lolium multiflorum*) growth with PGPR treatment (no PGPR, UW4 or CMH3) in NaCl spiked soil (10 dS/m) at 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of the plants grown in control soil (0.5 dS/m).

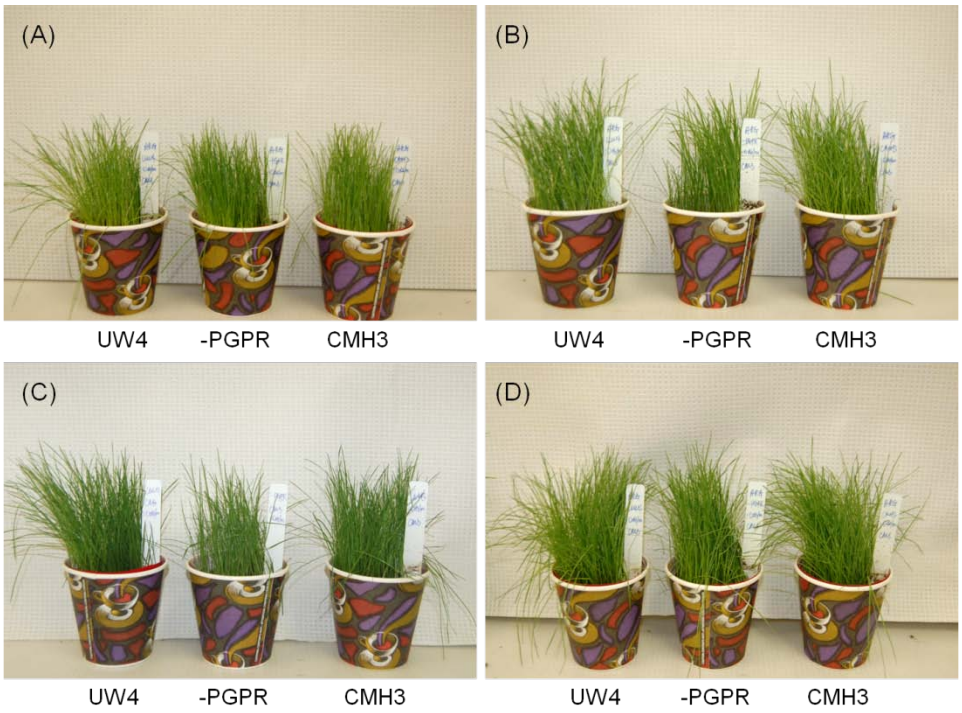


Figure 3.18: A comparison of annual ryegrass (*Lolium multiflorum*) growth with PGPR treatment (no PGPR, UW4 or CMH3) in CMS soil diluted with topsoil (10 dS/m) at 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of the plants grown in control soil (0.5 dS/m).

Shoot fresh weight significantly decreased as a result of salt stress. Plants growing in spiked salt soil had 74% less biomass and in diluted salt soil had 44% less biomass than control plants. NaCl spiked soil more severely inhibited plant growth than diluted CMS salt soil, even though they were of the same salinity. Both UW4 and CMH3 increased plant biomass by 9% relative to non-PGPR treated plants, though no significant improvement was observed at $P< 0.05$. (Figure 3.19 and Table 3.14). The trend showed that CMH3 treated plants produced more biomass in both un-impacted control and salt-impacted soil. UW4 increased plant biomass only in the salt-impacted soil, but not in un-impacted control soil. However, the biomass production of UW4 treated plants in salt soil was higher than CMH3 treated plants on day 12, 15 and 18 (Figure 3.19 B, C and D).

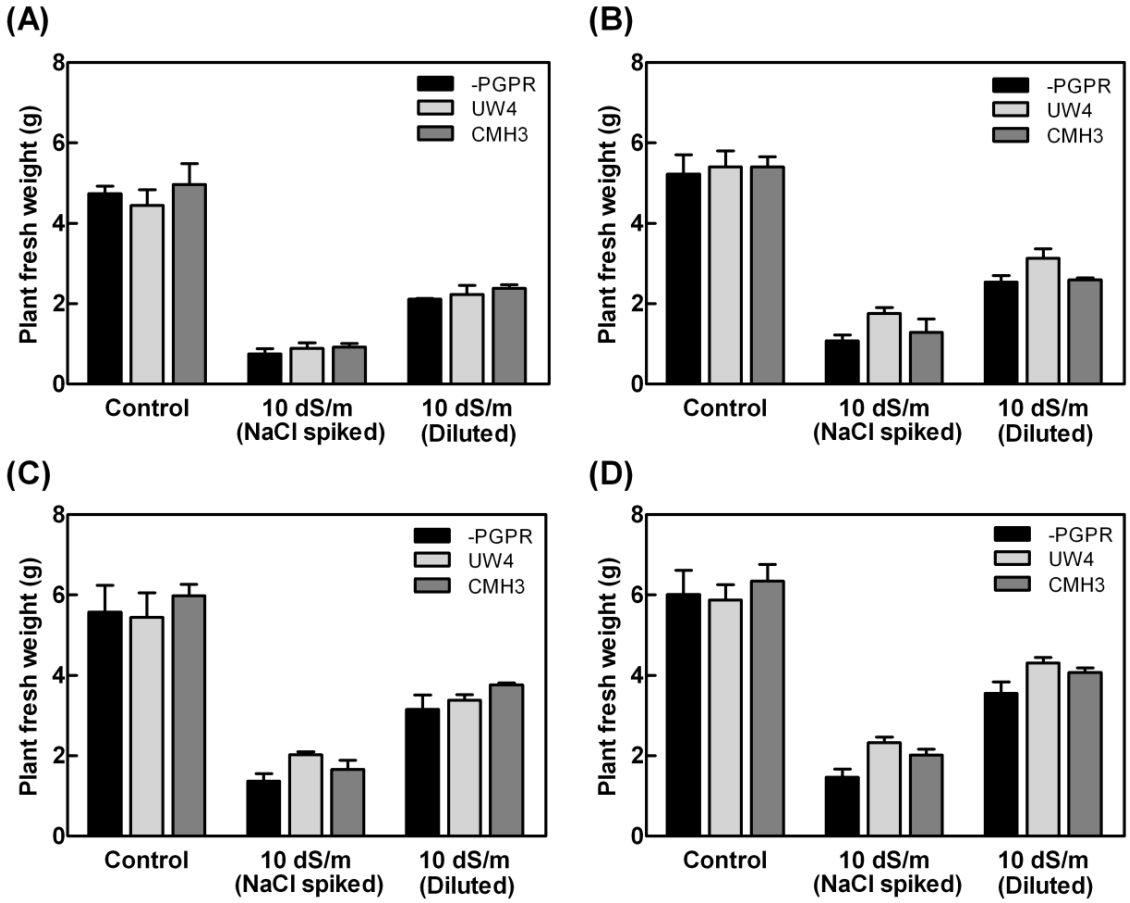


Figure 3.19: Shoot fresh weight of annual ryegrass grown in control soil (0.5 dS/m), NaCl spiked soil (10 dS/m) and diluted CMS salt soil (10 dS/m) at 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of plants grown in control soil. All measurements were performed in triplicates, and the whole experiment was repeated 3 times (N = 9). Error bars are standard error (S.E.). Statistical analysis was performed using two-way ANOVA and the Bonfferoni post-tests comparing the significance of PGPR treatment at each salt level.

Plants were sampled 9, 12, 15 and 18 days after the germination of the plants growing in the un-impacted soil for physiological assays. SOD, proline and membrane leakage were tested on fresh tissues.

3.3.2 The effect of salt stress and PGPR inoculation on SOD activity

In the control plants, SOD activity remained at the similar level in both non-PGPR and PGPR treated plants for the first 15 days, while slight increase was observed with UW4 and CMH3 treatment at the last sampling point (Figure 3.20). In the NaCl spiked soil, there is an increase in SOD activity for all the treatments over time. In the diluted CMS soil, SOD activity of non-PGPR treated plants did not change much over sampling period; while the PGPR inoculated plants exhibited a slight increase and then decrease at the last sampling day.

Overall, both spiked soil and diluted field collected salt-impacted soil significantly increased SOD level in plant tissues by approximately 50% (Figure 3.20 and Table 3.14) during the first 15 days. On the last sampling day (day 18), the salt induced SOD increase was lower. In NaCl spiked soil the increase was 44%, and diluted CMS soil 34%.

PGPR treatment did not significantly improve SOD activity ($P<0.05$); however, higher SOD activity was observed in PGPR treated plants through the experimental period. On average, UW4 enhanced SOD activity by 9.7%, and CMH3 by 10.1% (Figure 3.20). However, at the last sampling point (day 18), PGPR enhancement of SOD was decreased to less than 3%.

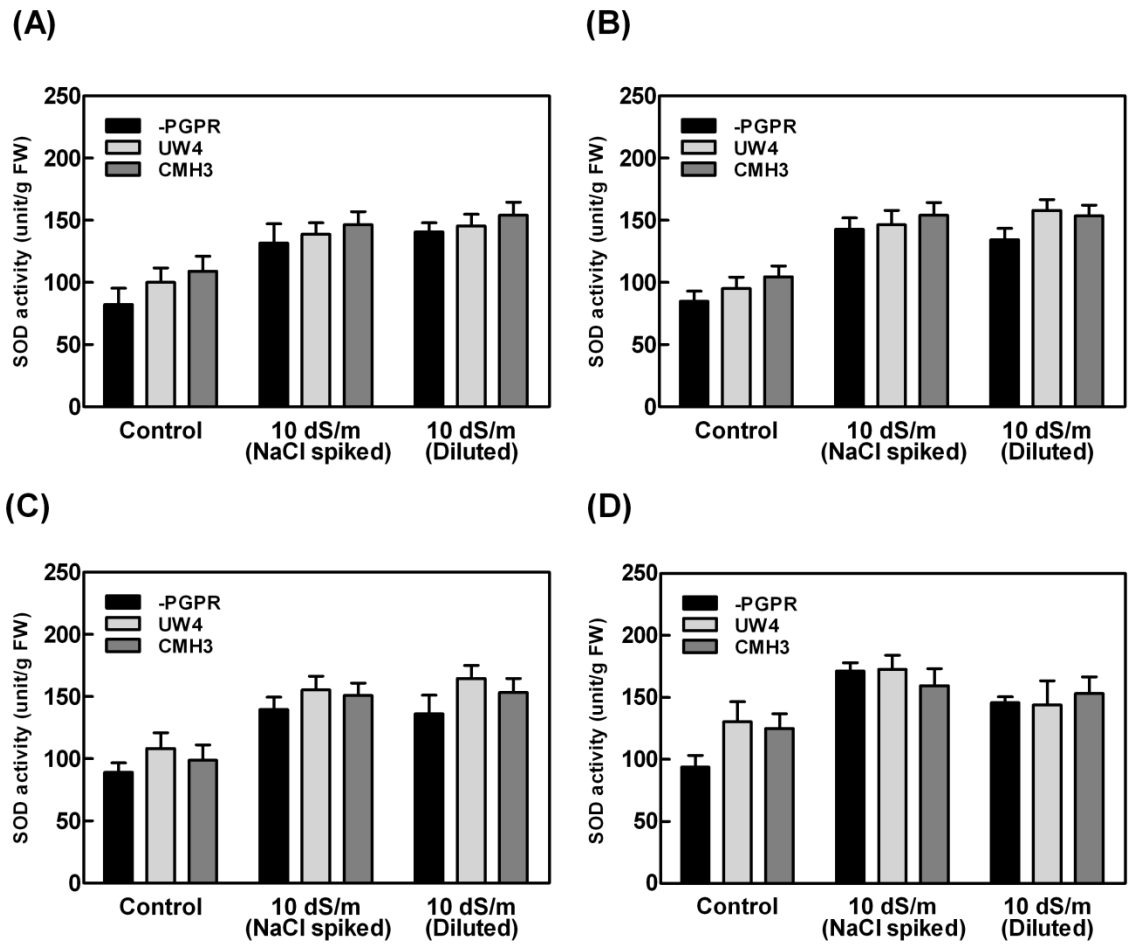


Figure 3.20: SOD activity of annual ryegrass grown in control soil (0.5 dS/m), NaCl spiked soil (10 dS/m) and diluted CMS salt soil (10 dS/m) at 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of plants grown in control soil. All measurements were performed in triplicates, and the whole experiment was repeated 3 times (N = 9). Error bars are standard errors (S.E.). Statistical analysis was performed using two-way ANOVA and the Bonfferoni post-tests comparing the significance of PGPR treatment at each salt level.

3.3.3 The effect of salt stress and PGPR inoculation on proline accumulation

In the control plants, the proline concentrations over the sampling period were fairly constant in both non-PGPR and PGPR-treated plants (Figure 3.21). In NaCl spiked soil and diluted CMS soil, proline accumulation in both non-PGPR and PGPR-treated plants reached a maximum on the day 12, and then declined, but remained significantly elevated compared to the control plants on the last sampling day (Figure 3.21). Both types of salt soil significantly increased the proline concentration in plant tissues by more than 20 fold (Figure 3.21 and Table 3.14).

Significant enhancement ($P<0.05$) of the proline content in plant leaves with UW4 and CMH3 treatment could be observed on days 12, 15 and 18 in both types of salt-impacted soil, except in UW4-treated plants in NaCl spiked soil on day 18 (Figure 3.21). On average, UW4 improved proline concentration by 25.9%, and CMH3 by 32.0%.

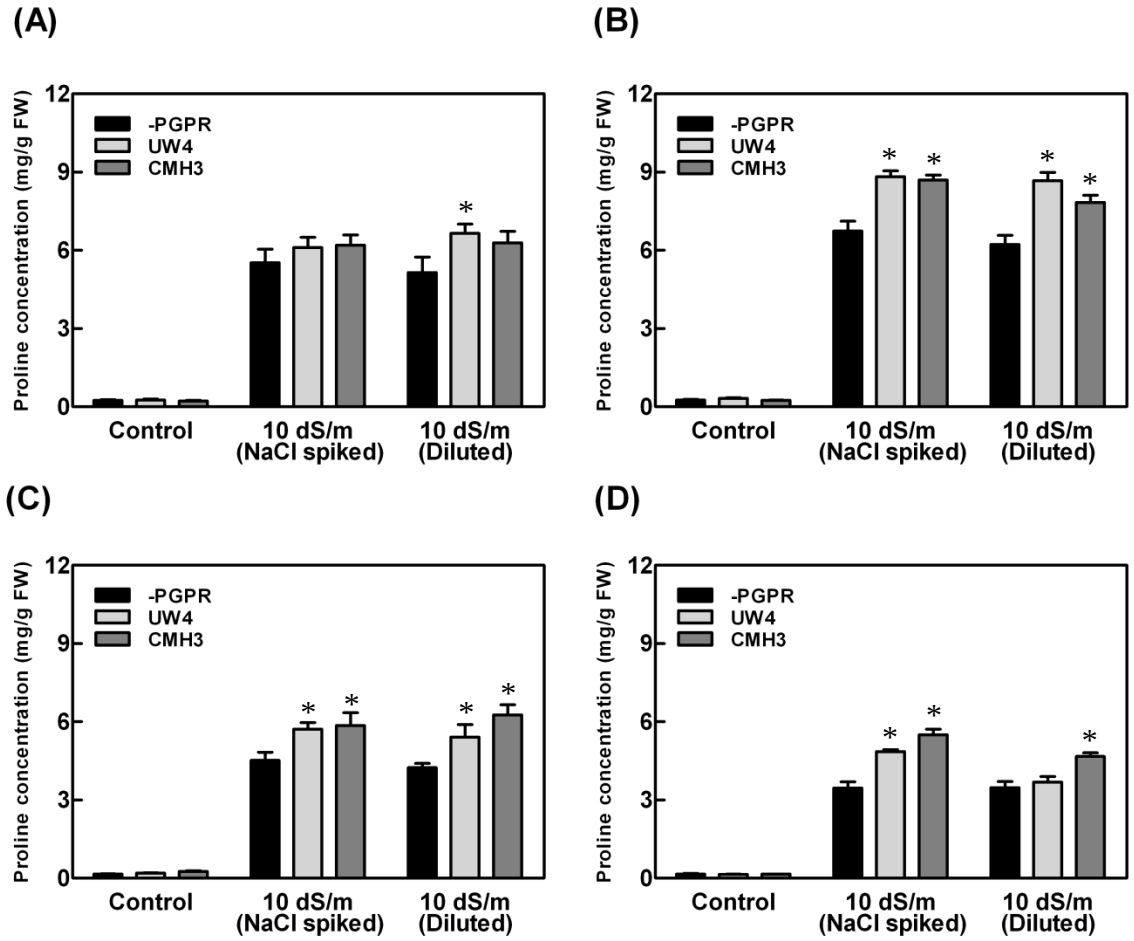


Figure 3.21: Proline concentrations of annual ryegrass grown in control soil (0.5 dS/m), NaCl spiked soil (10 dS/m) and diluted CMS salt soil (10 dS/m) 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of plants grown in control soil. All measurements were performed in triplicates, and the whole experiment was repeated 3 times (N = 9). Error bars are standard errors (S.E.). Statistical analysis was performed using two-way ANOVA and the Bonfferoni post-tests comparing the significance of PGPR treatment at each salt level. * indicates significant differences observed when comparing untreated (No PGPR) sample to PGPR treated samples on salt soil at $P < 0.05$.

3.3.4 The effect of salt stress and PGPR inoculation on electrolyte leakage

Overall, in control soil and both types of salt-impacted soil, plant cell membrane leakage increased until day 15 and then decreased on the last sampling day (Figure 3.22). Salinity stress, either spiked soil or diluted CMS soil, significantly increased membrane leakage in plant leaves at $P<0.05$ (Figure 3.22 and Table 3.14). The degree of cell membrane damage became increased over time, from 1.8 (day 9) to 2.8 fold (day 18) in NaCl spiked soil relative to the control, and from 1.6 (day 9) to 2.6 (day 18) fold in diluted CMS soil relative to the control.

The significant alleviation ($P<0.05$) of membrane damage by PGPR treatment could be observed on day 9 and day 12 in spiked salt soil, and in both types of salt soil on day 18 (Figure 3.22). On average, electrolyte leakage decreased by 9.1% and 15.4% with UW4 and CMH3 treatment, respectively.

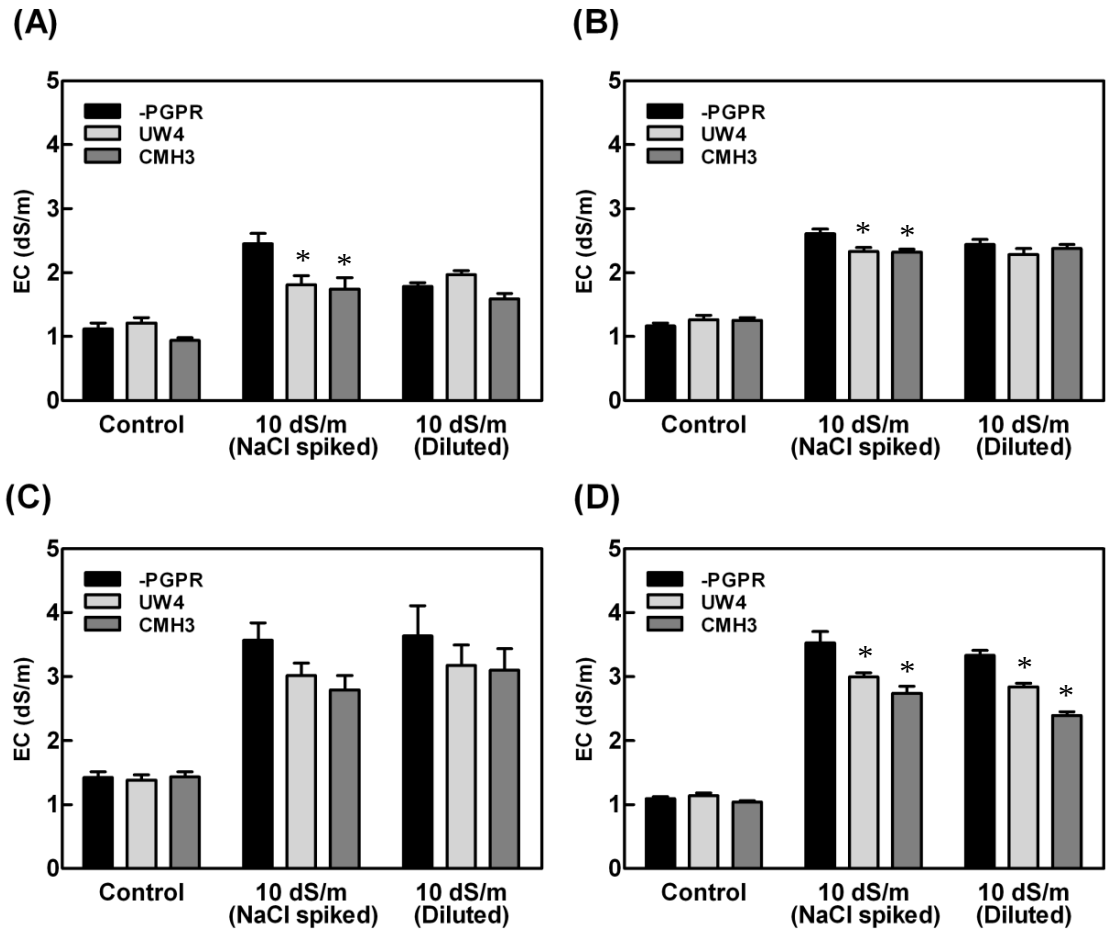


Figure 3.22: Cell membrane leakage of annual ryegrass grown in control soil (0.5 dS/m), NaCl spiked soil (10 dS/m) and diluted CMS salt soil (10 dS/m) 9 days (A), 12 days (B), 15 days (C) and 18 days (D) after the germination of plants grown in control soil. All measurements were performed in triplicates, and the whole experiment was repeated 3 times (N = 9). Error bars were standard errors (S.E.). Statistical analysis was performed using two-way ANOVA and the Bonfferoni post-tests comparing the significance of PGPR treatment at each salt level. * indicates significant differences observed when comparing untreated (No PGPR) sample to PGPR treated samples on salt soil at $P < 0.05$.

In summary, PGPR inoculation significantly decreased membrane leakage on day 9 and day 18, and increased proline accumulation on day 12, 15 and 18 (Table 3.14). However, no significant result could be observed in PGPR's promotion effect on the fresh leaf weight and the SOD activity (Table 3.14). Salinity significantly changed all the physiological indicators examined in this study. The significance of combined treatment of salinity (10 dS/m) and PGPR could be seen regarding proline levels and membrane leakage (Table 3.14).

Table 3.14: Two factors ANOVA (PGPR inoculation and saline stress) for all parameters studied on annual ryegrass (*Lolium multiflorum*) *p* significance values.

Time point	Index	PGPR inoculation (*I*)	Saline stress (*S*)	Interaction (*I*×*S*)
9 days	Fresh weight	0.4627	< 0.0001*	0.8403
	SOD	0.1436	< 0.0001*	0.9656
	Proline	0.0538	< 0.0001*	0.3488
	Membrane leakage	0.0006*	< 0.0001*	0.0033*
12 days	Fresh weight	0.1119	< 0.0001*	0.8312
	SOD	0.0775	< 0.0001*	0.8453
	Proline	< 0.0001*	< 0.0001*	< 0.0001*
	Membrane leakage	0.0956	< 0.0001*	0.0233*
15 days	Fresh weight	0.3463	< 0.0001*	0.7022
	SOD	0.0741	< 0.0001*	0.9840
	Proline	< 0.0001*	< 0.0001*	0.0285*
	Membrane leakage	0.1101	< 0.0001*	0.6626
18 days	Fresh weight	0.1178	< 0.0001*	0.5042
	SOD	0.9247	< 0.0001*	0.1521
	Proline	< 0.0001*	< 0.0001*	< 0.0001*
	Membrane leakage	< 0.0001*	< 0.0001*	< 0.0001*

- Only when $p<0.05$ could the differences being considered as statistically significant.
- * means that the p-values indicate significant differences.

3.3.5 Salt stress and PGPR effect on photosynthetic activity

As a measure of photosynthesis, chlorophyll fluorescence has been shown to be a physiological parameter that correlates with salinity tolerance (Flexas et al., 2004; Longstreth &

Nobel, 1979). Chlorophyll fluorescence was measured using pulse amplitude modulated (PAM) fluorometry to determine if salinity would negatively affect photosynthetic activity, and if PGPR treatment would alleviate stress on photosynthetic activity. Annual ryegrass was planted with and without PGPR treatment, in um-impacted control soil (EC_e = 0.5 dS/m) and CMS soil mixed with topsoil (EC_e = 10 dS/m). Chlorophyll α fluorescence was measured after 20 days of growth (Table 3.15). Representative fluorometry induction curves for each treatment were shown in Figure 3.23

By obtaining the minimal fluorescence in dark-adapted plant tissue (F_o) and the maximal fluorescence (F_m), the maximal quantum yield of PSII (F_v/F_m) ratio was calculated. The typical value of F_v/F_m for healthy plants is approximately 0.8 (Maxwell & Johnson, 2000), as indicated by control plants (Table 3.15). Salinity significantly decreased F_v/F_m ($P<0.05$) (Table 3.15). The effective quantum yield at steady state (yield) is a measure of the overall quantum yield of photochemical energy storage (Maxwell & Johnson, 2000). In this study, the photochemical yield for non-PGPR plants in salt soil decreased by 16% compared to the control plants (Table 3.15). Steady-state fluorescence (F_s) can also be used to study the degree of salt damage on photosynthetic electron transport. F_s increased in uninoculated plants in saline soil. This indicated possible impairments to photosynthetic apparatus (Figure 3.23).

Photochemical quenching (qP) and non-photochemical quenching (qN) usually range from 0 to 1. In healthy plants, qP typically stabilizes at a steady-state value which exceeds 0.8, while qN stabilizes at a lower value, usually less than 0.6 (Genty et al., 1989). In this study, qP decreased by 8% and qN increased by 36% in salt stressed plants compared to plants growing in control soil.

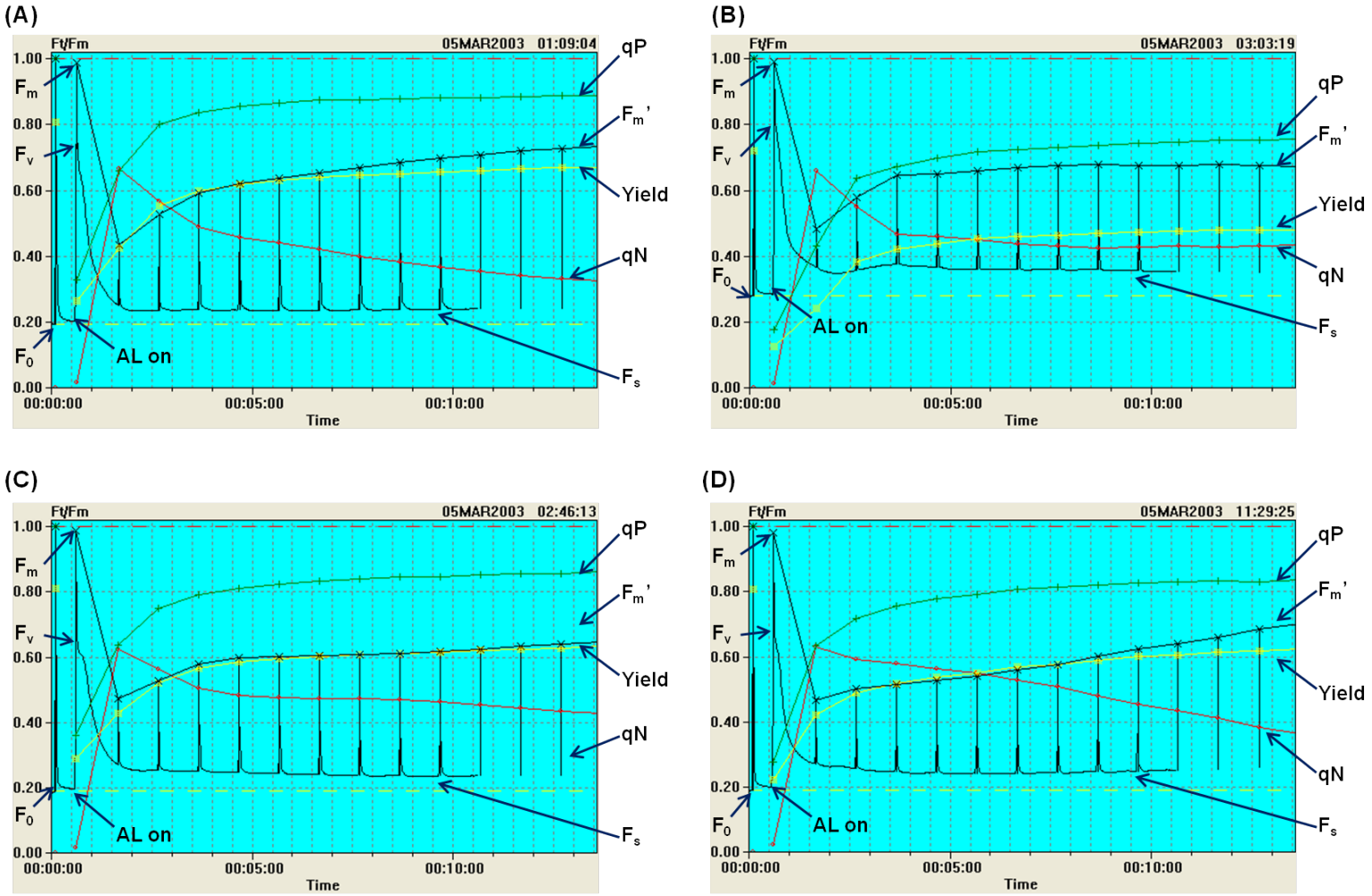


Figure 3.23: PAM induction curve of annual ryegrass grown for 20 days on topsoil (EC_e < 0.5 dS/m) without PGPR treatment (A), diluted salt-impacted soil from CMS site (EC_e = 11 dS/m) without PGPR treatment (B), with UW4 treatment (C), and with CMH3 treatment (D). All treatments were performed in 9 replicates; however, only a representative PAM fluorescence trace was presented.

PGPR inoculation of plants was found to alleviate the negative effect of salinity on photosynthesis, as indicated by the higher values of F_v/F_m, effective quantum yield, and qP, and lower values of qN and F_s in inoculated plants compared to non-PGPR treated plants (Table 3.15, Figure 3.23). The maximum quantum yield of PSII (F_v/F_m) was restored to the non-stressed value (≥0.8) with both UW4 and CMH3 treatment. The effective quantum yield at steady-state (Yield) significantly increased by 16% with UW4 and by 4% with CMH3 treatment ($P<0.05$).

Photochemical quenching (qP) and non-photochemical quenching (qN) were also improved in PGPR treated plants. A 6% increase in qP and a 20% decrease in qN was observed in UW4-treated plants. A 4% increase in qP and a 16% decrease in qN was observed in CMH3 treated plants. Furthermore, a decrease of fluorescence at steady-state (F_s) was observed for PGPR-treated plants compared to untreated plants (Figure 3.23).

Table 3.15: Chlorophyll α fluorescence parameters of annual ryegrass (20 days) in control (0.5 dS/m) and diluted CMS salt impacted soil (EC_e = 10 dS/m).

Chlorophyll α fluorescence parameters	Control (0 dS/m)	-PGPR (10 dS/m)	UW4 (10 dS/m)	CMH3 (10 dS/m)
F_v/F_m	0.810±0.001 a	0.779±0.008 b	0.810±0.001 a	0.811±0.001 a
Yield	0.657±0.004 a	0.550±0.016 b	0.636±0.008 a	0.629±0.005 a
qP	0.873±0.004 a	0.802±0.012 b	0.853±0.007 a	0.846±0.004 a
qN	0.345±0.010 b	0.470±0.030 a	0.377±0.021 b	0.397±0.016 a

- PAM measurements for annual ryegrass were performed in 9 independent replicates (N = 9).
- Error values were based on standard errors (S.E.).
- Statistical analysis was performed using one-way ANOVA and the post-hoc Tukey's test. Numbers followed by different letters in a row within the same plant are significantly different at $P < 0.05$.

Chapter 4 – Discussion

In this study, field and laboratory work was performed to examine phytoremediation of salt-impacted soils. For the field study, PGPR-treated plants grew well at both CMN and CMS sites. The average surface soil salinity levels at CMN and CMS during the 2009 season decreased by 15.2% and 11.3%, respectively. After the data were adjusted by a series of logical assumptions and simulations, the estimated remediation efficacy was 107.5% for CMN and 117.5% for CMS. In contrast, at the Alameda site, due to the strong inhibition of plant germination and seedling growth by the high salinity (>30 dS/m) in most part of the site and a heavy frost three days after planting, neither decent vegetation coverage nor salinity decrease was observed.

Salinity and PGPR effects on plant biomass production, proline accumulation, SOD activity, membrane permeability and photosynthetic activity were examined in greenhouse and laboratory studies on annual ryegrass. In greenhouse studies, treatments with UW4 and CMH3 promoted plant growth, enhanced proline accumulation, SOD activity and photosynthesis, as well as alleviated plant cell membrane damages caused by high salinity.

4.1 Salt mass balance study in phytoremediation of salt-impacted sites

4.1.1 Plant growth and soil salinity change

The initial average EC_e (3.7 dS/m) in surface soil at CMN was the lowest among the three research sites. What we planted at the beginning of the season successfully covered the entire planted area. Plants in plot 1 and 2 were taller than those in plot 4 and 5 (Figure 3.2). One explanation for that is the EC_e in the surface 25 cm in plot 4 and 5 were higher than those in plot 1 and 2 (Figure 3.1). In plot 5, an EC_e higher than 7 dS/m surpassed the tolerance limit of the moderately sensitive species annual ryegrasses, and was at the high end of the tolerance for oats. Second, an EC_e above 7 dS/m observed in plot 4 and 5 25-75 cm (Figure 3.1) might also be

detrimental to plant growth. The active absorption of salt into aboveground tissues lowered salt concentration in the surface soil. Salt in the lower horizons (below 25 cm) might migrate up to the surface and diffuse to lower concentration zones. Hence, a constant supply of high concentrations of salt ions in the root zone kept plants under stress in plot 4 and 5, and lead to stunted plant growth and lower biomass production. This is also supported by the fact that plant uptake of Na^+ and Cl^- in plot 5 was higher than that in other plots (Table 3.1). For most plant species, the more salt ions are accumulated in plants, the more plant growth is impaired (Munns & Tester, 2008).

Another reason for the under-performance of plant growth in plot 4 and 5 might stem from the high Sodium Absorption Ratio (SAR), which indicates the soil is both saline and sodic (alkaline) . Normally, in agricultural soil, Ca^{2+} is the major ion that takes up the cation exchange sites at the surface of negatively charged soil particles and keeps the particles aggregated (Bernstein, 1975). Due to calcium being a double valent ion, the soil particles are loosely packed and leave pores which facilitate air, water and nutrient transport, as well as root extension in between soil particles (Bernstein, 1975). However, in some salt-impacted soils, the presence of large amount of Na^+ leads to the gradual replacement of calcium by sodium as the dominant ions tied to soil particles (Bernstein, 1975). Because sodium is a monovalent ion, soil particles associated with sodium ions will become smaller in size and tightly packed, and result in the dispersion of soil particles and the degradation of soil structure (Bernstein, 1975). Sodium adsorption ratio (SAR) is a calculation of the amount of sodium (Na^+) relative to calcium (Ca^{2+}) and magnesium (Mg^{2+}) in soil solution (Bernstein, 1975; Läuchli & Luttge, 2002). The structure of the soil deteriorates as SAR increases, with the optimal values for SAR being less than 4.0. SAR values higher than 15 indicate that the soil quality is severely degraded (Läuchli & Luttge,

2002). The soil SAR was shown to be 23±10 in plot 4 and 5 and 12±3 in plot 1 and 2. Though gypsum was applied to ameliorate the sodic soil, the soil still had a high sodicity in 2009. At the beginning of the season, the surface soil was dry and loose due to tilling; the middle layer (25-50 cm) was wet and sticky, whereas the deepest sampling horizon (50-75 cm) became dry again. This is a typical sign of sodic soil, in which the soil pores in the middle layer were clogged and water infiltration to the lower layer was blocked. It is difficult for plant roots to break into the highly packed sodic soil, thus nutrient uptake was hindered to support biomass production.

The CMS site had the most uneven salt distribution of the three sites. Plots 3 and 4 (including the blank area) (Figure 3.6 and 3.8) were in a low area and were flooded every season. Salt might have been carried by rainwater from plots 1 and 2 to plots 3 and 4, resulting in uneven salt distribution and vegetation coverage. Plants thrived in plots 1 and 2 in spite that the average surface EC_e was as high as 5.9 dS/m and 14.0 dS/m, respectively. However, seed germination was strongly inhibited in plot 3A and 4A where the salinity level was close to 30 dS/m, which is above the tolerance limits of all the planted species (Miyamoto et al., 2004). Where plants did grow in plots 3A and 4A they were generally short and low in density. Therefore, salt accumulation in plant biomass was negligible, and these two plots were treated as a blank area in data processing.

Although salt in the surface soil decreased at the end of the season at both CMN and CMS, salinity in surface soil increased at midseason (Figure 3.3 and 3.9). During the summer, high temperature, low precipitation, and vigorous plant transpiration would lead to high water loss from the surface, which might pull water up from the subsoil along with the salt dissolved in it. After the water evaporated, any salt it contained would remain in the surface soil, resulting in a salinity increase at mid-summer. The surface soil EC_e decrease at the end of the season indicated

that plants might not only remove salt from the surface soil, but also take up a portion of salt ions from the subsoil that was brought up to the surface. Another reason for midseason EC_e increase might be the salt concentrating effect of roots. When plants were growing in saline soil, salt accumulated near the root surface because plants took up water faster than they took up salt.

No consistent results were observed in salinity change in subsoil (25-75 cm) at CMN and CMS (Figure 3.4 and 3.10). The subsoil water movement was not measured, so conclusions could not be drawn on the salt migration in the lower soil horizons. The salinity increase in the middle layer (25-50 cm) (Figure 3.4) might result from the leaching from the surface soil, as well as the replenishment from salt in layers lower than 75 cm.

In the blank area, the salt mass in the surface soil decreased at CMN and did not change at CMS (Figure 3.3 and 3.9). Without the vegetation to hold the rainwater close to the root zone, salt might be leached further down to soil layers beyond 75 cm. However, insufficient data have been collected to support this hypothesis. In the future, more sampling points from each plot, samples from deeper horizons, and the knowledge about soil water movement might provide more insights into the salt mass balance study.

An average surface soil EC_e of 30 dS/m at Alameda (Figure 3.14) is above the tolerance limits of most plant species. In previous years, inhibition of the germination and growth of PGPR-treated plants were also observed. This year, in addition to high salt, the Alameda site was stricken by a hard frost three days after planting (Figure 4.1). The frost was devastating to the selected plant species, because these non-cold-tolerant species could not survive the sudden drastic decrease in temperature during the germination and the fragile seedling stages. This allowed the highly cold and salt tolerant weed species kochia (*Kochia scoparia*) to grow instead (Steppuhn & Wall, 1993). The salt uptake by kochia and precipitation leaching might be the main

reason of the EC_e dropped at midseason (Figure 3.14). Unfortunately, we were unable to estimate the plant contribution to the salinity decrease, since the dry mass of most plant samples, including kochia, from both midseason and the end of the season did not provide sufficient sample dry weight to determine the sodium and chloride content in plant tissues according to the analysis methods used by the accredited laboratory (ALS laboratory). Only two kochia samples from midseason, one kochia and one grass sample from the end of season were sent to ALS. Analysis results (see Appendix Table 1) indicated that the ability of kochia to accumulate Na^+ and Cl^- was comparable to our selected species. However, because kochia lacks economic benefits and spreads quickly (Steppuhn & Wall, 1993), it is an unsuitable species for phytoremediation, especially when the site is required to be reclaimed for agricultural use.

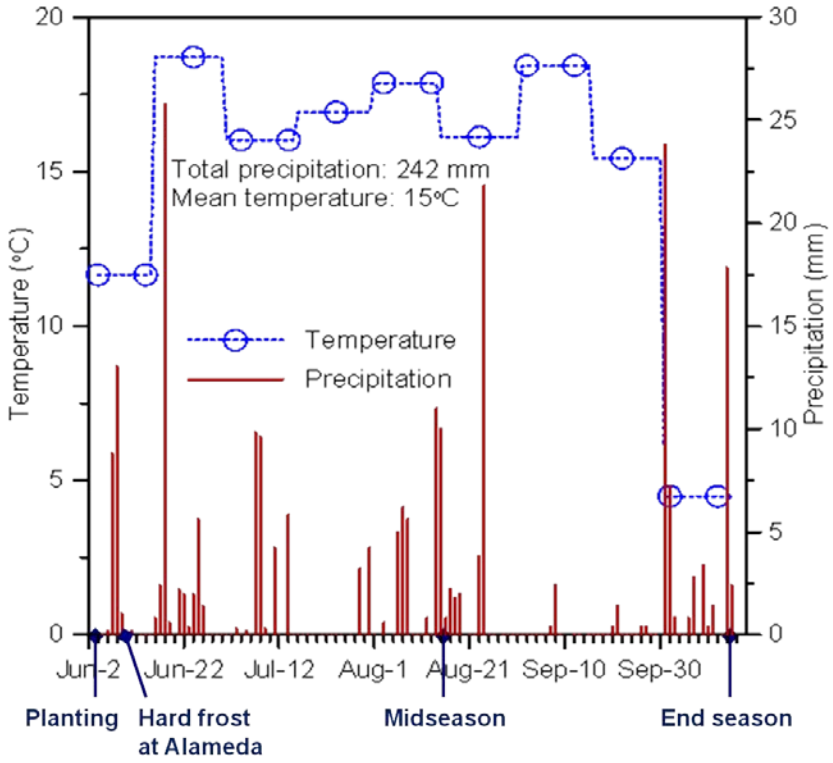


Figure 4.1: Temperature change and precipitation distribution in Estevan (the nearest weather station for three research sites) over the 2009 growing season.

4.1.2 Salt mass balance study and remediation efficiency assessment

The plant samples were shipped to Waterloo from Saskatchewan and washed before sending to the analytical laboratory for analysis. The purpose of washing was to remove the dirt and obtain more accurate results of plant dry biomass. However, due to the high solubility of sodium and chloride ions, washing plant samples rinsed off the salt that was secreted to the leaf surface and drew out more ions from the damages caused by destructive sampling, and thereby largely lower the accuracy of the determination of salt concentrations in plants. This was proven by the significant discrepancy of plant uptake data ($P<0.05$) between washed and unwashed samples (Table 3.3 and Figure 3.5).

After the adjustment for salt loss due to washing the tissues, the remediation efficacy was 59.9% for CMN and 56.1% for CMS. Therefore, approximately 60% of the EC_e decrease in the surface soil was accounted for by plant uptake of NaCl. The remaining 40% might result from either the plant assimilation of other ions like Ca^{2+} and K^+ that are also highly soluble in soil water extract, or the leaching of solutes by water to lower soil horizons. Though the remediation target was Na^+ and Cl^-, which are known to be detrimental to the soil structure and the crop growth, the quantification of plant accumulation of Ca^{2+} and K^+ can provide a clearer picture of plant contribution to EC_e decreases in the surface soil. Because high concentrations of Ca^{2+} and K^+ in plant tissues were observed in the 2007 and 2008 season, it was hypothesized that the other 40% of the EC_e decrease in the surface soil resulted from the plant uptake rather than leaching.

After the incorporation of Ca^{2+} and K^+, plant uptake was calculated to account for 117.5% and 107.5% of the EC_e decrease in surface soil at CMN and CMS, respectively. The higher than 100% values indicated that plants might not only accumulate salt ions from the surface soil, but also partially from lower layers horizons (25-75 cm). Soil solutes in different soil layers migrate

along with water movement. When evapotranspiration was high during low precipitation and high temperature period of summer, solutes from the lower horizons (25-75 cm) might have moved upwards to the surface layer and would be deposited there as water evaporated. The increased midseason EC_e indicated more solutes were present in the surface layer (Figure 3.3 and 3.9) than the beginning of the season. These solutes brought from the lower horizons to the root zone were absorbed by plants. Therefore, it can be concluded that the other 40% of EC_e drop was due to plant uptake of ions other than Na^+ and Cl^-.

However, the simulation was based on a series of assumptions that might lead to errors. First, the Ca^{2+} and K^+ concentrations in plants in the 2009 season were estimated by the ratios of $[Ca^{2+}]/[Na^+]$ and $[K^+]/[Na^+]$ calculated using data from 2008. These ratios may vary from midseason to the end of the season and from year to year. Second, since the concentrations of Na^+, Cl^-, Ca^{2+} and K^+ in plant samples were over 5000 mg/L, they were assumed to be the only contributors to EC_e in soil. However, all the exchangeable ions in the soil might be extracted by water during analysis. They would thus serve as components of the EC_e, as well as be taken up by plants (Rhoades et al., 1999). If all the ions accumulated in plants were taken into consideration, the value of $EC_{e\ plant}$ might be higher than estimated in this study. Third, calcium, depending on its counter ion, might not be readily soluble in soil water extraction and contribute to EC_e. Gypsum ($CaSO_4 \cdot 2H_2O$), which was applied in large amounts in 2007, was supposed to be the primary Ca^{2+} source in CMN and CMS. However, unlike the high solubility of sodium and potassium salts, $CaSO_4$ is only moderately water soluble. Hence, the water extraction in soil EC_e measurements might only contain a portion of soil calcium that is readily soluble. However, at the soil and plant roots interface, the amount of Ca^{2+} in solid phase and that is dissolved in water phase is in a dynamic equilibrium. As Ca^{2+} concentration in water phase is lowered by plant

assimilation, Ca^{2+} attached to soil particles will be released into water phase to maintain equilibrium. Whereas EC_e measurement can only give an indication of the portion of Ca^{2+} that is water soluble, plant contained Ca^{2+} might derive from both the soluble portion and the Ca^{2+} storage in the solid phase. Consequently, the contribution of plant uptake of Ca^{2+} to surface soil EC_e decrease might have been overestimated. In aged salt-impacted soil, similar to Ca^{2+}, excessive sodium ions may also be tied to soil particles and slowly released for plant absorption. Therefore, an overestimation of plant contribution to EC_e decrease might occur during mass balance calculation.

Compared to leaching, phytoremediation of salt is more advantageous. First, as mentioned above, phytoremediation is an irreversible process, when plants with salt are removed from the site. After the adjustment for washing loss, approximately 613 kg/ha of NaCl at CMN and 426 kg/ha at CMS would be removed if the foliage is removed from the site (calculated by converting total NaCl uptake data in Table 3.1 and 3.8 to per hectare data). In contrast, no salt would be actually removed from the site by leaching, and the potential harmful effect of salts always exists. Second, phytoremediation of salt-impacted soil is more cost effective and less labor intensive for the remediation of salt-impacted soil from sites in Saskatchewan. If leaching was implemented to remove the same amount of NaCl, a large quantity of fresh water would have to be applied, and effective drainage systems must be built to collect the salt water. Many salt sites were in the middle of relatively dry and under-populated area. The access to the fresh water and the construction of drainage system would be expensive and labor intensive. However, during phytoremediation, plants were gradually extracting salt from the soil without necessary human interference and management. Furthermore, the addition of amendments in order to compensate for the leaching loss of nutrients would result in extra cost. Third, leaching is not likely to be

effective in remediating the sodic soil, since the soil has been dispersed and compact. Leaching might result in waterlogging (Monteiro et al., 2009).

Compared to ex-situ strategies such as excavation of salt contaminated soil and disposal, phytoremediation is less destructive of the local environment. Furthermore, when the brine water spill covers a large area, and salt has been leached down by rainwater to deep horizons, excavation is labor consuming and expensive, and becomes impractical. Therefore, phytoremediation is more appropriate for the reclamation of large-scale salt-impacted soil.

4.2 Assessment of salinity and PGPR effects on physiological parameters

Salinity effects on stressed plants are reflected by the changes in physiological indicators compared to non-stressed plants (Ashraf & Harris, 2004). Frequently used indicators for salt stress include soluble osmolyte accumulation, antioxidant activities, membrane integrity, K^+/Na^+ ratio and photosynthetic activity (Ashraf & Harris, 2004; Parida & Das, 2005). PGPR ability to protect plants against the harmful effects of salinity can then be studied by examining its amelioration of these parameters. The plant species chosen for this study, annual ryegrass (*Lolium multiflorum*), is used as a pasture and forage crop with high nutritional value (Sagi et al., 1997). It has a densely fibrous root system that aids in holding soil, increasing water infiltration, and improving soil tilth (Sagi et al., 1997). Annual ryegrass is ranked as a moderately salt sensitive species with a tolerance limit of 6 dS/m (Miyamoto et al., 2004), and has previously used as a mulch species to decrease the surface soil salinity in Australia (Barrett-Lennard, 2003). A study of Australia saltland pastures showed that the removal of the grass caused the concentration of salt at the soil surface to increase by 140%. Cultivation and sowing with a mixture of cereals and annual ryegrass for four years re-established plant cover and decreased

soil salinity in the upper 30 cm by 50-75% (Barrett-Lennard, 2003). However, very limited information can be found in the literature about the physiological changes of annual ryegrass under salt stress and no study has been performed on the amelioration of the physiological performances of PGPR on this species (Ji & Huang, 2007). This is the first time that salinity stress indicators such as proline accumulation, SOD activity, electrolyte leakage and photosynthesis were evaluated in annual ryegrass. It is also the first study that partially attributes the alleviation of salt stress to the PGPR inoculation.

4.2.1 Plant growth

Annual ryegrasses were planted in control (0 dS/m), NaCl spiked salt soil (10 dS/m) and diluted salt-impacted soil (10 dS/m). An EC_e of 10 dS/m was well above its tolerance limit. It was expected that the germination and growth of annual ryegrass would be adversely affected by both types of salt soil, regardless of the PGPR treatment.

The germination of the salt treated pots was delayed by 2 to 3 days compared to the un-impacted control pots. PGPR did not accelerate the germination speed of the treated plants. The germination rate also decreased in salt soil as indicated by the visible low density of plants. These observations were in accordance with the studies showing that moderate levels of NaCl (2 to 6 dS/m) affected dormancy and germination percentages of annual ryegrass, but did not cause plant death (Alonso et al., 1999; Marcar, 1987). Germination might be blocked as a consequence of the difficulty of seeds to imbibe water under high osmotic pressure rather than NaCl toxicity (Alonso et al., 1999).

The growth of annual ryegrass in salt soil was obviously impaired as compared to the un-impacted control plants. The decreased plant growth might be due to drought induced loss of

turgor and outflow of intracellular water, and the disruption of metabolism induced by the overaccumulation of Na^+ and Cl^-. For example, a portion of energy might be allocated to produce and accumulate organic compounds in cells and to avoid the uptake and transport of toxic ions to leaf tissues (Greenway & Munns, 1980). As indicated in Figure 3.16, plants growing in spiked soil had less biomass and shorter leaves than plants growing in diluted salt soil, although these two types of salt soil were at the same salinity level. There are several reasons that the spiked salt soil was more inhibitory to plant growth. First, an EC_e of 0.5 dS/m of the un-impacted topsoil indicated a negligible amount of salt ions in the soil. Therefore, in the spiked salt soil, an EC_e of 10 dS/m was mostly contributed by the added NaCl, which is considered to be the primary toxin in saline soil. In contrast, in diluted CMS soil, other than sodium and chloride, other major contributors to salinity was potassium and the amended calcium, which is beneficial for plant growth. Second, in aged field soil that was collected from CMS, the salt cations are attracted to the negative charged surface of soil particles and not readily available to plants; whilst the salt ions in spiked salt soil was prepared one day before the planting day by mechanically mixing sodium chloride powder into the un-impacted topsoil. Both sodium and chloride would be dissolved in water that was applied to pots daily during the growing period. Consequently, plants growing in spiked soil were subjected to higher concentration of toxic salt ions than the diluted CMS soil, and thereby exhibited more inhibited growth.

Although no significant promotion of plant fresh weight with PGPR treatment ($P<0.05$) (Figure 3.19), PGPR-inoculated plants, especially UW4 treated plants in spiked salt soil, had less decreases in leaf biomass when compared to the non-PGPR plants. An average increase of 9% increase in fresh leaf biomass may seem a small change in greenhouse study. Nevertheless, if plants grown at remediation sites had an improved biomass of 9%, much more salt could be

removed from the site. The enhanced biomass production could be attributed to the alleviation of stress ethylene in plant tissues by ACC deaminase activity in PGPR. It has been well established that ethylene biosynthesis in plants is stimulated in response to salt stress (Cao et al., 2007; Ouaked et al., 2003). Ethylene regulates stress and defense responses and many key events of plant growth and development (Ouaked et al., 2003). Elevated levels of ethylene paralleled with impaired plant growth under salt stress have been found in rice (Lutts et al., 1996), wheat (El-Shintinawy, 2000), tomato (Botella et al., 2000; S. Mayak et al., 2004a; Mizrahi, 1982), lettuce (Zapata et al., 2004) and various other plant species (Zapata et al., 2007). The concentration of ACC, the immediate precursor of ethylene, in plants under salt stress was also examined by several studies. Gomez-Cadenas et al, 1998 found that ACC production in a citrus rootstock increased proportionally to the magnitude of salt stress. Plants watered with 90 mM NaCl had 18-fold higher in ACC concentration compared to control plants (Gomez-Cadenas et al., 1998). The ACC accumulation followed a two-phase response, with an initial increase which was related to osmotic onset of salt treatment, and an overlapping gradual and continuous accumulation caused by the buildup of salt ions in leaves (Gomez-Cadenas et al., 1998). Based on the observation of these studies, it was assumed that in our study, ACC and ethylene levels in annual ryegrass were enhanced by high salinity. The symptoms of excess ethylene production included the inhibition of seed germination, root elongation, seedling growth and biomass accumulation. UW4 and CMH3 with high ACC deaminase activity and salt tolerance ability can lower ethylene levels and thereby enhance biomass yield.

Transgenic plants that express bacterial ACC deaminase also have improved ability to tolerate high salinity (Sergeeva et al., 2006) and various other stresses (Farwell et al., 2006; Grichko & Glick, 2001; Robison et al., 2001). However, these transgenic plants responded

similarly to non-transformed plants treated with plant growth-promoting bacteria with ACC deaminase activity (Glick et al., 2007). The bacterially-treated plants generally outperform the transgenic plants (Glick et al., 2007). This might indicate the fact that in addition to lowering their ethylene levels, plant growth-promoting bacteria can do more for plants, such as the production of auxins (Glick et al., 2007).

4.2.2 Proline accumulation

As a sensitive indicator of drought and salinity stress, proline is one of the most common compatible osmolytes that has been discovered to accumulate in various crop and grass species (Colmer et al., 1996; Lutts et al., 1999; Marcum & Murdoch, 1994; Qian et al., 2001). Proline is not only derived from protein degradation, but also by de novo synthesis in the cytosol in response to stress (Hare et al., 1999). The 20 to 25 fold increases of proline in both types of salt soil in this study was observed. This is in accordance with studies that proline levels could increase up to 80% of the total soluble free amino acids as a result of salt treatment, while under non-stressed condition, less than 5% of the total pool of free amino acids is maintained as proline (Matysik et al., 2002; Thomas et al., 1992). However, the magnitude of the increase under salinity stress, more than 20 fold, was only previously reported in root tips (Colmer et al., 1996) prior to this study. The higher than usual increase in proline content might be due to the differences in the sampling method used in the colorimetric determination of proline.

The initial rise of proline on day 12 in both PGPR-treated and untreated plants indicated that there was a large demand of proline at the beginning of seedling growth. The following decline on days 15 and 18 was probably a sign that plants acclimated to the high salt environment. For example, osmotic adjustment had been achieved by gradual uptake of salt ions, or the overall

metabolism was weakened to a steady reduced level, and proline synthesis was shut down or decreased.

PGPR treated plants had higher proline levels than the uninoculated plants throughout the experimental period, and significant improvements were observed on day 12 and day 15. UW4 and CMH3 might protect the host plants against detrimental effect of salt by increasing proline level during active growing period by approximately 30%. This observation was in accordance with a previous study showing significant increase of proline accumulation in lettuce leaves treated with a *Pseudomonas* PGPR strain under drought stress (Kohler et al., 2010).

Though primarily recognized as osmolytes, the role of increased proline in annual ryegrass, like in other plants, should be multiple. In fact, the significance of proline contribution to the overall adjustment of osmotic pressure has been challenged. A recent study showed that up to 95% of osmotic adjustment in old leaves and between 80% and 85% of osmotic adjustment in young leaves of a quinoa (*Chenopodium quinoa Willd*.) was achieved by means of the accumulation of inorganic ions (Na^+, K^+, and Cl^-), whilst the contribution of organic osmolytes was very limited (Hariadi et al., 2011). Similar results were reported in another study of tomato under salt stress, where free proline contribution to psi did not exceed 5% (Shalata & Tal, 1998). Therefore, an abundance of proline in cytosol under salt stress might have other functions.

One of the proposed functions of proline was to act as a ROS quencher. Increased proline concentrations in salt-stressed plants have been frequently correlated with the less membrane electrolyte leakage and malondialdehyde (MDA) production (Bandurska, 2001; Jain et al., 2001; Jaleel et al., 2007), which is also supported by the observation in this study. PGPR treated plants with elevated amounts of proline showed decreased membrane injuries compared to the non-treated ones (Figure 3.23 and 3.24). It has been well established that membrane damage is due to

the active ROS attack of lipids (Bandurska, 2001; Dhindsa et al., 1981). The molecular mechanism of proline quenching of ROS is related to the chemical property of its pyrrolidine, which confers it ability to effectively react with singlet oxygen and hydroxyl groups, and thereby neutralize the destructive effects of ROS on important molecules, such as DNA and enzymes (Matysik et al., 2002). It was also found that proline can act as a potent antioxidant and inhibitor of apoptosis in a fungal pathogen using a CAT-dependent mechanism (Chen & Dickman, 2005). In turn, it has also been shown that abiotic stress induced excess ROS could signal the expression of anionic glutamate dehydrogenases, which could catalyze the formation of glutamate, a precursor of proline (Skopelitis et al., 2006).

It was also demonstrated in previous studies that exogenous addition of proline into plant culture medium or transgenic plants overproducing proline had enhanced biomass yield, K^+ uptake and chlorophyll contents (Gadallah, 1999; Kishor et al., 1995). The interaction between proline accumulation and various salt stress indicators indicated the chemical active status of proline in cytosol and its important role as an osmoprotectant. PGPR's protection of annual ryegrass against salt stress might be largely derived from the up-regulation of proline synthesis than the uninoculated plants and the action of proline to scavenge ROS, hence decrease membrane damage and diminish the stress perceived by plants growing in salt soil.

Though not assayed in this study, various other amino acids, betaine and soluble sugars can also act as osmoprotectants (Ashraf & Harris, 2004; Gilbert et al., 1998). For example, glycinebetaine, mainly synthesized in chloroplast, has been shown to stabilize many functional units such as the electron transport chain, membranes and proteins and enzymes such as RuBisCo (Makela et al., 2000). Our observation that PGPR significantly ameliorated photosynthesis parameters in salt-stressed plants is consistent with this. Therefore, PGPR might

also enhance the production of glycinebetaine, which protected enzymes involved in photosynthesis and thereby alleviated the salt induced damage of photosynthetic apparatus.

4.2.3 SOD activity and membrane leakage

Superoxide dismutase (SOD) activity is a widely used indicator of salt stress. When salt stress is imposed, superoxide anions ($O_2^{\bullet-}$) that are formed as by-products of the electron transport chain will increase due to the damage of chloroplast and mitochondria. SOD is the enzyme that catalyzes superoxide to H_2O_2. If not detoxified by CAT and APX, H_2O_2 would form extremely aggressive oxygen species hydroxyl groups (·OH) through fenton reaction. The efficient removal of superoxide by SOD is therefore very important to prevent the production of highly destructive hydroxyl groups. The induction of SOD is considered as one mechanism of salt tolerance in plants (Hernández et al., 2003).

In this study, a general trend of increased SOD activity over 18 days in salt soil implied the growing need of plants to scavenge superoxide during salt stress. A significant increase ($P<0.05$) of SOD level was observed when annual ryegrass was subjected to salt stress at 10 dS/m (Figure 3.20). This is in agreement with studies showing that SOD activity increased along with the increase of salinity levels (GuetaDahan et al., 1997; Hernandez et al., 2000; Meloni et al., 2003). The increased SOD was supposed to quench ROS and protect plant cell membranes from being damaged. However, membrane leakage was still significantly elevated ($P<0.05$) by salinity and showed a trend of steady increase over time (Figure 3.22). There might be several reasons for this. First, stress induced production of ROS might be too overwhelming for plants to generate enough antioxidants to eliminate them. Even with an overall elevation in the antioxidant production system, excessive ROS would still be yielded if the production rate is constantly

higher than the removal rate. As a vulnerable target of ROS, membrane lipids are readily damaged. Second, the increase in SOD activity sometimes might not be matched by the subsequent increase of enzymes like CAT and APX (GuetaDahan et al., 1997) that can detoxify H_2O_2, and result in the accumulation of destructive hydroxyl groups. Third, the overall low metabolism of plants under salt stress might limit the plant resources that can be used for enzyme synthesis.

This study also showed that for plants grown in non-saline soil, PGPR inoculated plants had higher levels of SOD than un-inoculated plants (Figure 3.21). This indicated that PGPR-treated plants had more tight control over ROS even under non-stressed condition. Under salt stress, PGPR treated plants exhibited an average of 10% increase in SOD activity and a parallel 12% less membrane leakage over 18 days relative to the control plants. For various plant species, it has been well-established that salt tolerant genotypes have inherently higher SOD activity and lower membrane damage than the sensitive genotype, indicating that tolerant genotype has a better protection mechanism against oxidative damage by maintaining a higher constitutive and induced level of antioxidant enzymes (Bor et al., 2003; Hernandez et al., 2000; Shalata & Tal, 1998). PGPR treatment could confer the moderately sensitive annual ryegrass tolerance to salt with enhanced SOD activity and lower level of membrane leakage.

SOD increases due to PGPR treatment were not significant ($P<0.05$) on any sampling day; however, significant lower membrane leakage ($P<0.05$) in PGPR treated plants was observed on day 9 and 12 in NaCl spiked soil and on day 18 in both types of salt soil. PGPR might help annual ryegrass adapt to oxidative stress and lower membrane damage through various other pathways in addition to increasing SOD activity. For example, increased proline levels to scavenge singlet oxygen and hydroxyl groups. In addition, among various antioxidants, only

SOD was assayed. However, other antioxidant enzymes, such as CAT which can detoxify H_2O_2, might also play an important role in protecting membrane integrity. PGPR have previously been shown to have the ability to elevate CAT and POX activities in response to severe salinity (Kohler et al., 2009). Furthermore, ROS quenching is also achieved by non-enzymatic reactions involving the activities of water soluble ascorbate (ASC) and glutathione (GSH), and the lipophilic α-tocopherol, which is also known as Vitamin E. α-tocopherol is worth mentioning, since it prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes (Munne-Bosch, 2005). A study of the concentration change of α-tocopherol might provide a better link between the antioxidant system and membrane damage under salinity and PGPR treatment. Therefore, PGPR effects on the change of a range of ROS scavengers should be further studied to elucidate which chemical is closely related to ethylene signaling transduction and the mechanism of PGPR protection of plants from activated oxygen species.

Proline, SOD and electrolyte leakage has been tested in both NaCl spiked soil and diluted CMS soil at 10 dS/m. The spiked soil showed a more inhibitory effect on plant growth than the diluted CMS soil. Nevertheless, plants grown in NaCl spiked soil only showed slightly higher levels of proline and membrane leakage than those in diluted CMS soil, while SOD activities did not differ. The small variation of physiological parameters between the two different salt treatments suggests that physiological responses were sensitive to overall salinity level rather than to the specific ions that contribute to salinity. Salt-induced osmotic stress might play a leading role in changes of these physiological parameters, as similar responses have been observed in drought-stressed plants (Bandurska, 2001; Flexas et al., 2004; Mittler & Zilinskas, 1994; Verslues et al., 2006).

4.2.4 Photosynthesis

Chlorophyll fluorescence measurements can give an accurate, rapid and minimally invasive assessment of changes in photosynthesis. This study demonstrated that in annual ryegrass, a salinity level of 10 dS/m led to significant decreases of fluorescence parameters like maximum quantum yield of PSII (F_v/F_m) ratio, effective quantum yield (Yield) and photochemical quenching (qP), and a paralleled significant increase in non-photochemical quenching (qN).

The maximum quantum yield (F_v/F_m) reflects the intactness of PSII. Decreased F_v/F_m is often associated with unrepaired damage to PSII, which can also correlate to a rise in heat dissipation (qN) within the reaction center (Maxwell & Johnson, 2000). Yield measures the proportion of absorbed energy being used in sustained electron transport, which indicates the light using efficiency of PSII in photochemistry (Maxwell & Johnson, 2000). A decrease in yield would indicate possible damage to the electron transport chain, which can result in the alternative usage of the absorbed photons, such as exciting triplet oxygen to its singlet form, which is a type of ROS (Blokhina et al., 2003; Maxwell & Johnson, 2000). The parameter of qP gives an indication of the proportion of PSII reaction centers that are open (Maxwell & Johnson, 2000). A decrease in qP under salt stress might result from the salt induced closure of reaction centers (Maxwell & Johnson, 2000; Schreiber et al., 1995). In this study, the significant decrease of F_v/F_m, yield and qP, accompanied by the increase of qN, are indicative of the salt-induced damage to PSII of annual ryegrass. First, the damage might be due to salt induced inhibition of enzymes in the electron transport chain and the Calvin cycle. Previous studies described that the accumulated salt ions could alter thylakoid structure, and impair the activities of enzymes in the electron transport chain of PSII (Barber et al., 1980). A possible structural change of PSII is also suggested by the increase of thermal dissipation of light energy (qN) (James et al., 2002). Second,

PSII damage might come from ROS attack of the membrane system. The chloroplast is the main organelle that produces ROS in plants under stressed conditions, and therefore becomes the primary target of deleterious ROS (Abogadallah, 2010). A study of two turfgrass species growing in salt-affected soil found a decline in leaf photochemical efficiency (F_v/F_m) alongside an increase in electrolyte leakage (Liu et al., 2011). This observation, together with the similar results from the present study, would suggest the potential correlation between ROS damage and decreased photosynthesis. Third, salt stress has been reported to result in significant decline of leaf chlorophyll content, which might also contribute to the overall decrease in photochemistry (Ayala-Astorga & Alcaraz-Melendez, 2010; Gadallah, 1999; Sairam et al., 2002). The loss of chlorophylls in salt stressed plants was correlated to an overproduction of ROS in chloroplast as a result of restricted CO_2 supply and an increased leakage of electrons from photosystems to O_2 (Mittler & Zilinskas, 1994; Streb & Feierabend, 1996).

In addition to the injuries of PSII and the resulting inhibition of light-dependent reactions as indicated by the PAM study, the dark reactions might also be subjected to salt-induced restrictions, which in turn down-regulate the light reactions. This is observed in the lower yield values. Salt induced osmotic stress can lead to stomatal closure and the subsequent reduced rate of CO_2 assimilation, and thereby limits the carboxylation efficiency and the production of photosynthates under stressed condition (Delfine et al., 1999; Flexas et al., 2004). Previous studies have provided evidences that the decrease in stomatal and probably also mesophyll conductance is the predominant factor that affects the diffusion and fixation of CO_2 in the leaves of drought and salt-stressed plants (Flexas et al., 2004). The gas exchange rate was not measured in this study; the stomata condition under salt stress was therefore not clearly known. However, it could be assumed that a salinity of 10 dS/m, which is higher than annual ryegrass's tolerance

limit, should be able to cause stomatal closure that would occur to constrain plant water loss. Furthermore, from the daily control of the soil moisture content, it was observed that the amount of water loss from the salt treated pots was much lower than the water loss in control pots, indicating lower transpiration rate in salt stressed plants. Plants growing in salt soil were perceived to be less hydrated than control plants based on visual observation. The examination of leaf water content and water use efficiency might provide further evidence of changes in stomata closure caused by the osmotic pressure. In addition, salt ions could also negatively affect the dark reactions. As K^+ is a major ion involved in stomatal control, the inhibited K^+ uptake under high salinity condition might also result in stomata closure. Also, the high concentration of Na^+ and Cl^- has been shown to inhibit the activity of the key enzyme RuBisCo in dark reaction, and therefore result in a decreased rate of the CO_2 assimilation (Davies et al., 1983).

PGPR treatment successfully restored all the fluorescence parameters tested in this study close to the non-stressed levels. The PGPR's improvement effect of these photosynthesis indices was previously observed in barley, oats, tall wheat grass and tall fescue (Wu, 2009). It is not completely clear how PGPR protect the photosynthetic apparatus from being impaired by high salinity, or how PGPR inoculation signals downstream factors to adjust the photosynthetic activity. However, based on the mechanisms of salt damage to photosynthesis summarized above, we know that all the salt induced immediate and secondary stresses, including osmotic, ion-specific and oxidative stresses, could have negative effects on photosynthesis. Combined the results of physiological assays with PAM results, it is speculated that PGPR might enhance photosynthesis through osmoregulation to maximize stomatal conductance while maintain proper water potential under salt stress. The effect of PGPR inoculation on wheat under salt stress was shown to significantly increased the plant relative water content, suggesting PGPR's ability to

regulate osmotic pressure, maybe through the elevation of proline concentrations (Nadeem et al., 2010). PGPR might also help mitigate the effect of salinity on photosynthesis by lowering ROS concentration to a level that was less harmful to the chloroplast structure and enzymes involved in photosynthesis. The increased production of the ROS quencher proline and decreased membrane leakage in inoculated plants in this study supported this assumption. Therefore, though direct evidence of PGPR's protection of photochemical apparatus was not found, PGPR's amelioration of some physiological parameters that were negatively affected by salinity was expected to positively adjust photosynthesis.

4.3 Conclusions

In field studies, the phytoremediation of salt-impacted soil was successfully performed. Mass balance calculation and remediation efficacy assessment suggested that salinity declined in the surface soil by15% at CMN and by 11% at CMS. After adjusting for the amount of salt lost as a result of washing plant samples, plant accumulation of NaCl accounted for 60% of surface soil EC_e decreases at CMN and 56% at CMS. When K^+ and Ca^{2+} uptake were also taken into consideration by a simulation study, EC_e decrease that was caused by plant uptake of salt ions would be 107 % at CMN and 118% at CMS, confirming the significant role of plants in remediation process, as well as precluding the likelihood of considerable solute loss in surface soil by leaching.

The actual soil salt movement through the whole season is difficult to elucidate with the data at hand, especially in the subsoil, because interactions among factors such as weather, soil, groundwater and plants form a dynamic and variable system. Increasing sample numbers per treatment plot and more careful sampling design may mitigate the problems we encountered.

However, because most field studies have constraints including time and resources, the required extensive sampling is often not a realistic option. Insufficient sampling can lead to highly variable data that are difficult to interpret. In the future, an understanding of the water movement in the subsoil at these sites might provide insight into the salt distribution and migration in the soil profile. A better understanding of the vegetation effect on salt movement and plant contribution to salinity change can then be attained.

The evaluation of physiological parameters of annual ryegrass under salinity stress and PGPR treatment was also successfully performed. A salinity level of 10 dS/m led to significant increases ($P<0.05$) in proline, SOD activity and membrane leakage, which can be viewed as salt-induced damage to leaf tissues. PGPR's improvement of proline, SOD and photosynthesis, as well as repression of membrane leakage, indicated its protective role on annual ryegrass exposed to prolonged salt stress. Though isolated from different sites, UW4 and CMH3 exhibited similar promoting ability of annual ryegrass. With photosynthesis being excluded, all the parameters were only tested in aboveground tissues, but not roots. It is suspected that the changes in roots would be more drastic than in leaves, since the roots are in direct contact with the salt in the soil. It has been well established that the sodium concentration in the roots is higher than it is in xylem and the leaves, resulting from the plant ability to retain Na^+ in roots to mitigate damage to photosynthetic tissues (Tester & Davenport, 2003). Therefore, salinity and PGPR effects on physiological indicators might be better presented in roots. Other parameters such as nutrient uptake (N, P, and K), glycine betaine, CAT and APX activity and water use efficiency might be further studied to provide a more thorough picture of plant responses to salt stress, and PGPR's amelioration effect on these parameters. In addition, ethylene and ACC concentrations in PGPR treated and untreated plants under salt stress can also be determined to provide support to the

theory of PGPR’s enhancing effects of phytoremediation.

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Appendix

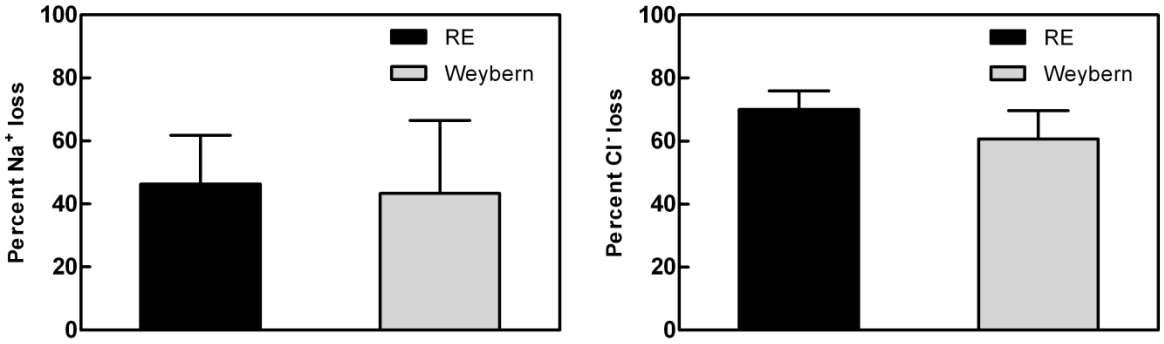

Figure 1: a comparison of the average loss of Na and Cl between washed plant samples from Red Earth (RE) and Weybern.

Table 1: a comparison of Na and Cl uptake in mixed grasses and kochia samples.

Plant sample	Na uptake (mg/kg DW)	Cl uptake (mg/kg DW)
Mixed grasses	2180	15500
Kochia	3200	6390

- DW stands for plant dry weight.