The Use of Plant Growth-Promoting Rhizobacteria (PGPR) and an Arbuscular Mycorrhizal Fungus (AMF) to Improve Plant Growth in Saline Soils for Phytoremediation

by

Pei-Chun Chang

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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.

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ABSTRACT

Upstream oil and gas production has caused soil salinity problems across western Canada. In this work we investigated the use of ACC (1-aminocyclopropane-1-carboxylate) deaminase-producing plant growth-promoting rhizobacteria (PGPR) and the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* to enhance the efficiency and feasibility of phytoremediation of saline soils. This work involved laboratory and field research for three sites in south east Saskatchewan, Canada. The three research sites were Cannington Manor South (CMS), Cannington Manor North (CMN) and Alameda (AL). CMS and AL were highly saline, while the CMN site had moderate salinity.

Indigenous PGPR were isolated from these sites and tested in greenhouse experiments using authentic salt-contaminated soils taken from the research sites. Increased plant biomass by PGPR and/or AMF was observed. This growth promotion effect varied with plant species, soil salinity and soil fertility. The combination treatment of two previously isolated PGPR *Pseudomonas putida* UW3 and UW4 (noted as UW3+4) from farm soil in Ontario consistently promoted shoot growth of both barley and oats grown in saline soils by approximately 100%. The indigenous PGPR *Pseudomonas corrugata* (CMH3) and *Acinetobacter haemolyticus* (CMH2) also promoted plant growth on par with UW3+4. In addition, in one experiment where alfalfa was tested, UW3+4, CMH2 and CMH3 treatments not only enhanced shoot biomass but also increased root nodulation. For AMF effects, *G. intraradices* enhanced biomass of oats and barley. Furthermore, the AMF+CMH3 was effective in promoting growth of Topgun ryegrass, while AMF+CMH2 was beneficial for Inferno tall fescue growth in salt impacted soils. The concentration of

NaCl in the plants grown in salt-impacted soils ranged from 24 – 83 g/kg. There was no evidence of an increase in NaCl concentrations of plant tissue by PGPR and/or AMF treatments. In addition, to determine the importance of nutrient addition to research sites, liquid fertilizer was applied to 2-week old plants. Results demonstrated that fertilizer effectively increased biomass, and more importantly the biomass of PGPR treated plants supplied with fertilizer was approximately 20% higher than that of plants treated with fertilizer alone. Therefore, research sites were then amended with compost before planting of the 2007 field trial.

Plant growth promotion by UW3+4 and CMH3 was tested in the summer of 2007 in the field. Prior to planting, soils were sampled from each site for soil salinity analysis. Barley, oats, tall fescue and ryegrass treated with and without PGPR were sown in plots. The plant coverage condition, NaCl concentrations and biomass of plant shoots were assessed to evaluate the PGPR effect. The results showed that PGPR promoted shoot dry weight by 30% - 175%. The NaCl concentrations of barley, oats and tall fescue averaged 53 g/kg, 66 g/kg and 35 g/kg, respectively. There was no evidence of an increase in NaCl concentrations of plant tissue by PGPR in the field. The salt removal of the CMN site was the highest among three sites due to the large amount of shoot biomass produced. The amount of salt accumulated in the shoots on the CMN site is estimated to be 1580 kg per hectare per year when both barley and ryegrass are planted together as a mix and treated with PGPR. Based on the field data, the estimated time required to remove 50% salt in the top 50 cm soil is seven years with PGPR treatments, while it takes fifteen years to do so without PGPR. In conclusion, PGPR-promoted phytoremediation was proven to be a feasible and effective remediation technique for soils with moderate salinity.

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ABBREVIATIONS

PGPR	plant growth-promoting rhizobacteria
AMF	Arbuscular mycorrhizal fungi
ACC	1-aminocyclopropane-1-carboxylic acid
EC	Electroconductivity
EC_e	Electroconductivity of a saturated soil paste extract
$EC_{1:2}$	Electroconductivity of a soil extract from fixed-ratio extract methods
K	The EC value based on fixed-ratio extraction can be related to the EC_e with the K
SAR	Sodium adsorption ratio, indicating the extent to which sodium contributes to the total salinity.
CEC	cation exchange capacity
OM	organic matter
UW3+4	The combination of PGPR UW3 and UW4

1. Introduction

The amount of salt-affected land worldwide is estimated to be 900 million ha, 6% of the global total land mass (Flowers, 2004). Salinity can result from the intrinsic salt and limited rainfall; however, salinization also commonly occurs as a result of human activities. For example, upstream oil and gas production have been causing soil salinity problems across western Canada.

Leaching salt downward in to the deeper layer with excess water is the most common method to lower soil salt content in the root zone (Qadir et al., 2003). However, soil leaching is not feasible for sites that are distant from water resources or for those with poor drainage. In such cases, more feasible *in situ* remediation techniques, such as phytoremediation, are in great need.

Phytoremediation is defined as the use of plants to remove contaminants, such as salt. Plants that are tolerant to salinity can yield aboveground biomass that accumulates salt and can be removed from the site through harvesting. Phytoremediation is particularly useful for remote, semi-arid, large-scale sites because this technique can be carried out *in situ* and does not need large amounts of water required by the salt leaching technique.

The research topic of this thesis is the phytoremediation for salt contaminated soils. In particular, it examined the growth promotion effect of plant growth-promoting rhizobacteria (PGPR) and an arbuscular mycorrhizal fungus (AMF) on plants grown in salt-impacted soils.

1.1. Soil Salinity

Soil salinity is defined as the concentration of dissolvable mineral salts extracted from soil by water (Richards, 1954). The extracted salts consist mostly of cations Na^+ , Ca^{2+}, Mg^{2+}, and K^+, as well as anions Cl^-., SO_4^{2+}, HCO^{3-}, CO_3^{2-} and NO^{3-} (Tanji, 2002). The electrical conductivity (EC) of the soil extract is often used as an integrated parameter for quantifying its salinity. The EC of a soil sample is mostly reported as the EC measurement of the extract of a saturated soil paste (EC_e). However, fixed-ratio extraction methods, e.g. $EC_{1:2}$ or $EC_{1:5}$, are often used due to its ease of measurement (Janzen, 1993). The EC value based on fixed-ratio extraction can be converted to the EC_e with an empirically determined K factor by Equation 1. K values range between two and four, depending on soil properties (Richards, 1954).

$$EC_{1:2} \times K = EC_e \qquad \text{Equation 1}$$

In addition to EC, sodium adsorption ratio (SAR) is used to assess soil sodicity (Equation 2). It is an important determinant of soil properties and plant growth. SAR indicates the extent to which sodium contributes to the total salinity. SAR is defined by the following equation:

$$\text{SAR (sodium adsorption ratio)} = \frac{[Na^+]}{\sqrt{([Ca^{2+}]+[Mg^{2+}])/2}} \qquad \text{Equation 2}$$

where the ionic concentrations are expressed in milliequivalents per liter in soil extract solution in equilibrium.

This equation was developed empirically by inoculating soils with salt solutions containing a mixture of a monovalent cation and a divalent cation until equilibrium between the soil and

solution established. Direct measurement showed that the proportions of monovalent and divalent cations present on the cation exchange sites of soils (exchangeable cations) had a linear relationship with the outcome of Equation 2, where concentrations are soluble cations in the soil solution (Richards, 1954). With this relation, the molar ratio of Na^+ to divalent cations (predominantly Ca^{2+} and Mg^{2+}) on the soil exchangeable sites can be easily estimated by simply measuring the cations in the soil solution.

Soils can be categorized into non-saline, saline, sodic and saline-sodic based on EC_e, SAR and pH (Table 1.1). Soils with an EC_e higher than 4 dS/m are consider saline (Richards, 1954). Soil with SAR more than 13 is defined as sodic soil. Sodic soils (SAR > 13) are inclined to have water infiltration problems due to the dispersion of clay particles in soil pore space that is previously available for drainage, resulting in a hard crust or soil cracking on the surface as the soil dries. This soil hardening can inhibit seedling emergence and growth. Consequently, soil leaching, a remediation approach for saline soils, is not suitable to sodic soils because excessive watering may further deteriorate soil properties and impede plant growth. This problem can be in part corrected by providing readily available source of calcium (Ca^{2+}) such as gypsum ($CaSO_4 \cdot 2H_2O$) or $CaCl_2$, which can provide Ca^{2+} to replace excess Na^+ on the cation exchange complex of soil (Qadir et al., 1996; Qadir et al., 2003).

Table 1.1. Classification of salt-affected soils and distinguishing properties.

Class	EC_e	SAR	pH
Nonsaline	< 4	< 13	< 8.5
Saline	> 4	< 13	< 8.5
Sodic	< 4	> 13	> 8.5
Saline-sodic	> 4	> 13	< 8.5

EC_e: electroconductivity (dS/m) of extract of saturated soil paste
SAR: sodium adsorption ratio
pH: pH of saturated soil paste

1.2. Salinity effects on plants and plant salt tolerance

1.2.1. Plant responses to soil salinity

Soil salinity inhibits plant growth and development with adverse effects such as osmotic stress, Na^+ and Cl^- toxicity, ethylene production, plasmolysis, nutrient imbalance, production of reactive oxygen species, and interference with photosynthesis (Sairam and Tyagi, 2004). The review herein focuses on the first three effects: 1) lower water potential of the root environment, 2) toxicity of excess Na^+ and Cl^- ions and 3) stimulated ethylene production. It should be noted here that throughout this thesis, 'salt' and 'NaCl' are used interchangeably.

Osmotic Stress

Plants can respond to water stress in a few seconds, whereas it takes days for plants to show salt-specific effects. Munns (2002) summarized the sequence of physiological responses of a plant that is exposed to salinity. Cells first shrink in the first second or minutes. Over hours, cells restore their original shape but their elongation rates slow down. Over days, the changes in the ability for cell elongation and division then lead to a decrease in expansion rate and final size; leaf growth is usually more sensitive to salinity than roots. After weeks, plants that accumulate salt at a higher rate may start losing the old leaves.

Early responses of plants to drought and salinity are very similar; both are attributed to water stress. Water stress-induced metabolic processes include a decrease in photosynthesis, the production of reactive oxygen species and generation of the plant hormone abscisic acid (ABA) (Bartels and Sunkar, 2005). When plants are exposed to high salinity, a decrease in growth rate is followed by a gradual recovery to a new reduced rate as the first response to the decrease in

water potential of soil rather than to the NaCl-specific toxicity (Verslues et al., 2006). This was supported by an experiment with polyethylene glycol (PEG) (Yeo et al., 1991) where PEG, a non-penetrating osmotic agent, caused a similar rapid decline of leaf growth as did salt. It was then further confirmed by Passioura and Munns (2000), who used a pressurization technique to maintain the water potential of plants while the soil was salinized. The results showed that the rapid growth inhibition was due to water stress rather than ion-specific toxicity.

Toxicity of excess Na^+ and Cl^- ions

Sodium is essential as a micronutrient for a limited number of C4 plants, but not for most C3 plants (Hopkins and Hèuner, 2004). Sodium deficiency symptoms of the bladder salt-bush (*Atriplex vesicaria*) are chlorosis and necrosis. Plants respond to the Na^+-specific effects with more intra-species variation than to osmotic effects (Munns, 2002; White and Broadley, 2001). Subbarao et al. (2003) argued that sodium should be categorized as a 'functional nutrient', defined as an requirement for maximum biomass growth for all plants (Subbarao *et al.*, 2003). Na^+ is generally assumed to compete with K^+ for absorption by plant roots through a mechanism that does not discriminate K^+ from Na^+ and thus Na^+ can inhibit the absorption of K^+. In addition, this mechanism requires no energy and is thought to operate by diffusive force, which involves ion channels (Epstein, 1979).

Once taken up, Na^+ may be translocated. Species that take up and translocate Na^+ freely to the shoot are 'natrophiles', while 'natrophobes' take up little Na^+ and usually retain Na^+ in the root with relatively insignificant tranlocation to the shoot (Smith *et al.*, 1980). Natrophiles transport Na^+ to shoots in the rapid moving transpiration stream in the xylem. Although Na^+ can

also return to roots *via* the phloem, the downward moving stream is essentially irrelevant. As a consequence, leaves or shoots accumulate higher concentrations of Na^+ than roots (Tester and Davenport, 2003). Most crops translocate little Na^+ to the reproductive or storage structure such as seeds because they are fed mainly through phloem. On the contrary, vegetative tissues are supplied mainly by the xylem flow and tend to be higher in Na^+ levels.

The salt tolerance of natrophiles is related to their ability to compartmentalize Na^+ in vacuoles because the cytoplasm can not tolerate high levels of Na^+. For example, enzymes isolated from salt tolerant plants such as *Atriplex* and *Salicornia* are equally sensitive to Na^+ (Greenway, 1972). The typical K^+ concentration is about 100 mM, while Na^+ rarely exceeds 20 mM. The metabolic toxicity of Na^+ is mainly attributed to the Na^+ competition with K for binding sites essential for cellar function including enzyme activation and protein synthesis (Blaha et al., 2000; Tester and Davenport, 2003).

Chlorine (Cl) is an essential micronutrient for higher plants. Chloride ion (Cl^-) is involved in the oxygen-evolving reactions of photosynthesis, maintaining electrical neutrality across membranes, and adjusting the vacuolative osmotic condition. Deficiency symptoms include reduced growth and wilting, followed by chlorosis, bronzing and necrosis. However, because Cl^- is mobile and can be readily taken up by most plants, Cl^- deficiency rarely occurs in the field (Hopkins and Hèuner, 2004).

Root cells take up Cl^- from soil solution through anion channels under saline conditions. Cl^- then traversus the root by a symplastic pathway to reach the xylem. Chlorine accumulates to higher concentrations in older leaves than in the newly mature leaves. The critical tissue Cl^-

concentration of leaves for toxicity is about 4-7 and 15-50 mg/g dry weight for Cl^- -sensitive and Cl^- -tolerant plants, respectively. Like Na^+, floral tissues generally have lower Cl^- levels than other shoot parts. Moreover, tissues that are fed predominantly through the phloem, e.g. fruits and seeds, tend to have the lowest Cl^- concentrations (White and Broadley, 2001).

To summarize, both Na^+ and Cl^- are taken up by plants primarily through passive symplastic pathways, driven by gradients and respiration fluxes. Plants translocate Na^+ and Cl^- mainly upwards in the xylem and accumulate in shoots or leaves although a small portion of these ions are in the phloem and can travel downward to the roots. Thus, tissues fed by phloem such as seeds and fruits tend to contain the lowest NaCl concentration.

Stimulated ethylene production

Salinity-induced stress on plants is in part the result of ethylene production (Blumwald, 2000; Cuartero and Fernandez-Munoz, 1999; O'Donnell et al., 1996; Shibli et al., 2007). For instance, ethylene production was stimulated by more than two-fold in tomato (*Lycopersicon esculentum*) and *Arabidopsis* that were exposed to salinity stress (Hall and Smith, 1995; Richard and El-Abd, 1989). Kukreja et al. (2005) also observed the salinity-induced increase in ethylene evolution, ACC (1-aminocyclopropane-1-carboxylate) content and ACC oxidase activity in chickpea. The relationship between salinity stress and ethylene production was demonstrated by an experiment where aminoethoxyvinylglycine (AVG), an ethylene biosynthesis inhibitor, alleviated salinity-induced plant responses such as increased hook closure and thickness of seedlings (El Beltagy et al., 1979).

1.2.2. Salt tolerance of plants

Salt resistant plants are categorized into two groups: salt-excluders and salt-includers. The former group of plants adapt to a saline environment by avoiding salt, whereas the includers take up salt and sequester it. Biochemical strategies to cope with salt stress include 1) selective accumulation or exclusion of ions, 2) control of ion uptake by roots and transport into leaves, 3) compartmentalization of ions at the cellular and whole-plant levels, 4) synthesis of compatible solutes, 5) alteration of membrane structure, 6) induction of antioxidative enzymes, and 7) induction of plant hormones (Khan and Rizvi, 1994; Parida and Das, 2005).

The plant responses to various salinity levels are listed in Table 1.2. Salt tolerance is usually assessed as the percent biomass production in saline versus control conditions over a prolonged period of time. In Table 1.3, salt tolerance of crops, forages, vegetables and woody plants are listed. The salt tolerance of any one species, however, varies with growth phases, ionic constitution of the soil solution, and soil properties. For example, some crop species that are very salt tolerant during later stages may be sensitive to salinity during germination. Sugar beets are highly salt tolerant during late growth stages, but extremely sensitive during germination. Barley has high salt tolerance during all stages, although it is also more sensitive during germination (Richards, 1954). Similarly, Verslues (2006) pointed out that the germination rate of *Arabidopsis* under salt stress was not well correlated with salt tolerance later in development.

Table 1.2. Degree of salinity and plant responses (Manitoba Agriculture-Food and Rural Initiatives, 2007).

EC_e (dS/m)	Degree of salinity	Hazard for crop growth	Plant response
0-2	Non-saline	Very low	Negligible
2-4	Slightly saline	Low	Restricted yield of sensitive crops
4-8	Moderately saline	Medium	Restricted yield of many crops
8-16	Severely saline	High	Only a few tolerant crops yield satisfactorily
> 16	Very severely saline	Very high	Only a few salt tolerant forage species grow satisfactorily

Table 1.3. Salt tolerance of crops, forages, vegetables and woody plants (Alberta Government, 2001; Richards, 1954).

EC_e (dS/m)[a]	Field Crops	Forages	Vegetables	Trees, Shrubs
High	kochia	beardless wildrye		Siberian salt tree
16	sugar beets	fulks altai grass		sea buckthorn
		levonns alkaligrass		silver buffaloberry
		alkali sucatan		
		salt grass		
		Nuttal alkaligrass		
		Bermuda grass		
		Rescue grass		
		Canada wildrye		
		western wheatgrass		
		altai wildrye		
		tall wheatgrass		
		Russian wildrye		
		slender wheat grass		
		perennial ryegrass		
		mountain brome		
8	6-row barley	birdsfoot trefoil	garden beets	hawthorn
	sunflower	sweetclover	asparagus	Russian olive
	oats	Dallis grass	spinach	American elm
	2-row barley	Sudan grass		Siberian elm
	fall rye	alfalfa		villosa lilac
	winter wheat	tall fescue		laurel leaf willow
	spring wheat	bromegrass		
Moderate	yellow mustard	orchardgrass	tomatoes	spreading juniper
		blue gramma	broccoli	poplar
		crested wheatgrass		
		intermediate wheatgrass		
	flax	reed canary grass	cabbage	ponderosa pine
	canola	meadow fescue		apple
		reed canarygrass		mountain ash
		big trefoil		
		smooth brome		
		milk vetch		
4	corn		sweet corn	common lilac
			potatoes	Siberian crab apple
				Manitoba maple

[a] the salinity range at which crops can be expected to yield 50% of normal yield.

1.3. Phytoremediation of saline soil

Phytoremediation is a non-destructive *in situ* remediation technique that used plants to clean up contaminated soil, water or air (Willey, 2006). Phytoextraction, a phytoremediation technique, is the use of plants to take up contaminants from the environment into the plant biomass that can be removed from the site (Raskin and Ensley, 2000). Throughout this article, phytoremediation and phytoextraction are used interchangeably. Unlike chemical or physical soil remediation methods that might result in deterioration of soil properties, phytoextraction holds great promise as a non-destructive salt removal technique. The efficiency of salt phytoremediation is determined by the total amount of salt accumulated in plant tissues, which is the product of the concentration of salt in the plant tissue and the amount of harvestable biomass.

Halophytes are plants that can grow well at high concentrations of salt in the rhizosphere. Some obligate halophytes' growth rates are simulated when exposed to salinity as high as 50% seawater, equal to 31 dS/m (Parida and Das, 2005). However, many of them require specific growth conditions or grow slowly with little biomass. Moreover, high levels of NaCl accumulation is not always observed in halophytes because some halophytes are 'salt-excluders'. Thus, the usefulness of halophytes for phytoremediation is limited.

In this study, the approach to increase salt phytoremediation efficiency is to use non-halophyte (glycophyte) plants that produce high biomass. The total amount of salt extracted by high-yield salt-tolerant non-halophyte plants is likely to exceed the amount of salt taken up by halophytes that produce little biomass. Salt-tolerant crops include oats, barley, wheat, sunflower

and fall rye. Grasses that have high salt resistance include ryegrass, fescue, Canada wildrye, and wheatgrass (Table 1.3). Although grasses do not generate as much biomass as crops, they generally have higher drought resistance, better adaptation to various soil environments and have a longer canopy period. Nonetheless, high salinity can inhibit or completely impede plant germination and growth. Hence, it is critical to promote plant growth under saline conditions in order to achieve successful salt phytoremediation using salt tolerant glycophytes.

Various methods have been developed to improve the salt tolerance of crops so that they can grow in highly saline areas, including traditional breeding (Colmer et al., 2006; Munns et al., 2006), genetic engineering (Farwell et al., 2007; Grichko et al., 2000; Ma et al., 2004; Sergeeva et al., 2006; Stearns et al., 2005; Yamaguchi and Blumwald, 2005), and the use of growth regulators (Khan et al., 2004; Rabie, 2005). This study focuses on the inoculation of PGPR (Cheng et al., 2007b; Mayak et al., 2004b) and endophytic fungi (Sannazzaro *et al.*, 2006; Waller *et al.*, 2005) to promote plant growth in saline soils. This method requires less time than breeding or genetic modification of plant species. In addition, it is more economical than the application of plant growth regulators, especially in a large scale.

1.4. Effects of PGPR on plant growth in saline soils

Ethylene is required by many plants for seed germination but high levels of ethylene can impede plant growth. The PGPR tested in this study are able to inhibit ethylene production in plants by hydrolyzing the ethylene precursor, ACC (Glick et al., 1998).

1.4.1. Ethylene and ACC deaminase

PGPR promotes plant growth by either mitigating adverse effects in the environment or directly improving growth (Glick, 1999). A group of PGPR of particular interest produces the enzyme ACC deaminase. Glick et al. (1998) have postulated that a significant proportion of ACC produced by plants may be exuded from plant roots or seeds and then hydrolyzed by ACC deaminase produced by PGPR. As shown in Figure 1.1, the ACC deaminase-producing PGPR lowers the ethylene concentration and prevents the inhibition of root elongation by ethylene. ACC that is exuded from plant roots is taken up by the PGPR and hydrolyzed by the enzyme ACC deaminase to ammonia and α-ketobutyrate. This uptake and cleavage of ACC decreases the amount of ethylene inside the plant and thereby alleviates ethylene-induced stress. In this research, *Pseudomonas putida* UW3 and UW4 (hereinafter may be referred to as UW3 and UW4) are among the PGPR tested in this research. These two strains were isolated from farm soil in Ontario and have been tested for its growth promotion effect on plants in several laboratory experiments and field trials (Cheng et al., 2007a; Hontzeas et al., 2004a; Huang et al., 2004a; Huang et al., 2004b; Patten and Glick, 2002). The characterization of ACC deaminase and its gene from UW4 is discussed in detail elsewhere (Hontzeas et al., 2004b; Li et al., 2001; Penrose, 2000)

How exactly salinity regulates ethylene biosynthesis remains unclear, the pathway of ethylene biosynthesis itself is well defined. The ACC synthase converts *S*-adenosylmethionine (AdoMet) into ACC, which thereafter is converted to ethylene by ACC oxidase (Hall and Smith, 1995). The ACC synthase and ACC oxidase are specific to the ethylene pathway, where the

ACC synthase is inducible and ACC oxidase may be constitutive or inducible (El Beltagy et al., 1979).

The ACC deaminase has been widely reported in numerous microbial species of gram negative and gram positive bacteria, rhizobia, endophytes and fungi (Saleem *et al.*, 2007). ACC deaminase-producing bacteria have been shown to be able to promote plant growth under various kinds of stress including salinity, drought, water logging, heavy metal and petroleum exposure. Penrose (2000) showed that the canola (*Brassica napus*)seedlings treated with the PGPR, *Enterobacter cloacae* CAL3, contained less ACC. It was suggested that the ACC was transported from plant root cells to PGPR and consumed in the PGPR.

Consequently, the adverse effects of ethylene on the growth of plants were alleviated by PGPR inoculation, resulting in longer root length, shoot length, early seedling establishment or nodulation of legumes. Belimov et al. (2001) suggested that PGPR containing ACC deaminase are present in most soils and offer promise as a bacterial inoculum for improvement of plant growth, particularly under unfavorable environmental conditions. Arshad et al. (2007) reviewed how inoculation with ACC deaminase-producing bacteria may promote plant growth for a more effective phytoremediation for metals and organic contaminants.

Burd et al. (1998; 2000) reported on the potential of the ACC deaminase-producing bacterium *Kluyvera ascorbata* SUD165 to protect canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) seeds from the heavy metal toxicity induced by high concentrations of nickel (Ni), lead (Pb) and zinc (Zn). Further studies have demonstrated that when exposed to high cadmium (Cd) contamination in soil, the growth of Indian mustard (*Brassica juncea*) and

rape (*Brassica napus* var. oleifera L.) was promoted by ACC deaminase-producing PGPR from contaminated soils including *Pseudomonas brassicacearum*, *Pseudomonas marginalis*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Pseudomonas* sp., *Alcaligenes xylosoxidans*, *Alcaligenes* sp., *Variovorax paradoxus*, *Bacillus pumilus*, and *Rhodococcus* sp. (Belimov et al., 2001). For organic contaminants, Huang et al. (2004a; 2004b) reported that the PGPR *Pseudomonas putida* alleviated toxic effects on tall fescue and enhanced root growth in creosote and PAH contaminated soils.

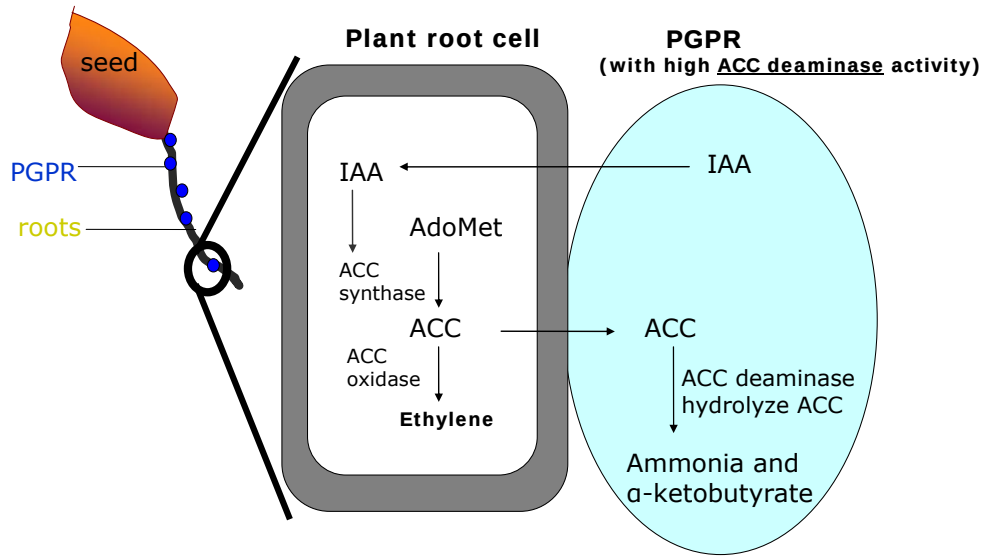


Figure 1.1. The ACC deaminase in PGPR degrades the ethylene precursor ACC. The ACC deaminase in PGPR lowers ethylene level in plants by degrading ACC to ammonia and α-ketobutyrate. Lowering ethylene in plants can alleviate stress and thereby improve plant growth. Some PGPR can also produce plant regulator IAA and further stimulate plant growth (derived from Glick and Pasternak, 2003).

1.4.2. ACC deaminase-producing PGPR and salt stress

In addition to metal and organic contaminants, growth inhibition caused by high salinity can also be alleviated by ACC deaminase-containing PGPR. Mayak et al. (2004a) isolated seven strains of PGPR that had high ACC deaminase activity from the Arava region of Northern Israel. The PGPR lowered ethylene production in tomato seedlings and increased the fresh and dry weight of tomato seedlings grown in the presence of up to 172 mM NaCl. Saravanakumar and Samiyappan (2007) reported that *Pseudomonas fluorescens* TDK1 possessing ACC deaminase activity enhanced the saline resistance of groundnuts and observed increase yields over the groundnuts treated by *Pseudomonas* spp. that lacked ACC deaminase activity. Cotton seedling growth was promoted by ACC deaminase-producing *Klebsiella oxytoca* Rs-5, with individual plant height and dry weight increasing by 14.9% and 26.9%, respectively. Nutrient analysis has exhibited the bacterium's ability to increase the cotton's absorption of N, P, K, and Ca , while Na uptake by plants decreased (Yue et al., 2007).

The ability of PGPR strains of *P. putida* to promote plant growth under saline conditions has been reported. Cheng et al. (2007a) found that *P. putida* UW4 significantly improved canola shoot dry weight by 5 fold at 20 ℃, whereas a mutant strain of UW4 lacking ACC deaminase activity (UW4/AcdS$^-$) did not promote plant growth. The same result was also reported by Li et al. (2000) where the ACC deaminase minus mutant (UW4/AcdS$^-$) no longer promoted root elongation of canola roots. Earlier, Glick (1997) also found that another *P. putida* strain GR12-2 promoted canola root and shoot growth in saline soils but the minus ACC deaminase mutant GR12-2/acd68 did not do so. These results are consistent with the proposed model that the

bacterial ACC deaminase activity of PGPR can lower the plant ethylene levels and hence promote plant growth. It should be noted that Cheng et al. (2007a) found that the Na concentrations in shoots were also increased by UW4 inoculation by 3 – 6 fold.

ACC deaminase-producing rhizobia can also enhance nodulation and hence the nitrogen fixation efficiency. Ma et al. (2003a; 2003b) reviewed the existence of ACC deaminase in a number of rhizobial strains and found that the inhibitory effect of ethylene on plant root nodulation can be reduced by the activity of ACC deaminase. For example, the ACC deaminase-producing *Sinorhizobium meliloti* showed 35 to 40% greater efficiency in nodulating alfalfa, likely by lowering ethylene production in the host plants (Ma *et al.*, 2004). Similarly, *Rhizobium leguminosarum* bv. viciae 128C53K enhanced the nodulation of pea, and the minus ACC deaminse mutants showed lower nodulation efficiency (Ma et al., 2003a).

1.4.3. Auxin production by ACC deaminase-producing PGPR

Some PGPR synthesize and secrete the plant growth regulator IAA (Indo-3-acetic acid), which can enter plant cells and stimulate root growth. Primary roots treated with wild-type strain *P. putida* GR12-2 were on average 35% to 50% longer than the root of canola seeds treated with an IAA-deficient PGPR strain (Patten and Glick, 2002).

In addition to stimulating plant growth as plant growth regulator, IAA can also stimulate ACC synthase (Figure 1.1) to produce more ACC, which can be transformed into ethylene by ACC oxidase (Mayak *et al.*, 1999). On the other hand, the simultaneously produced ACC deaminase can hydrolyze ACC and inhibit ethylene production. As a consequence, the final effect on ethylene production or root growth depends on the balance of the IAA and ACC

deaminase produced in concert by *P. putida*. Moreover, plants respond to IAA differently. The effect of the treatment on the root growth depends on their initial elongation rate; slow growing roots were inhibited by exogenous IAA and ABA at any concentrations used. Whereas for fast growing roots their elongation was promoted by these two hormones at low concentrations (Pilet and Saugy, 1987). In a recent study, Gravel et al. (2007) used *P. putida* to alleviated the detrimental effect of excess exogenous IAA on tomato seedlings, possibly through repressed ethylene production resulted from microbial degradation of IAA in the rhizosphere (and the resultant decrease in ACC) and/or by ACC deaminase activity present in both microorganisms. In conclusion, plant responses to IAA exuded from ACC deaminase-producing PGPR vary with plant species, root growth rates and its balance with ACC deaminase activity.

Glick et al. (2007) proposed a model that explains how ethylene and IAA interact as a feedback loop. The decrease in ethylene levels by ACC deaminase not only down regulates the plant stress responses but also relieves the ethylene repressed auxin responses factor (ARF) synthesis, leading to plant growth promotion resulted from both stress alleviation and growth simulation. However, with the increase in ARF synthesis, ACC synthase is also simulated to produce more ACC and ethylene, which represses the ARF synthesis. In this way, ethylene limits its own production.

1.5. Effects of AMF on plant growth in saline soils

Arbuscular mycorrhizal fungi (AMF) are also known as vesicular-arbuscular mycorrhizae (VAM). The hyphae can enter into the plant cell walls and invaginate the cell membranes, forming structures that are either balloon-like (vesicles) or dichotomously-branching (arbuscules) invaginations. These structures increase the surface area for nutrient transfer

between the cell cytoplasm and hyphae. AMF can change mineral nutrient composition, hormonal balance, water use efficiency, and production of osmoregulators (Auge and Stodola, 1990; Ruiz-Lozano and Azcon, 2000). As well, some AMF can secrete a glycoprotein, glomalin, which contributes to soil aggregation stability and improved drainage, nutrient movement and aeration. These traits are potentially beneficial to plants grown in saline soils, especially soils with high SAR.

AMF can enhance salt tolerance of plants. Waller et al. (2005) reported the potential of using the AMF *Piriformospora indica* to induce salt tolerance in barley. The beneficial effect was associated with an elevated antioxidative capacity and an overall increase in grain yield was observed. Ouziad et al. (2006) found colonization of tomato by AMF under salt stress resulted in a drastic increase of the mRNA of three aquaporin genes. Aquaporins are known to regulate water movement in plants and may facilitate water uptake in soil with high salinity. Copeman et al. (1996) found tomato plants inoculated with AMF had lower Cl^- concentrations in roots than the non-AMF plants but the P level remained unchanged. In another study, the growth promotion effects of two isolated AMF on lettuce were tested. Both AMF strains protected the host plants against salt, but the symbiotic efficiencies differed. *Glomus* sp. protected plants from high salinity by stimulating root development, while *G. deserticola* treated planted has higher N and P in plant tissue (Ruiz-Lozano and Azcon, 2000). In addition to the increase in N and P levels in plants, Feng (2002) reported that AMF not only increased the shoot and root dry weight but also led to higher concentrations of chlorophyll, electrolytes and soluble sugars of maize (*Zea mays*) . The colonization of AMF can also affect Na^+ and Cl^- uptake. Indian sesbania (*Sesbania aegyptiaca* and *Sesbania grandiflora*) treated with *G. macrocarpum* had

lower Na in the tissue and significantly higher root and shoot biomass, chlorophyll, number of nodules and increases in the concentrations of N, P and Mg (Giri and Mukerji, 2004).

The AMF *Glomus intraradices* has been tested for its growth promotion effect on plants under salt stress. In a study with sorghum, maize, cotton and *Pennisetum* sp, the *G. intraradices* inoculated 20-day old seedlings had higher fresh and dry shoot weight than the non-inoculated ones (Jalaluddin, 1993) when plants were grown in saline soil (16 dS/m). Acroca et al. (2007) found the hydraulic conductance of kidney bean (*Phaseolus vulgaris*) plants inoculated with *G. intraradices* was twice as high as that of untreated plants. *G. intraradices* was also beneficial to the halophyte *Atriplex nummularia* (saltbush); the phosphorus levels, dry shoot and root biomass were increased by the AMF (Plenchette and Duponnois, 2005). In Sannazzaro et al. (2006), *G. intraradices* developed an effective symbiosis with the tolerant genotype of *Lotus glaber* in saline soil, and enhanced the net growth, shoot/root and K^+/Na^+ ratios, chlorophyll levels and protein concentrations in plants. It was associated with prevention of Na^+ accumulation in plants and enhancement of K^+ concentrations in roots. Diouf et al. (2005) showed that the *G. intraradices* inoculation improved the growth of the salt-stressed *Acacia* species and recommended the concomitant inoculation of the AMF and rhizobia because of the increased foliar proline accumulation that resulted in better water retention of plants. An increase of proline upon AMF inoculation was also reported in Cho et al. (2006). However, Sannazzaro et al. (2007) later reported increased amounts of total free polyamines in AMF-inoculated plants, while proline levels remained unchanged.

1.6. Fertilization to improve plant growth in saline soils

Soil salinity may cause nutrient imbalances and inhibition of plant growth. High soil Na^+ levels lead to deficiency of other nutrients. For example, elevated Na^+ disrupts transporters such as K^+-selective and Ca^{2+} ion channels on the root cell membranes. Moreover, nitrate and ammonium uptake and assimilation are inhibited by salinity (Ullrich, 2002), and excess Na^+ also interacts with various ions in soil solution, altering availability of cations to plants (Glenn et al., 1999; Hu and Schmidhalter, 2005).

Therefore, change of soil nutrients can alter the effect of high salinity on plants. For example, calcium ameliorated Na^+ toxicity in plants by changing the formation of ion channel proteins and decreasing Na^+ influx through nonselective cation channels (Cramer, 2002; Subbarao et al., 2003). In addition, Shabala (2006) reported that elevated external Ca^{2+} inhibited Na^+-induced K^+ efflux through outwardly directed K^+-permeable channels. Phosphorus fertilizer also alleviated chloride toxicity in wheat, resulting in a significant yield increase. The lower Cl^- concentrations in plants at higher P levels in the soil were attributed to a dilution effect that was caused by increased growth rate due to better P nutrient (Chauhan and Chauhan, 1985).

Ward (1986) found that the presence of additional calcium enhanced the nitrate uptake and growth of barley seedlings under saline conditions, effectively promoting plant growth. Direct addition of nitrogen (N) alleviated salt-inhibited N uptake and improved plant health (Ullrich, 2002). The alteration of N species also improves salt toxicity. It was found that alteration of the ratio of ammonium to nitrate in the nutrient solution had an ameliorating effect on tomato fruit yield under salinity (Ben-Oliel et al., 2004). Similarly, In Irshad et al. (2002), the mixed application of both ammonia and nitrate forms of N enhanced the total dry biomass (shoots and

roots together) of wheat more than did the single source. The application of gypsum, farmyard manure, Mg and NPK fertilizer in combination was the most effective way to enhance yields of rice and wheat that were irrigated with sodic ground water (Yaduvanshi and Swarup, 2005).

A concern should be noted here that nitrate in the vicinity of roots may stimulate ACC oxidase activity and hence increase ethylene production, so the application of N fertilizer might decrease the efficiency of ACC deaminase-producing PGPR. In Shaharoona et al. (2006), the increases in plant height, root weight and total biomass of maize in response to ACC deaminase-producing *P. fluorescens* was higher in the absence of N-fertilizer application. However, the application of N-fertilizer with a lower ratio of ammonium-N to nitrate-N could lead to different results (Ben-Oliel et al., 2004). It is generally accepted that nutrient supply improves plant growth only when the nutrient is deficient in the soil and when the salt stress is not severe (Hu and Schmidhalter, 2005).

1.7. Objectives

Upstream oil and gas production has caused soil salinity problems across western Canada. The main objective in this study was to investigate the use of phytoremediation to remove excess salt from salt-contaminated soil. The efficiency of this method largely depends on the amount of harvestable biomass, especially shoots. However, plant growth can be severely inhibited at high salinity and result in unsuccessful remediation. From previous research, it is clear that salt tolerance can be increased by inoculation of PGPR and AMF. This study thus explored the effect of PGPR and AMF on growth and salt accumulation of plants grown in saline soil. The hypothesis was that PGPR and/or AMF can increase plant biomass, and hence increase total salt uptake from saline soils, resulting in more salt removal. The objectives of

this study are to 1) establish a reliable in-house soil salinity measurement procedure; 2) isolate indigenous ACC deaminase-producing PGPR; 3) examine the effect of PGPR and AMF on plant growth and salt accumulation under salt stress; 4) examine the effect of fertilization on plant growth, and 5) conduct phytoremediation field trials.

Table 1.4. Five objectives of this study.

Objective	Content
1. Establishment of a reliable in-house soil salinity measurement procedure	Soil salinity measurement
2. Isolation of indigenous ACC deaminase-producing PGPR	PGPR isolation Strain identification Determination of inoculation efficiency
3.Examination of the importance of nutrient addition	Percent germination Biomass determination Salt accumulation in biomass
4 Examination of the effect of PGPR and AMF on plant growth and salt accumulation under salt stress	Root elongation pouch assay Plant species selection for PGPR tests Greenhouse tests Biomass determination Root colonization by AMF Salt accumulation in biomass
5. Field test on salt contaminated sites	Tests of PGPR effects in the salt-impacted sites

2. Material and Methods

2.1. Research sites

The high salinity of soil is contributed to upstream gas and oil production. In this research, these sites were named after the location. Soils for greenhouse tests were taken from the top 30 cm soil from these sites. Table 3.2 contains details of each soil sample taken from these sites.

2.1.1. Cannington Manor South site (CMS) and North sites (CMN)

Both Cannington Manor South site (CMS) and North sites (CMN) are located in Cannington Manor, Saskatchewan. It is suspected that the leakage of a brine water storage tank was the contamination source. The leak may have occurred in winter and the brine water spread over a wide area on the frozen ground. Attempts have been made to re-establish vegetation over the past 40 years. The land has been treated with gypsum (CaSO4) and planted with several plant species, including foxtail and barley. Before the planting in May 2007, a 4-inch layer of compost was mixed into the top soil of both sites. The compost is manure from a feedlot in High River (Alberta, Canada) that was piled and allowed to heat to over 60 ℃ to cook all of the weed seeds.

The CMS site is 107 m long and 15 m wide, approximately 0.16 hectare. The CMS site is on a lower ground level and often experiences flooding in summer. The North site (CMN) is 75 m long and 50 m wide, nearly 0.38 hectare. It is 400 m apart from the CMS site.

2.1.2. Alameda Site (AL)

The Alameda site (AL) is located in Alameda, Saskatchewan. The history of the AL site and source of salt is unknown; however, it is inferred that the saline soil came from a flare pit years ago. The AL site is an irregularly-shaped 0.21 hectare area, approximate 85 m long and 25 m wide. A 4-inch layer of compost was mixed into the top soil of the site before planting in May 2007. The compost is manure from a feedlot in High River, (Alberta, Canada) that was piled and allowed to heat to over 60 ℃ to cook all of the weed seeds

2.2. Measurement of salinity and other soil parameters

The measurement of EC_e and $EC_{1:2}$ was performed as follows (Janzen, 1993). For EC_e, soil was air dried, ground and then sieved. An aliquot of 200g - 400 g of soil was mixed with sufficient deionized water to reach saturation when the soil paste glistened and slid cleanly from the spatula. The sample was allowed to stand for at least 4 h and was checked to ensure the saturation criteria were still met. If free water had accumulated on the surface, a known amount of soil was added and remixed. If the soil had stiffened or did not glisten, distilled water was added and mixed thoroughly. The paste was allowed to sit for another 4 h. It was then filtered through a Buchner funnel or centrifuged at 800 ×g. EC_e of this soil extract or the supernatant was then measured using an electroconductivity meter (Oakton Instruments, IL).

$EC_{1:2}$ was measured in a 1:2 (w/w) ratio of soil to water. Soil and deionized water were mixed in a 250 mL Erlenmeyer flask and shaken at 100 rpm for 30 min. This soil suspension was then centrifuged at 2000 rpm for 5 min. The electroconductivity and pH of the supernatant were then measured by the electroconductivity meter. Each measurement was carried out in triplicates. The K values were determined according to equation 1.

An aliquot of 400 g soil of selected samples was sent to ALS Laboratory (Waterloo, Ontario; hereinafter referred to as ALS) for cation exchange capacity (CEC), electroconductivity ($EC_{1:2}$), sodium adsorption ratio (SAR), sodium (Na), chlorine (Cl), available boron (B-avail), calcium (Ca), potassium (K) and magnesium (Mg). The $EC_{1:2}$ values reported by ALS (labeled as $EC_{1:2}^{ALS}$) were primarily acquired to compare the data resulted from the in-house procedure. Agri-Food Laboratories (Guelph, Ontario; hereinafter referred to as Agri-Food) analyzed the pH, organic matter (OM) and soil texture.

2.1. Isolation of indigenous ACC deaminase-producing PGPR

The isolation of ACC deaminase-producing PGPR required four steps: 1) screening for indigenous bacteria using ACC as a sole nitrogen source, 2) quantifying ACC deaminase activity of individual isolates, 3) measuring the auxin production and 4) species identification. The formulae and methods for preparing DF minimal salt medium, Tris-HCl, Salkowski's reagent, 0.5M ACC (1-aminocyclopropane-1-carboxylate) stock, 0.2% 2,4-DNP(dinitrophenyl-hydrazine) and 2mg/mL L-trp (L-tryptophan) stock solution were described in the **Error! Reference source not found.**.

2.1.1. Screening with ACC as a sole nitrogen source

The detailed procedure is described in Shah et al. (1998). An aliquot of 20 g of soil from each sample was transferred into sterile tryptic soy broth (TSB) rich medium and incubated. An aliquot of this culture was then washed and transferred into sterile DF minimum salt medium (Dworkin and Foster, 1958). After incubation, the pellet was spun down and resuspended in 1.0 mL DF salt medium that contains no nitrogen. A loopful of this culture was streaked onto 1.5% agar DF minimum salt agar medium with 300 mM ACC (referred as ACC plates hereafter) and

incubated upside down for at least 3 days. The individual colonies were then randomly selected. The ACC deaminase activity of each isolate was then determined by the ACC deaminase activity assay.

2.1.2. ACC deaminase activity assay

The ACC deaminase activity assay is based on the method in Penrose and Glick (2000). Individual isolate was incubated in sterile TSB medium overnight. The culture was centrifuged at 1600 ×g for 10 min. The pellet was washed twice with 15 mL DF minimum salt medium without nitrogen. The final pellet was resuspended with DF minimum salt medium with ACC as the sole nitrogen source. The ACC deaminase activity was induced at this step. The culture was then centrifuged and the pellet was then washed twice with Tris-pH7.6. The pellet was then resuspended with Tris-pH8.0, followed by adding 300 µL toluene and vortexing. Two aliquots of 200 µL of the mixture were transferred into two microcentrifuge tubes. The remaining 230 µL in the tube was used for the protein assay. An aliquot of 20 µL of 0.5M ACC was added into one of the tubes containing 200 µL mixture, and 20 µL milli-Q water were added into the other one. Both tubes were incubated at 30 °C in a water bath for 30 min, after which 0.56 M HCl was added to stop the enzymatic activity. After centrifugation for 5 min 6400 ×g, 1.0 mL supernatant was transferred into a glass tube, and 0.56 M HCl and 2,4-DNP were added. After incubation at 30 °C for 30 min in a water bath, 2 N NaOH was added. The true absorbance values were obtained by subtracting the absorbance reading of the cuvette without ACC from the absorbance reading of the cuvette with ACC. The total amount of cells of each culture varies, thus it is necessary to measure the total protein content of each culture to normalize the

ACC deaminase enzyme activity. In order to normalize the ACC deaminase activity based on protein levels, total protein content of each sample was measured.

Two aliquots of 40 µL of the lysate were transferred into two new microcentrifuge tubes as duplicates. To each one, Tris-pH8.0, and 0.1 N NaOH were added, the tubes were then mixed using a vortex stirrer and incubated. Standard solutions were prepared in triplicate using 0.136 µg/µL bovine serum albumin (BSA, BioRad™) stock. After cooling, the protein in the solutions was colorized by BioRad™ reagent and the optical density at 595 nm (OD_{595}) was recorded.

2.1.3. Auxin production assay

The auxin production assay followed the procedure in Khalid et al. (2004). An aliquot of 20 µL of isolate stock was pipetted into 10 mL sterile DF minimum salt medium [+$(NH_4)_2SO_4$, +glucose] and incubated. Five sterile tubes with 8 mL of DF minimum salt medium [+$(NH_4)_2SO_4$, +glucose] containing 500 µg/mL L-Trp were prepared. An aliquot of 20 µL of the 48 h-culture was transferred into the DF minimum salt with L-Trp, followed by incubation for another 48 h at room temperature. After incubation, the OD_{600} reading was recorded. The remaining solution was then transferred into a centrifuge tube and spun at 2000 ×g for 20 min. The pellet was discarded. IAA standard solutions were prepared from 0.1 mg/mL IAA stock. An aliquot of 0.5 mL of the supernatant of the sample and standards was transferred into tubes. Two milliliters of Salkowski's reagent were added and the tube was incubated for 20 min. The OD_{535} was measured. The reported auxin production assay data were normalized to be in µg per mL per OD.

2.2. Root elongation pouch assay

The root elongation pouch assay followed the procedure in Patten and Glick (2002). Growth pouches (Mega International, Minneapolis, Minnesota, USA) were soaked with 1% NaCl solution prior to autoclaving. For each treatment, ten pouches were prepared. For Ranger barley and common oats, six seeds were placed into each pouch, whereas ten seeds were placed into a pouch for Topgun ryegrass and Excalibur tall fescue. The pouches were incubated at room temperature ranging from 20℃ - 25 ℃. For the first 2 days, the seeds were kept in the dark by covering the pouches with aluminum foil. Germination, shoot and primary root length were then measured after three days for crop species and five days for grass species after germination.

2.3. PGPR inoculation method and its efficiency

Seeds were inoculated with PGPR with the following method developed by Aaron Khalid (2007). An aliquot of bacteria glycerol stock was added into 50 mL sterile TSB medium (30 g/L milli-Q water) contained in a 250 mL Erlenmeyer flask. This culture was grown at room temperature on a shaker table (80 rpm) for 20 – 22 h. The culture was then centrifuged at 800 ×g for 10 min, washed and resuspended with 0.1% (w/v) sodium pyrophosphate to remove secondary metabolites. The final pellet was re-suspended in 0.25% methylcellulose (adhesive reagent, Sigma, USA) to reach an OD_{600} of 2.0 – 3.0. The 0.25% methylcellulose was prepared as follows. Methylcellulose (Sigma, USA) 2.5 g was slowly dissolved in 1.0 L of milli-Q water with vigorous stirring until the polymer powder completely dissolved. The solution was then autoclaved for 30 min at 121 ℃, after which a gelatinous solid was formed. The gel then liquefied into a viscous solution upon cooling. A blue colorant (Color Coat Blue, Becker Underwood Canada, Saskatchewan) was then stirred into the bacteria-polymer slurry at a ratio

of 1.75 mL colorant to 100 mL slurry. The presence of colorant meets safety regulations requiring all treated seeds to be visibly colored to avoid use for animal consumption. An aliquot of 20 mL of this blue slurry was inoculated onto 600 mL of seeds using a seed treater (HEGE 11, Wintersteiger Inc., Austria). The dried seeds were immediately transferred into sealed bags and stored at 4 °C. For seeds treated with the combination of two PGPR, a 10 mL aliquot of each bacterial slurry was applied. For PGPR combination treatment, such as UW3+4, seeds were inoculated with 5 mL of each bacterial slurry. It should be noted here that the label 'UW3+4' means the combination of UW3 and UW4 as the PGPR treatment.

After inoculation, the number of viable bacteria cells on seeds treated with UW4 was measured to ensure proper coating procedure. Twenty treated seeds for crop species or 0.2 g (~100 seeds) seeds for grass species were added into 20 mL sterile DF minimum salt medium and shaken for 1.5 h. The count of cfu/seed was determined by the spread count method on a DF minimum salt agar medium containing ACC as a sole nitrogen source at 30 °C for three days.

2.4. Plant species selection for PGPR greenhouse experiments

The goal was to select plant species that could germinate and produce reasonable amounts of aboveground biomass in highly saline conditions. Eight salt tolerant and/or native plant species were tested: common wheat (*Triticum sativum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), Excalibur tall fescue (*Festuca arundinace*), Topgun ryegrass (*Lolium perenne*), blue gramma (*Bouteloua gracilis*), buffalo grass (*Buchloe dactyloides*) and creeping red fescue (*Festuca rubra*). Seeds of these plants were purchased from Ontario Seed Company (Waterloo, Ontario). Saline soil (EC_e = 49.5 dS/m) was sieved through 0.25 inch mesh and homogenized before use.

Two-inch square hard plastic pots (0.25 inch × 0.5 inch) had a rectangular hole at each side of the bottom for drainage. Each pot was filled with approximately 80 – 100 cm^3 sieved soil. Ten crop seeds or 0.2 g (~100 seeds) grass seeds were planted in each pot on July 15th, 2007. Plants were grown in the greenhouse with daily watering at 25 ℃ - 40 ℃ without supplemental lighting. The growth and germination were assessed after 30 days. Species that had higher percent germination and growth in saline soil were selected for further greenhouse tests with PGPR.

2.5. The effects of fertilizer on plant growth in saline soil

Two-week old barley and oats seedlings were fertilized with Plant-Prod 20-20-20 water soluble fertilizer (Plant Products, Brampton, Ontario). Plants were watered with 30 mL of the 1g/L (200 mg/L total nitrogen) fertilizer solution and reverse osmosis water on alternate days for three weeks, followed by two weeks of watering without the fertilizer before the harvest. The fertilizer contains 20% total nitrogen, 20% phosphorus pentaoxide, 20% potassium oxide, 0.02% boron, 0.05 chelated copper, 0.1% chelated iron, 0.05% chelated manganese, 0.0005% molybdenum, chelated zinc and 1% EDTA (ethylene diamine tetraacetate).

2.6. Greenhouse experiment conditions

The plants, PGPR, AMF, soil, watering, and the type of pots used varied in experiments, and the specifications were noted at the beginning of each experiment in Section 3. Methods, material and growth conditions that were constant throughout experiments are described here. Ten PGPR treated crop seeds (i.e. barley, wheat and oats) or 0.2 g of smaller seeds (i.e. fescue, ryegrass and alfalfa) were sown in the soils sampled directly from the contaminated sites. Pots were 80% filled with soil; the amount of soils varied with the size of the pot. An aliquot of 0.1 g

- 0.2 g of the granule product of the AMF, *Glomus intraradices,* (Myke-pro, PremierTech™, Rivière-du-Loup, Quebec) was applied in the soil alone or in combination with PGPR treated seeds. The fresh or dry weight of shoots and roots were measured after the growth period. Each treatment included four replicates. For controls, seeds were sown in plots containing wet unsterile ProMix™ BX general purpose growth medium (Premier horticulture, Riviere-du-Loup, Quebec). ProMix™ BX contains sphagnum peatmoss (75% - 85% by volume), perlite, vermiculite, macronutrients (calcium, magnesium, nitrogen, phosphorus, potassium and sulphur), micronutrients (boron, copper, iron, manganese, molybdenum and zinc), dolomitic limestone, calcite limestone and a wetting agent. Plants were irrigated with reverse osmosis water at 25 ℃ - 40 ℃ in the greenhouse without supplemental lighting. Experiments were conducted during the period of May 2006 to September 2007. Seeds of AC Ranger barley (referred as to Ranger barley hereinafter) were purchased from the Cribit Seeds (West Montrose, Ontario), and CDC Baler oats (referred to as Baler oats hereinafter) were from the Wagon Wheel Seed (Churchbridge, Saskatchewan). The rest of the seeds were purchased from Ontario Seed Company (Waterloo, Ontario). The local species in Saskatchewan, including alfalfa (*Medicago sativa*), red spring wheat (*T. aestivum*), Vivar barley (*H. vulgare*) and orchardgrass (*Dactylis glomerata*), were provided by a research partner.

2.7. AMF colonization

Roots were washed and chopped into 2 – 4 cm long fragments. One to two grams of cleaned root sample were autoclaved in 10% KOH (w/v) 125 mL in a 500 mL beaker by a liquid cycle for 20 min. After cooling, roots were rinsed with water and autoclaved in the Chlorazaol black E staining solution for 20 min. Excess stain was then removed by immerging roots in 50%

glycerol for 3 days. The images of vascules and arbuscules were taken by an electrical microscope. Root length colonized (RLC) is estimated by using the gridline intersection method (Brundrett et al., 1996). Stained root samples are dispersed on a dish with grid lines. Mycorrhizal colonization as RLC was then assessed under a dissection microscope by dividing counts of mycorrhizal roots by non-mycorrhizal roots.

2.8. Measurement of concentrations of Na, Cl, B, K, P and Mg in plant tissues

At the end of growth period, plant shoots were harvested and oven dried at 60 ℃ for 72 h. The concentrations of Na, B, K P and Mg in the dried plant shoot tissues were analyzed by method USEPA 6020, where plant tissue was completely decomposed in nitric acid and analyzed by ICP-MS (Inductively Coupled Plasma Mass Spectroscopy). Chlorine was analyzed by IC (Ion Chromatography) according to APHA method 4110B. The analysis was conducted in ALS Laboratory (Waterloo, ON).

2.9. Identification of indigenous isolates

In order to apply newly isolated indigenous bacteria in the field, identification was necessary. Two Year-2006 isolates, CMH2 and CMH3, were classified by the 16s rDNA sequences and BioLog™ assay. Total DNA extraction was conducted with the Wizard Genomic DNA Purification Kit (Cat. no.A120, Promega). The 16s rDNA then was amplified by PCR with the forward (1F, AGCGGCGGACGGGTGAGTAATG) and the reverse (R1509, AAGGAGGGGATCCAGCCGCA) primers (Young et al., 2004). The PCR product was extracted with QIAGEN Gel Extraction Kit (Cat no. 20021, Qiagen) and sequenced directly. The sequencing data were edited with BioEdit. The edited 16s rRNA gene sequences were submitted for comparison and identification to the GenBank database using BLAST

(http://130.14.29.110/BLAST/) and/or the Ribosomal Database Project II (RDP, http://rdp.cme.msu.edu/) and/or EMBL (www.ebi.ac.uk/fasta33/nucleotide).

In addition, the biochemistry-based BioLog™ assay (GN2 MicroPlate™ , BioLog™) was also conducted for further verification (Biolog, 1999). Gram staining, oxidase tests and TSI (Triple Sugar Iron) slant were performed first for choosing the culture media and the type of MicroPlate™ . Each bacterial culture was then incubated for 16-24 h before resuspended in the inoculating fluid to reach certain turbidity and transferred into the MicroPlates. The plates were then incubated at 30℃ overnight before read by the Biolog MicroLog 3 (release 4.0). The bacterium was then identified according to the pattern of colorization of the wells.

2.10. Field trials on salt contaminated sites

Plant species and PGPR were then selected for the field trial. All three sites were tilled and mixed with a layer of 4-inch thick compost before seeding. The fields were divided into plots for each plant species and PGPR combination. Each plot was divided into two subsections on the Alameda (AL) site and three subsections on the Cannington Manor South (CMS) and North (CMN) site. From each subsection, three top soil samples (~ 20 cm) were randomly taken by an auger and mixed to form a composite sample for salinity analysis. The $EC_{1:2}$ and EC_e were measured for ten soil samples for each site for the determination of the K^{LAB}. The K^{LAB} was then used to calculate the EC_e values from $EC_{1:2}{}^{LAB}$ for the rest of the samples. Seeds were sown at a density of three passes run by a Brilliant™ drop-spreader at a setting of 5 for crops and 6 for grasses at the CMS and CMN site. A portable seed dispenser was use in the AL site, and the seeds were harrowed into soil afterwards. After a growth period of two months, plant

tissue was sampled for analysis of concentrations of Na, Cl, B, Ca, K and Mg. The percent vegetation coverage of the contaminated sites was also recorded. To evaluate the PGPR effect in the field, biomass of plants on an area of 0.25 m^2 with similar soil salinity and vegetation coverage (> 80%) was collected.

2.11. Statistical analysis

Root length data of the pouch test (n = 10) and biomass data (n = 4) of the greenhouse tests were analyzed by one-way analysis of variance (ANOVA) and the post-hoc Dunnet test (*P <0.01 versus -PGPR), where replicates were considered as random. The analysis of concentrations of elements in plant tissue was conducted using one-way analysis of variance (ANOVA). The software used for analysis was STATISTICA (StatSoft, Inc.).

3. Results and discussion

The purpose of this study was to investigate the growth promotion effect of plant growth-promoting rhizobacteria (PGPR) and an arbuscular mycorrhizal fungus (AMF) *Glomus intraradices*. Indigenous PGPR were isolated from soil of the research sites based on their ACC (1-aminocyclopropane-1-carboxylate) deaminase activity. Indigenous and non-indigenous PGPR were tested in the greenhouse and the field for their effect on plant biomass and NaCl levels in plant tissue. The AMF was tested in the greenhouse only. For assessment of soil salinity of the field samples, the in-house procedure for salinity measurement was developed. In addition to PGPR and AMF, the effect of fertilizer was also investigated.

3.1. Establishment of a reliable in-house soil salinity measurement procedure

Eight soil samples (Figure 3.1) were analyzed in the lab for both $EC_{1:2}$ and EC_e (referred to as the $EC_{1:2}^{LAB}$ and EC_e^{LAB}) (Table 3.1). Each sample was also sent to ALS (Waterloo, ON) for $EC_{1:2}$ measurement to verify the in-house $EC_{1:2}$ measurement results.

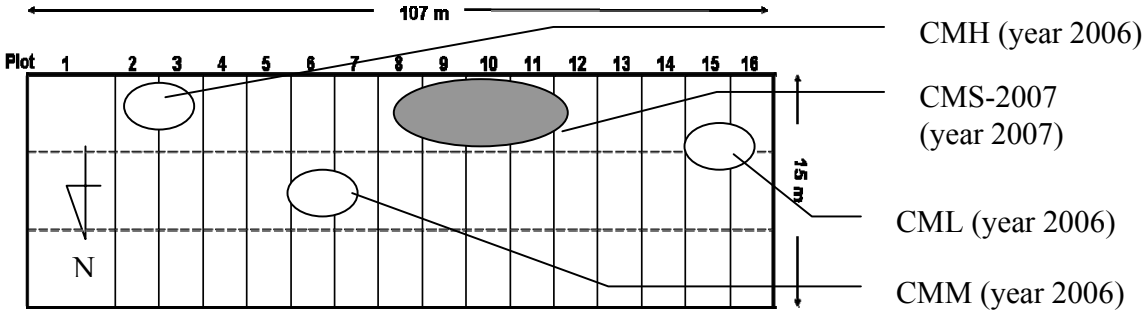
The $EC_{1:2}$ values acquired by the in-house procedure ($EC_{1:2}^{LAB}$) were consistently < 10% higher than the $EC_{1:2}$ values reported by ALS ($EC_{1:2}^{ALS}$) (Table 3.1). This result means that the in-house $EC_{1:2}$ procedure was reliable and accurate. However, the $EC_{1:2}$ measurement is affected to a great extent by the soil texture. For example, sandy soils tend to give higher $EC_{1:2}$. Therefore, the EC_e values are required to compare the salinity of different soils.

The EC_e of soil can be empirically determined (see Section 2.1) or calculated with a measured $EC_{1:2}$ and the conversion factor K (equation 1). The K of these eight samples (K^{LAB}),

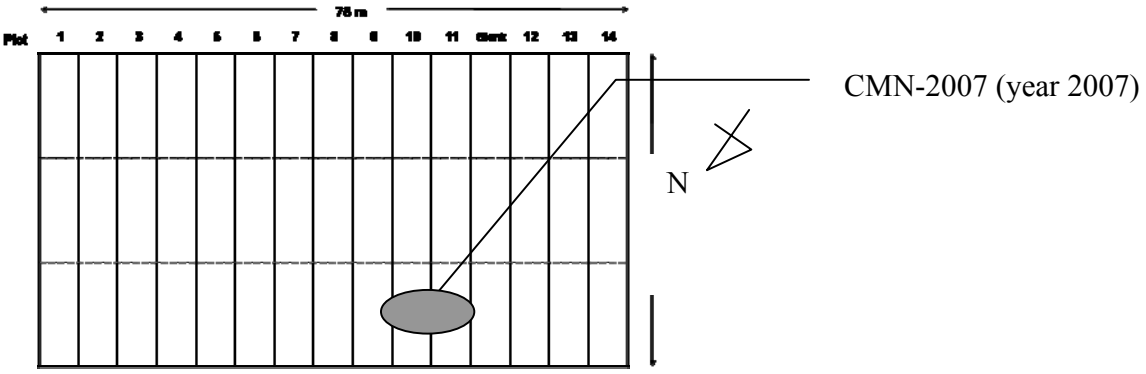
were determined by the $EC_{1:2}$ ($EC_{1:2}^{LAB}$) and EC_e (EC_e^{LAB}) measured in the lab. The K^{LAB} values of all these eight samples fell in the common range (from two to four) of K values (Richards, 1954). The consistent $EC_{1:2}$ and accurately ranged K values indicated that the in-house procedure developed in the lab was reliable. The K value varies with soil properties such as particle size and the amount of organic matter.

For field salinity assessment that requires a large number of samples, the $EC_{1:2}$ method is particularly useful. The $EC_{1:2}$ values can give a quick estimation of the spatial variation of salinity levels of the site. With the measurement of both EC_e and $EC_{1:2}$ of relatively small number of samples, the average K value can be determined and used to calculated the EC_e values of the rest of the samples with presumably similar soil properties. Therefore, this method was adopted for field salinity assessment (Section 3.9).

a) Cannington Manor South site (CMS), 0.16 hectare



b) Cannington Manor North site (CMN), 0.38 hectare



c) Alameda site (AL), 0.21 hectare

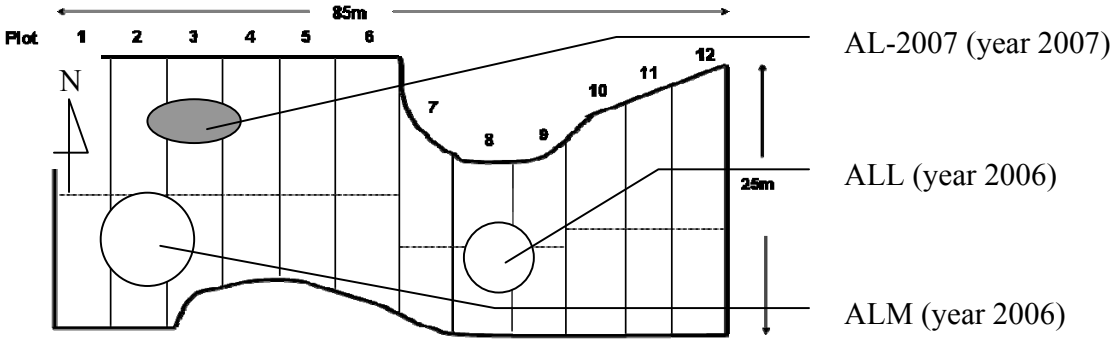


Figure 3.1. The maps of the sampling spots of each soil sample in this study. The detailed soil properties were listed in Table 3.2. Open circle: sampled in year 2006; solid circle: sampled in year 2007.

Table 3.1. $EC_{1:2}$ and EC_e values of soil samples. K is the conversion factor of $EC_{1:2}$ to EC_e. The lab analysis was done in triplicate.

No.	Soil[a]	Soil Texture[c]				Electroconductivity (dS/m)			
		Particle size			O.M (%).	ALS[b]	LAB		
		Sand	Silt	Clay		$EC_{1:2}^{ALS}$	$EC_{1:2}^{LAB}$	EC_e^{LAB}	K^{LAB}
1	CMH	36	53	11	-	15.0	15.2	51.7	3.4
2	CMM	49	44	7	-	15.0	16.0	52.8	3.3
3	CML	34	36	30	-	2.7	2.6	9.3	3.6
4	CMS-2007	48	37	15	14.6	4.3	4.5	9.0	2.0
5	CMN-2007	44	31	25	10.2	2.8	3.1	7.8	2.5
6	ALM	37	35	28	4.7	7.2	7.9	21.3	2.7
7	ALL	28	38	34	2.7	3.9	4.3	11.2	2.6
8	AL-2007	35	34	31	7.9	5.7	6.1	15.3	2.5

[a]See Table 3.2 for more details of each soil.
[b]ALS laboratory (Waterloo, Ontario)
[c]The soil texture and organic matter (O.M.) were analyzed by Agri-Food (Guelph, Ontario)

3.2. Properties of soils sampled from the CMS, CMN and AL site

Detailed properties of the soils (Figure 3.1) used in this research were listed in Table 3.2, including the exchangeable cation capacity (CEC), electroconductivity (EC in dS/m), sodium adsorption ratio (SAR), sodium (Na), chlorine (Cl), available boron (B-avail), calcium (Ca), potassium (K) and magnesium (Mg).

In general, soil with EC_e of 4 dS/m is considered saline, and the yield of most crops dramatically declined at 16 dS/m (Table 1.2). The EC_e values of soil samples are listed in Table 3.2, where the EC_e values are the products of $EC_{1:2}^{ALS}$ and K^{LAB}. The CMM and CMH soils sampled from the CMS site in 2006 were extremely saline (EC_e > 50 dS/m). Even after blending in compost and tilling in 2007, the CMS site was still highly saline, with an average EC_e of 18 dS/m (Figure 3.28). ALM soil was both saline (19 dS/m) and sodic (SAR = 16). However, the other soil sample from the same site, ALL, not only had a much lower EC_e (10.1 dS/m) but also lower SAR (4). This implied that distribution of salinity might be uneven on the AL site.

The soil samples taken from CMS site (CMH, CMM and CML) in 2006 are named according to the vegetation density. Soil sampled from spots of less vegetation cover was assumed to be higher in salinity. CMH, CMM and CML are soils taken from spots of CMS site with presumably high, medium and low salinity (Table 3.2). The analysis results partly supported this assumption. The EC_e of the CML soil was much lower than that of the CMH and CMM soil (Table 3.1). However, CMH and CMM had similar EC_e values, suggesting that the vegetation development was affected by other variables besides soil salinity.

The pH values showed that all soils were moderately alkaline (> 8.0); however, most plants prefer a soil pH between 5.5 and 7.5 and do best at the middle range. In addition, most bacteria proliferate at the pH range 6.3 – 6.8, hence the soil is too alkaline for both plants and the rhizosphere bacteria community. Soil alkalinity also affects the availability of various nutrients including nitrogen (N) and phosphorus (P) (Ajouri et al., 2004; Ullrich, 2002). When soil pH > 7, P may form less soluble minerals with Ca. Additionally, N is subject to greater losses at higher pH through volatization of NH_3. Similarly, micronutrients such as manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and boron (B) tend to decrease as soil pH increases (Chaignon et al., 2003; Gallardo-Lara et al., 1999; Ministry of Agriculture and Food, 1991; Zheljazkov and Warman, 2004).

The CEC is the capacity of a soil for ion exchange of positively charged ions between the soil and the soil solution. The CEC is determined prominently by the amount of negative charged clay and organic matter. The more clay or organic matter, the higher capacity the soil has to retain cations including Na^+. The CEC of soil can range from less than 5 to 35 meq/100g for agricultural type soils.

CMN-2007 soil contained more than 10% organic matter and CMS-2007 soil contained 15% organic matter. It should be pointed out that organic mater (OM) contributed around 90% of the total CEC for soils sampled from CMS site in 2006 (CMH, CMM and CML). Therefore, adding more organic matter (compost) into the soil might result in more sodium retention (less leaching out) in the top soil although adding OM can also improve soil drainage and dilute the soil salinity, and hence assist plant establishment.

Total concentrations of Na, Cl, Ca, K and Mg in plant shoot tissue were also listed. For B, the available fraction, rather than the total concentration, was listed because total B is an unreliable index for the bioavailability of B to plants (Adriano, 2001). Chlorine concentrations highly correlated ($r^2 = 0.99$) with the soil EC_e (Figure 3.2). Hence, Cl was a better indicator for soil salinity, but more data points in the range of 20 – 50 dS/m are needed to support this notion. The Cl and Na concentrations of the soils were not correlated. The calcium concentration of CMN-2007 soil was 2-6 times higher than soil from other sites. This result is was consistent with the fact that CMN site was treated with gypsum in the past years.

Table 3.2. Properties of soil samples taken from the research sites.

Soil	CMH	CMM	CML	ALM	ALL	CMS-2007	CMN-2007	AL-2007
Site	CMS	CMS	CMS	AL	AL	CMS	CMN	AL
Year	2006	2006	2006	2006	2006	2007	2007	2007
Month	August	August	August	October	October	May	May	May
pH	7.9	7.9	8.2	7.8	7.9	8.1	7.9	8.1
OM (%)	14.1	15.7	12.7	4.7	2.7	14.6	10.2	7.9
CEC (meq/100g)	26.3	23.3	36.8	20.8	21	21	24.8	22.8
Sand (%)	36	49	34	37	28	35	44	48
Silt (%)	53	44	36	35	38	34	31	37
Clay (%)	11	7	30	28	34	31	25	15
Texture	Silt Loam	Loam	Clay Loam	Loam	Clay Loam	Loam	Loam	Loam
$EC_{1:2}^{ALS}$ (dS/m)	15	15	2.6	7.2	3.9	4.3	2.8	5.7
K^{LAB}	3.4	3.3	3.6	2.7	2.6	2.0	2.5	2.5
EC_e (dS/m)	50.4	49.5	9.4	19.4	10.1	8.6	7	14.3
SAR	12	10	11	16	4	10	12	7
Na (mg/kg)	5090	4780	2580	3320	560	2710	2370	920
Cl (mg/kg)	15000	15400	1880	4950	2450	1400	1120	3710
B-avail (mg/kg)	-	-	-	2.8	1.9	4.3	2.8	1.7
Ca (mg/kg)	-	-	-	24800	32200	12900	72800	15400
K (mg/kg)	-	-	-	4000	4530	1290	3270	3580
Mg (mg/kg)	-	-	-	8550	9570	19000	21000	5710

CEC: Cation Exchange Capacity
SAR: Sodium Adsorption Ratio
EC_e is the product of $K^{LAB} \times EC_{1:2}^{ALS}$.
K^{LAB} was determined by the $EC_{1:2}$ and EC_e values produced in the lab.
B-avail stands for the available B determined by hot-water extraction method (Richards, 1954).
Na, Cl, Ca, K and Mg are total concentrations determined with the method US-EPA 3050B by ALS (Waterloo, Ontatio).

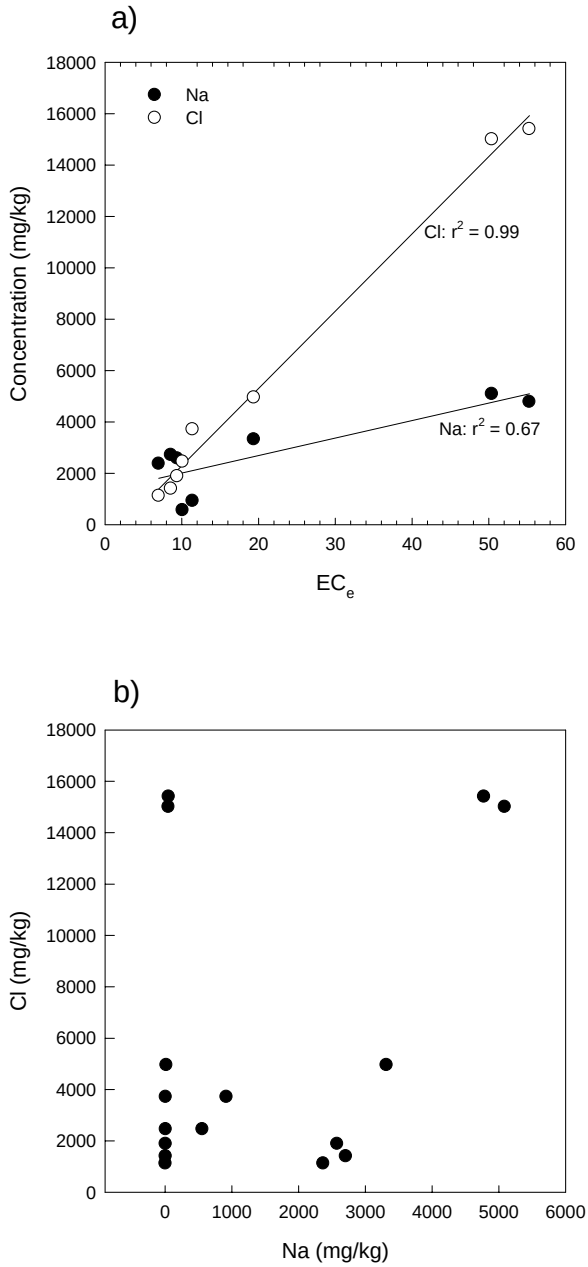


Figure 3.2 The relations between Cl, Na and ECe of soil samples. Cl concentrations highly correlated (r^2= 0.99) with the EC_e (a). The Cl and Na concentrations (b) of soils were not correlated. Figures were derived from Table 3.2. Data were analyzed by linear regression of STATISTICA (StatSoft, Inc.).

3.3. The ACC deaminase activity and auxin production of isolated potential PGPR

The ACC deaminase activity of isolates that were capable of utilizing ACC as the sole nitrogen source is listed in Table 3.3. The ACC deaminase activity was normalized on a per mg protein basis. UW3 (Huang et al., 2004a; Huang et al., 2004b) and UW4 (Huang et al., 2005) have successfully improved plant growth and hence enhanced the petroleum degradation in several greenhouse and field trails.

From Year-2006 soil samples, i.e. CML, CMM and CMH, eighteen potential PGPR were isolated and tested for their ACC deaminase activity. Among them, CMH2 and CMH3 not only had the highest ACC deaminase activity but also had high salt tolerance since they were isolated from very saline soils (EC_e = 50.4 dS/m). These two isolates would therefore be tested in soils sampled from the site of origin (CMS) in the greenhouse (Experiment #4 and #5) for their plant growth promotion effect, and the results would provide evaluation for the use of these potential PGPR in the Year-2007 field trials.

From Year-2007 soil samples, CMS-2007 and AL-2007, fifteen more potential PGPR were isolated. Their ACC deaminase activity and auxin production were quantified (Table 3.3). Among them, CM6, CM7, CM8, AL1, AL2 and AL7 were selected for a greenhouse experiment (Experiment #6, page 101). Further greenhouse tests of these isolates are required in order to select more effective strains for the field trial in 2008.

Figure 3.3 and Figure 3.4 indicate the ACC deaminase activity and the IAA-equivalents concentration of eight isolates that produced high ACC deaminase activity: CMH2, CMH3, CMR6, CMR7, CMR8, ALR1, ALR2 and ALR7. To avoid false results, controls were included.

The positive controls were UW3 and UW4, which are PGPR with high ACC deaminase activity. The negative control accD^{-} was an ACC deaminase gene knockout mutant of UW4.

UW3 had the highest ACC deaminase activity of all isolates, while the IAA-equivalents concentration of UW4 was the highest. Therefore, the combination of UW3 and UW4 would be applied as one combination PGPR treatment in the following greenhouse tests. Isolates that showed the highest ACC deaminase activity were CMH2, ALR1, ALR2 and UW4, of which UW4, CMH2 and ALR1 also had the highest IAA-equivalents concentrations. ALR2, however, produced relatively low IAA-equivalents. CMH3 was moderate in both ACC deaminase activity and IAA-equivalents production. The results indicated no correlation between the auxin production and ACC deaminase activity.

Table 3.3. ACC deaminase activity and IAA-equivalent concentrations of colonies that used ACC as the sole nitrogen source. UW3 and UW4 were positive controls, whereas accD- was the negative control.

Soil	EC_e (dS/m)	Name	ACC deaminase activity (μmoleα-KA/mg protein/h)	IAA- equivalents (μg/ml/OD_{600} unit)	Gram Stain
		UW3	10.7±0.01	0.66±0.04	-
		UW4	6.12±0.03	3.35±0.09	-
		$accD^-$	0.05±0.02	3.01±0.03	-
			Year-2006 Isolates		
CMH	50.4	CMH1	1.00±0.02	2.10±0.21	-
		CMH2	6.63±0.03	2.51±0.11	-
		CMH3	4.22±0.01	1.50±0.03	-
		CMH4	0.50±0.01	0.01±0.22	-
		CMH5	0.30±0.02	0.51±0.11	-
		CMH6	0.25±0.02	0.23±0.25	-
CMM	49.5	CMM1	0.20±0.01	0.69±0.39	-
		CMM2	0.10±0.00	0.19±0.11	-
		CMM3	0.03±0.01	0.91±0.15	+
		CMM4	0.02±0.01	1.01±0.94	-
		CMM5	1.11±0.02	2.34±0.76	-
		CMM6	1.50±0.06	1.51±0.31	-
CML	9.4	CML1	0.11±0.01	0.99±0.12	-
		CML2	1.21±0.01	0.54±0.05	-
		CML3	2.44±0.02	0.22±0.01	-
		CML4	1.01±0.03	0.32±0.07	-
		CML5	0.78±0.03	0.11±0.06	-
		CML6	0.81±0.02	0.07±0.04	-
			Year-2007 Isolates		
CMS-2007	8.6	CMR1	1.12±0.02	1.29±0.11	+
		CMR2	0.96±0.22	0.71±0.01	-
		CMR3	1.17±0.01	2.86±0.20	-
		CMR4	0.87±0.11	0.91±0.04	-
		CMR5	0.92±0.03	0.34±0.06	-
		CMR6	3.76±0.62	0.33±0.12	-
		CMR7	4.21±0.21	1.22±0.11	-
		CMR8	3.42±0.87	1.09±0.06	-
AL-2007	14.3	ALR1	8.50±0.94	3.21±0.14	-
		ALR2	7.77±0.28	0.22±0.01	-
		ALR3	1.51±0.11	0.39±0.12	-
		ALR4	1.22±0.31	3.12±0.26	+
		ALR5	0.83±0.11	1.77±0.12	-
		ALR6	0.97±0.11	3.18±0.33	-
		ALR7	2.70±0.95	2.93±0.17	-

UW3 and UW4 are *Pseudomonas putida* as positive controls.
The negative control $accD^-$ is an ACC deaminase gene knockout mutant of UW4

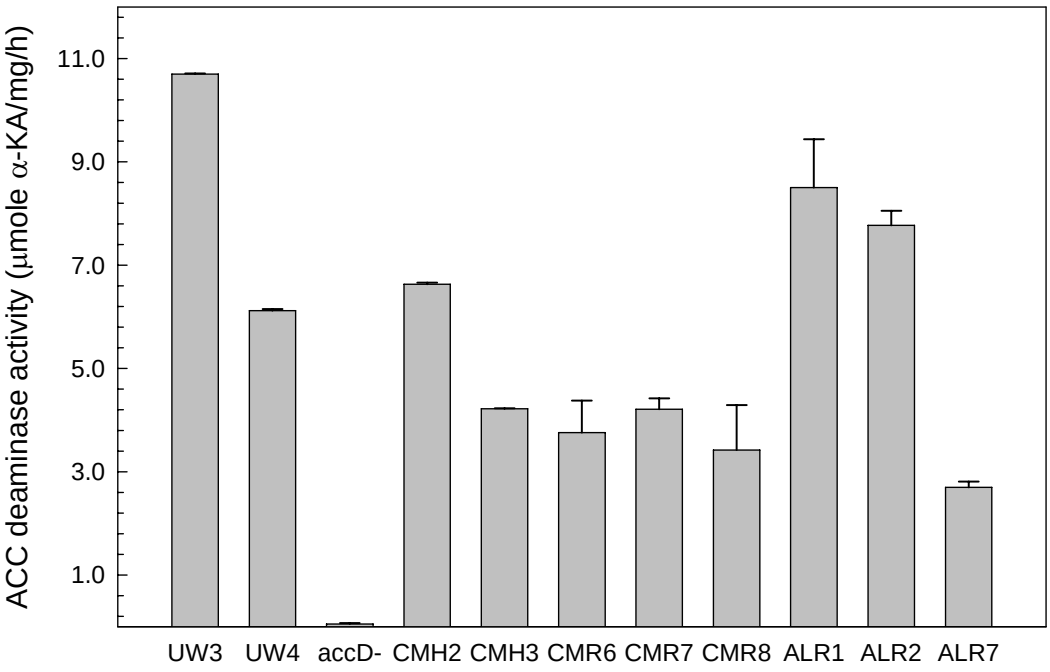


Figure 3.3. The ACC deaminase activity of selected isolates. UW3 and UW4 were positive controls, whereas accD$^-$ was the negative control. See Table 3.3 for detailed information.

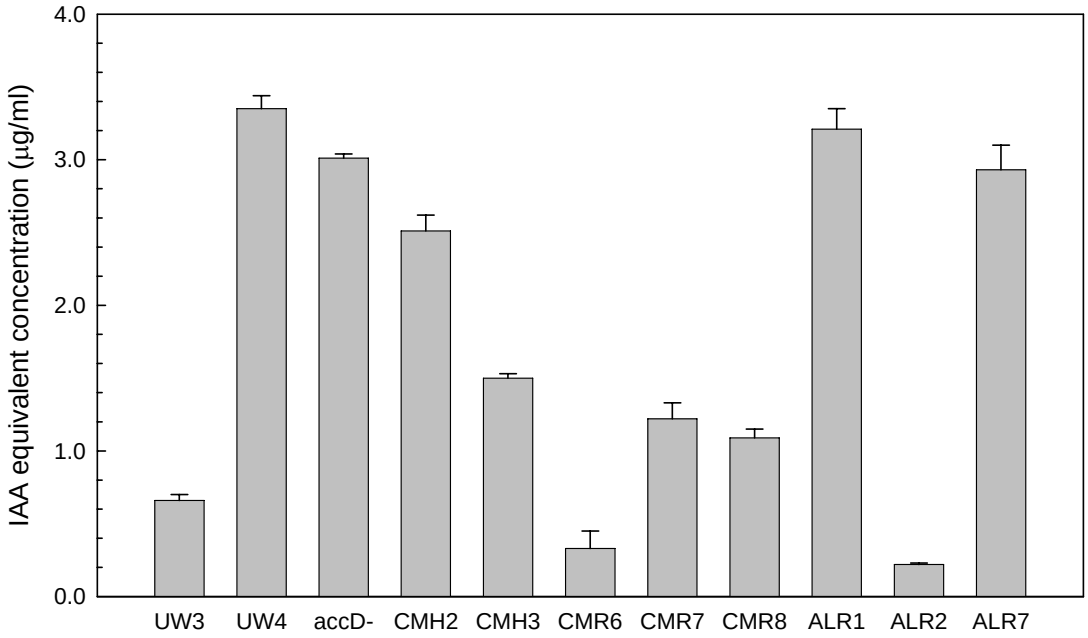


Figure 3.4. The IAA-equivalents concentrations of isolates that had high ACC deaminase activity. The ACC deaminase gene knockout mutant accD$^-$ had similar IAA level as UW4. See Table 3.3 for detailed information.

3.4. Viable bacteria counts of the PGPR inoculated seeds

Isolates with high ACC deaminase activity were coated onto the surface of seeds as the inoculation method. Prior to testing their plant promotion effect in the soil, the efficiency of inoculation was examined.

The results of plate counts method indicated that the average number of viable PGPR of *Pseudomonas putida* UW4 on seeds was 10^8-10^9 cfu/seed for oats and barley, and 10^6-10^7 cfu/seeds for ryegrass and fescue. These inoculation rates approximated to those in Germida and Walley (1996) where pseudomonad *Pseudomonas cepacia, P. aeruginosa, P. fluorescens* and *P. putida* were inoculated on winter wheat at a rate of 10^7 - 10^8 cfu/seed and effectively enhanced plant growth and the yield. Hence, all PGPR tested in this study was inoculated onto seeds with the same inoculation method.

3.5. Root elongation pouch test

Before testing the growth promotion effect of the Year-2006 isolates CMH2 and CMH3 in soil, a pouch assay was conducted to examine their root elongation effect in a more controlled environment. At 0% salt, no PGPR treatment enhanced root elongation (Figure 3.5). However, at 1% salt (Figure 3.6) improved root elongation was observed ($P < 0.01$). CMH3 enhanced the root length of barley, Topgun ryegrass and Excalibur tall fescue. The root length of barley and oats treated with UW3 and UW4 in combination (labeled as 'UW3+4' hereafter) were significantly longer than that of the untreated (-PGPR). CMH3 promoted the root length of barley, ryegrass and fescue. CMH2 enhanced root growth of Excalibur tall fescue. In

conclusion, PGPR enhanced root growth of plants under saline conditions but not under non-saline conditions.

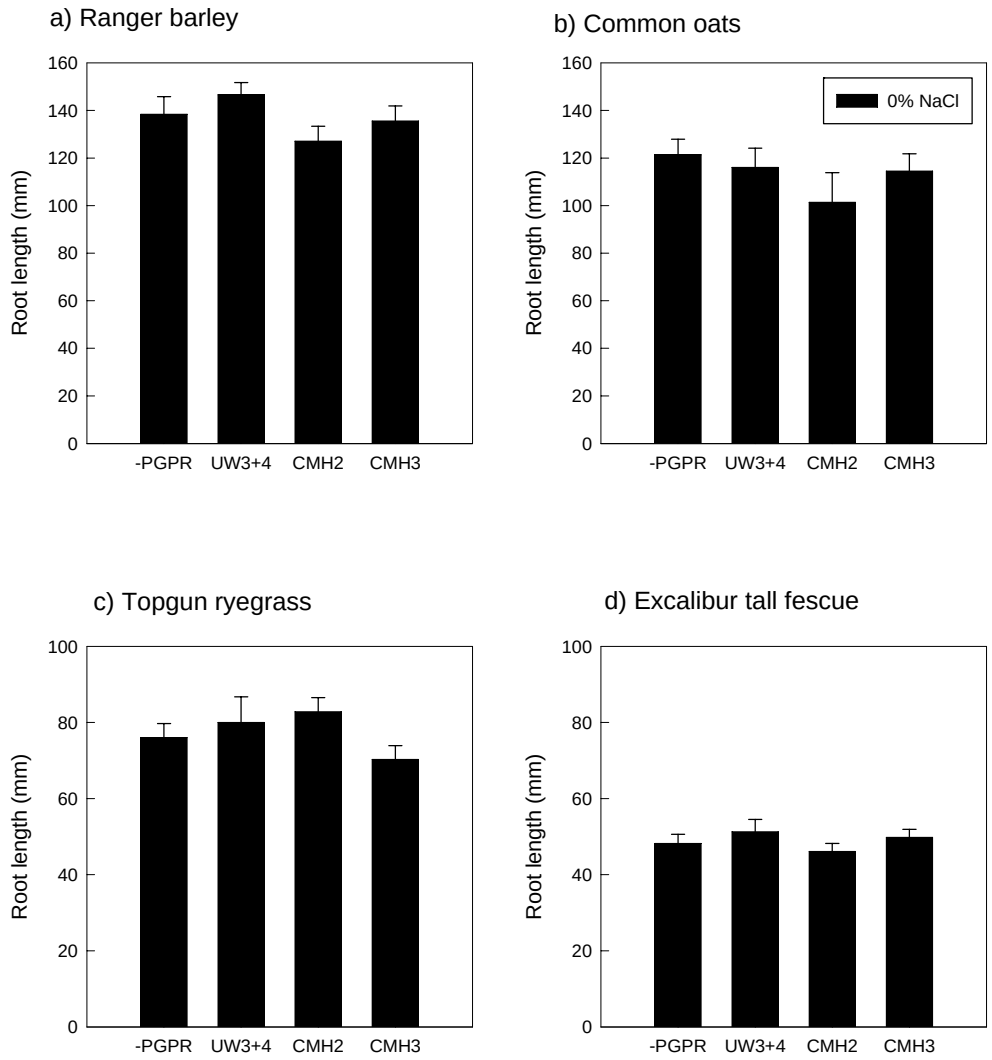


Figure 3.5. The root length of a) Ranger barley, b) Common oats, c) Topgun ryegrass and d) Excalibur tall fescue at 0% salt. No PGPR treatment enhanced root elongation. Root length was measured 3 days after germination for crops and 10 days after germination for grasses. The label 'UW3+4' means the combination of UW3 and UW4 as the PGPR treatment. Results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR).

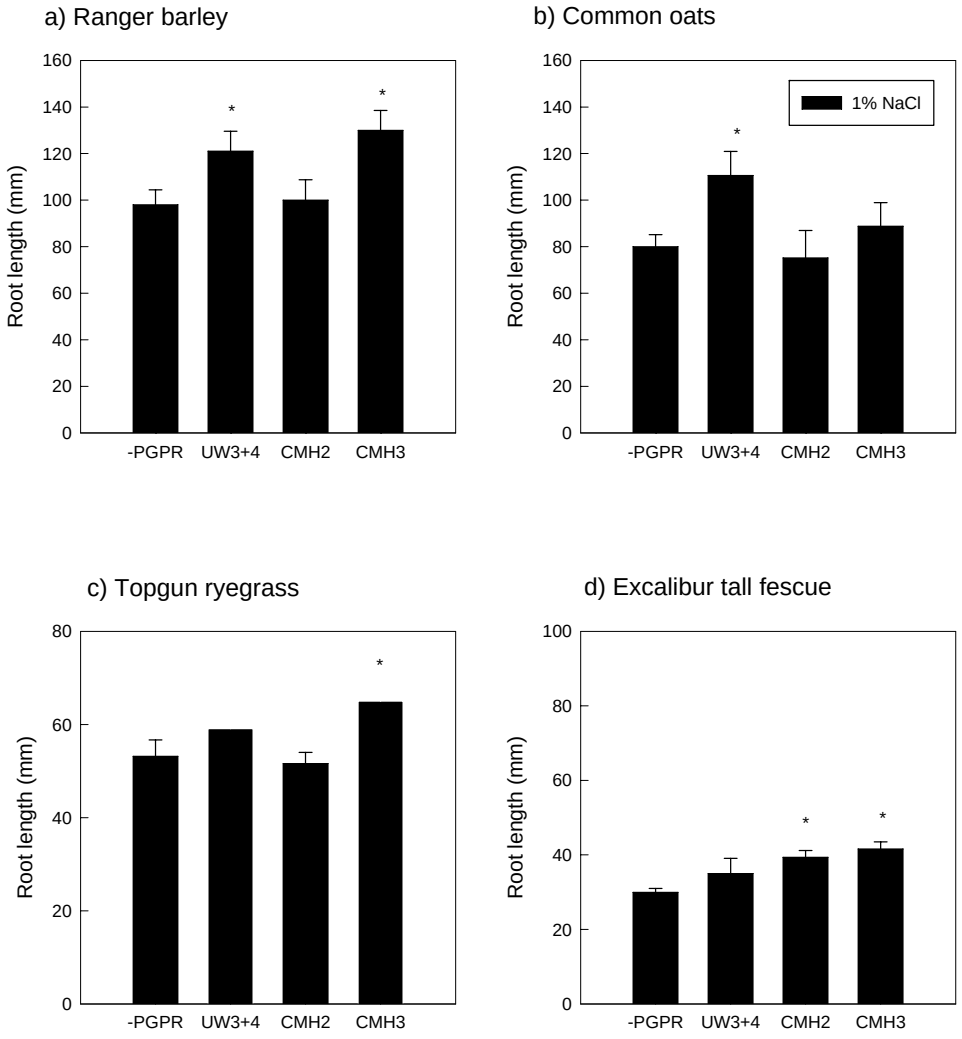


Figure 3.6. The Root length of a) Ranger barley, b) Common oats, c) Topgun ryegrass and d) Excalibur tall fescue at 1% salt (17 dS/m). At 1% salt, the radicle emergence of both crops and grasses occurred 5 days later than the 1% salt treatment. Root length was measured 3 days after germination for crops and 10 days after germination for grasses. The label 'UW3+4' means the combination of UW3 and UW4 as the PGPR treatment. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR).

3.6. Plant selection for PGPR greenhouse tests

Three crop and five grass species were tested for salt tolerance based on their percent germination and growth in 6 weeks. The goal was to select plants that are salt tolerant, capable of producing high biomass under local climatic conditions because the efficiency of salt phytoremediation (removal) largely depends on the shoot biomass.

Barley, oats and wheat are common crops in western Canada. Barley is a salt-tolerant crop that was cultured as a substitute for wheat due to soil salinity in Mesopotamia centuries ago. Oats and wheat have moderate to high salt tolerance (Richards, 1954). Perennial cool season grasses such as fescue and ryegrass were chosen because they can overwinter and start to grow early in the year. Additionally, grasses also have more extensive and dense roots than crops so that they can be more resistant to osmotic stress (drought). Blue gramma and buffalo grasses were chosen because they are native species in western Canada and are moderately salt tolerant. It is common for land reclamation practices to grow annual crops together with perennial grasses in a mix in order to prolong the canopy period.

Based on the results in Table 3.4, barley, oats, fescue and ryegrass were chosen for greenhouse tests with PGPR because of their superior germination condition and growth. The variety of oats and fescue used in the following greenhouse experiments may be different in part due to the availability of the seeds or the preference to local varieties.

It must be noted that the soil used was so saline (EC_e = 49.5 dS/m) that these non-halophyte (glycophyte) plants would not have been able to produce such amounts of biomass, therefore salt leaching away from the soil caused by excessive watering was suspected. This issue will be further discussed in Section 1.1.1.

Table 3.4. Germination and growth of eight plants for the preliminary selection. Soil used was CMM (EC_e = 49.5 dS/m). The experiment was conducted in four replicates.

Species	Performance		Select
	Germination	Biomass	
Common barley *Hordeum vulgare* (Annual)	+++++	++++	√
Common oats *Avena sativa* (Annual)	+++++	+++++	√
Winter wheat *Triticum sativum* (Annual)	++++	+++	√
Excalibur tall fescue *Festuca arundinace* (Perennial)	++++	++	√
Topgun ryegrass *Lolium perenne* (perennial)	++++	++	√
Blue gramma *Bouteloua gracilis* (perennial)	-	-	-
Buffalo grass *Buchloe dactyloides* (perennial)	++	+	-
Creeping red fescue *Festuca rubra* (perennial)	++	+	-

3.7. Greenhouse tests: Effects of PGPR and AMF on plant growth in saline soils

Six greenhouse experiments were conducted. All these greenhouse experiments were carried out with soils sampled directly from the research sites to replicate the *in situ* rhizosphere condition and hence give better prediction of plant growth promotion effects of PGPR for the field trials. The soil used for each experiment varied, in part, due to the availability of soil. More importantly, by using different soils, the consistency of growth promotion effects of PGPR in soils with various salinities could be examined. Moreover, in Experiment #3 and #5 the sampled soils were diluted with the general purpose potting soil Tri-Mix™ (Kengrove Inc., Ontario) for adjusting soil salinity to certain salinity levels.

Experiment #1 examined the necessity of fertilizing the Alameda (AL) site. In Experiment #2, UW3+4 and AMF were tested with oats and wheat. Experiment #3 showed how plant growth was affected by a gradient of soil salinity, and the variation in salt tolerance between plant varieties. Experiment #4 included the indigenous isolate CMH2 and CMH3 as the PGPR treatment for selected crops and grasses, while local plant varieties were tested in Experiment #5. Finally, in Experiment #6, CMH2, CMH3 and the newly isolated Year-2007 isolates were tested.

The percent germination rate, biomass and salt accumulation in plant tissue were measured to evaluate the phytoremediation efficiency. In addition, AMF colonization in Experiment #4 and nodulation of alfalfa in Experiment #5 were quantified. The brief description of the experimental purposes and conditions of these six experiments are listed in Table 3.5. For the

ease of referencing, specific experiment conditions are also listed at the beginning of each experiment.

Table 3.5. The purposes and experimental variables of six greenhouse experiments.

Exp	Soil (EC_e in dS/m)	Purpose	Plant	PGPR/AMF	Measurement
1	ALM (19.4) and ALL (10.1)	Examination of the importance of nutrient addition	Ranger barley, Baler oats	UW3+4	Shoot dry weight Plant salt uptake
2	CMH (50.4)	Examination of PGPR and AMF effects on oats and wheat	common oats, winter wheat	UW3+4, AMF, UW3+4+AMF	Shoot fresh weight Germination
3	Diluted CMH (12.5, 17, 25, 50)	Determination of the soil salinity range for plant growth	Ranger barley, Baler oats, common oats, Inferno tall fescue, Tomcat tall fescue	None	Germination Root dry weight Shoot dry weight
4	CML (9.4)	Examination of PGPR and AMF effects (including Year-2006 isolates)	Common oats, Ranger barley, Excalibur tall fescue, Topgun ryegrass	UW3+4, CMH2, CMH3, AMF, UW3+4+AMF	Root dry weight Shoot dry weight AMF colonization Plant salt uptake
5	Diluted CMM (13)	Examination of PGPR effects on local varieties	Alfalfa, red spring wheat, Vivar barley, Orchardgrass	UW3+4,CMH2, CMH3	Root dry weight Shoot dry weight Nodulation
6	AL-2007 (14.3) and CMS-2007 (8.6)	Examination of PGPR effects on barley and oats (including Year-2007 isolates)	Ranger barley, Baler oats	UW3+4, CMH3, ALR1, ALR2, ALR7, CMR6, CMR7, CMR8	Shoot dry weight

'UW3+4' means the combination of UW3 and UW4

3.7.1. Greenhouse experiment #1: The importance of additional nutrient supply

The purpose of this experiment was to examine the importance of nutrient addition for alleviating salt stress. For this experiment, soils from the AL site, ALM (EC_e = 19.4 dS/m, SAR = 16) and ALL (EC_e = 10.1 dS/m, SAR = 4), were used. Baler oats and Ranger barley were the plant species tested. The inoculated PGPR was UW3+4. The growth period of this experiment was 45 days.

The biomass of plants grown in the ALM soil was less than that in the ALL soil. This could be due to higher soil salinity of the ALM soil. In addition, for both soils chlorosis of mature leaves of oats and barley occurred in 2 weeks, while new leaves were green. The growth was stagnating as well (Figure 3.7, -PGPR, -Fertilizer). Therefore, liquid fertilizer (20-20-20) was applied in the third week as an amendment. The application of fertilizer alone increased shoot biomass of both barley and oats by up to 300% (Figure 3.8, -PGPR). More importantly, when fertilizer was applied to PGPR treated plants, plant shoot biomass was approximately 20% higher than that of plants treated with fertilizer alone (i.e. without PGPR) (Figure 3.8). In fact, the PGPR treated plants were taller and greener than the -PGPR plants regardless of the fertilizer application. Although fertilizer enhanced plant growth, some typical NaCl phytotoxicity symptoms remained when plants were not treated with PGPR. These symptoms included leaf tip burning, necrosis and less biomass. When plants were treated with both fertilizer and PGPR, those symptoms were less prominent (Figure 3.7).

The concentrations of Na, Cl, B, Ca, K, P and Mg of shoot plant tissue were listed in Table 3.6. The salt (NaCl) levels of plants with various treatments were fairly constant. That is, NaCl

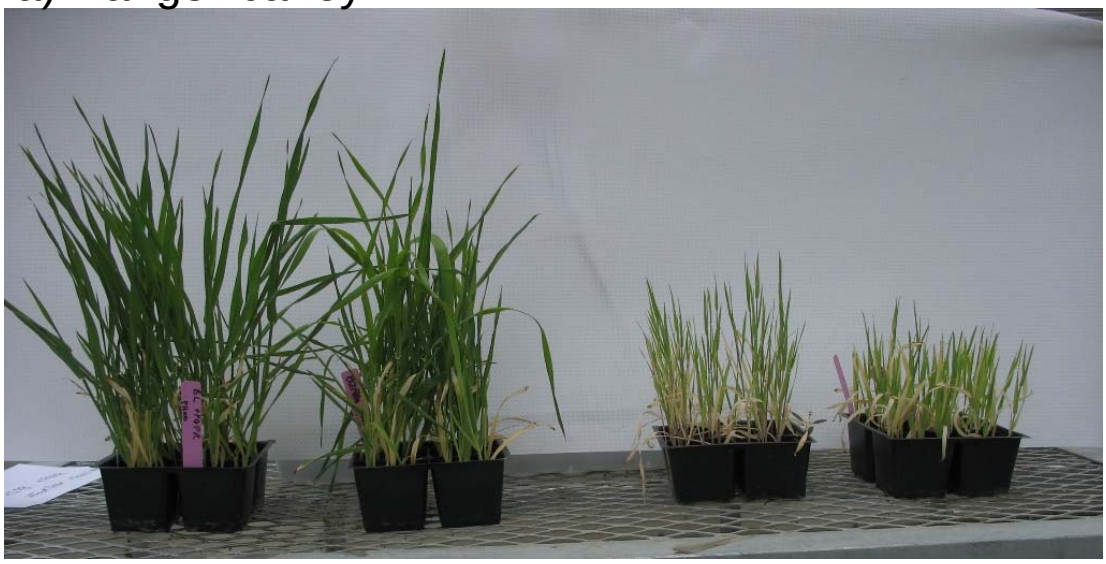
concentrations in plant tissue (~ 62000±5000 mg/kg) were not greatly altered by PGPR or fertilizer treatments. Consequently, the total amount of salt accumulation in plant biomass, i.e. removable salt, is proportional to plant biomass. The symptoms of oats and barley growth resembled nitrogen or phosphorus deficiency. The barley plants lacked tillering and the stems were thin with red basal portions. The leaves were pale green and older leaves turned yellow with purple tints, followed by premature withering. For oats, the tiller number was low and stems were thin. As well, the leaf sheaths turned purple, which is a typical phosphorus deficiency symptom. Elemental analyses of plants also implied P deficiency (Table 3.6). The phosphorus concentrations in fertilized oats and barley were significantly higher than those in unfertilized plants ($P < 0.05$). The K and Ca concentrations also were moderately higher in the plant tissue.

Saline soils sometimes also contain high levels of boron that can inhibit plant growth, and hence boron toxicity to plants was suspected. Soil with available boron more than 2 mg/kg might be toxic to plants (Richards, 1954). In Table 3.2, the available boron levels of two types of soil used in this experiment were 2.8 (ALM) and 1.9 mg/kg (ALL), which might be high enough to result in boron toxicity. Moreover, the boron levels in shoot tissue ranged from 24 to 93 mg/kg with an average of 53 mg/kg (Table 3.6), approximating the level of boron at which plant toxicity symptoms start to appear for oats and barley (Adriano, 2001). Therefore, boron in the soil might have, in part, inhibited plant growth.

In summary, UW3+4 inoculation was effective in promoting plant growth in soils regardless of fertilizer application. Moreover, the results here suggested the importance of supplying nutrients to soils in the field. Nonetheless, due to the impracticality of applying liquid fertilizer

in the field, the three research sites were amended with a layer of compost before planting seeds inoculated with PGPR for the year 2007 field trials.

a) Ranger barley



+Fertilizer -Fertilizer

+PGPR -PGPR +PGPR -PGPR

b) Baler oats

+Fertilizer -Fertilizer

+PGPR -PGPR +PGPR -PGPR

Figure 3.7. Pictures of 45-day old a) Ranger barley and b) Baler oats grown in ALL soil with fertilizer supply. PGPR: UW3+4.

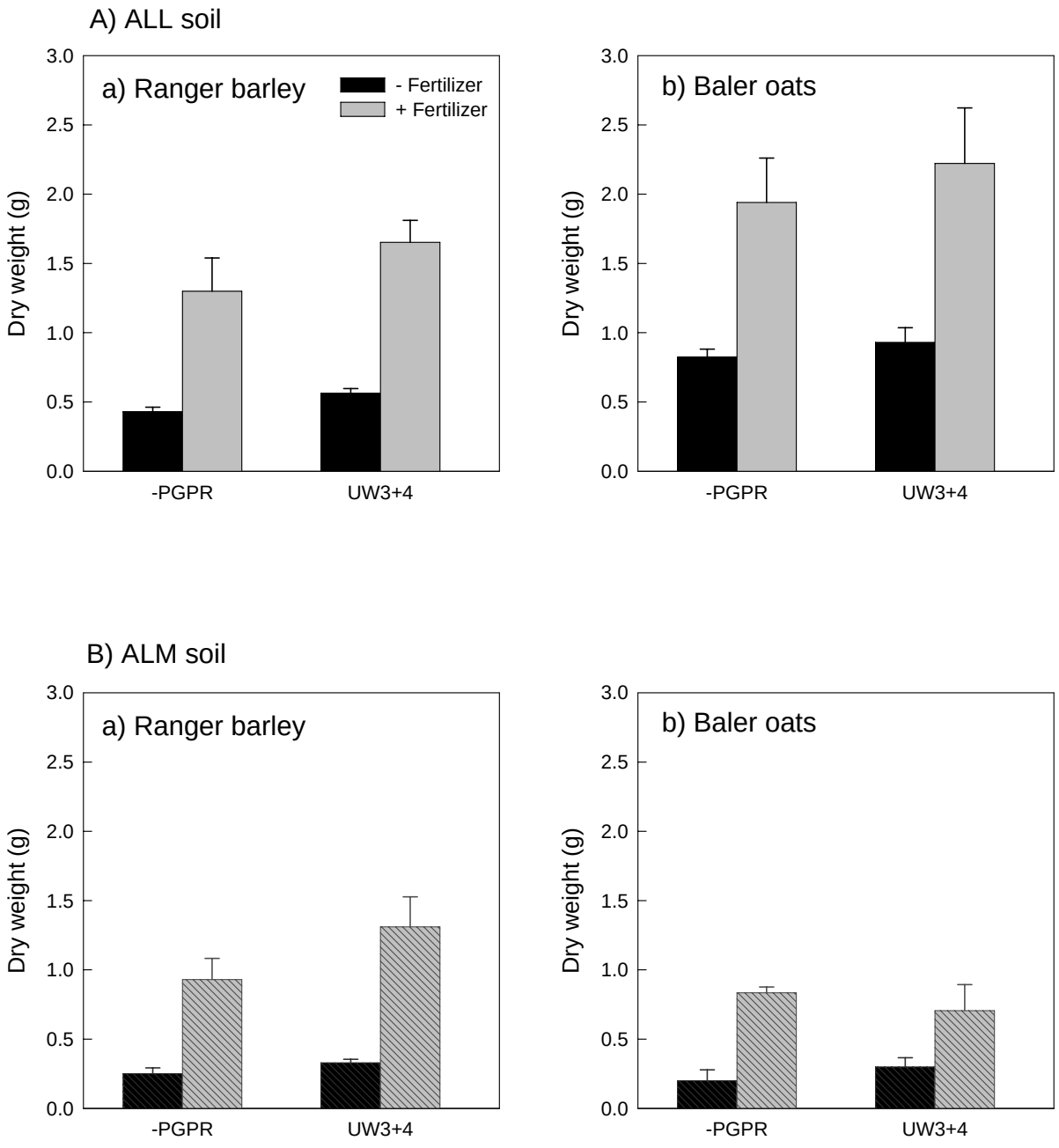


Figure 3.8. Shoot dry weight of a) Ranger barley and b) Baler oats in ALL and ALM soils in 45 days. The results are expressed as means ±1 SE of four replicates. The 20-20-20 fertilizer alone (-PGPR, + Fertilizer) effectively increased shoot biomass of both barley and oats by 150% to 300%.

Table 3.6. Concentrations of Na, Cl, B, Ca, K, P and Mg of 45-day old Ranger barley and Baler oats grown in ALL and ALM soils with fertilizer. The shoot biomass of four replicates was collected as one composite sample for analysis. Unit: mg/kg.

Soil	Fertilizer	Species	PGPR	Na	Cl	NaCl	B	Ca	K	P	Mg
ALL											
	- Fertilizer	Barley	-PGPR	12700	44200	56900	82	8560	21200	1320	17000
			UW3+4	6640	38700	45340	63	5710	20400	1170	11400
		Oats	-PGPR	9300	35700	45000	57	5510	18600	1210	11500
			UW3+4	8810	30400	39210	76	6640	21100	1210	10300
	+ Fertilizer	Barley	-PGPR	30100	47800	77900	93	9280	26700	5460	65000
			UW3+4	18000	36900	54900	57	6040	33300	4540	8250
		Oats	-PGPR	13000	26100	39100	32	4720	18700	1830	7450
			UW3+4	14700	33100	47800	51	7350	18100	1600	10400
ALM											
	- Fertilizer	Barley	-PGPR	16500	30300	46800	25	5790	16900	2020	4980
			UW3+4	26800	44800	71600	33	6620	16300	1970	5550
		Oats	-PGPR	36300	63800	100100	35	13000	9710	1210	6730
			UW3+4	29600	52500	82100	24	9490	13500	1680	5020
	+ Fertilizer	Barley	-PGPR	30900	50900	81800	54	8640	28700	6190	6230
			UW3+4	16900	40900	57800	68	8720	27500	3690	12200
		Oats	-PGPR	32300	49500	81800	53	11700	13200	2350	6870
			UW3+4	30600	43400	74000	38	10600	15106	6555	6640
			Mean	20822	41813	62634	53	8023	19939	2750	12220
			SE	2460	2458	4681	5	597	1588	474	3611

3.7.2. Greenhouse experiment #2: PGPR and AMF effects on oats and wheat

The purpose of this experiment was to examine the growth promotion effect of UW3+4 and AMF on plants grown in saline soil. For this experiment, soils from the CMS site, CMH (EC_e = 50.4 dS/m, SAR = 12) was used. Common oats and winter wheat were the plant species tested. The PGPR/AMF treatments included UW3+4, AMF and UW3+4+AMF. The growth period of this experiment was 35 days.

As shown in Figure 3.9 and Figure 3.10, UW3+4 completely reverted the root and shoot growth inhibition caused by salinity. In fact, the shoot biomass of plants treated with UW3+4 exceeded that of the control. Compared to -PGPR, UW3+4 improved the shoot and root fresh weight of oats by 100%. For wheat, the increase in fresh weight was 20% for roots and 80% for shoots. AMF enhanced root growth of both plants by 30%. The UW3+4 and AMF combination did not show an additive or synergistic effect. Although both oats and wheat growth were promoted by PGPR, oats are the better crop for phytoremediation than wheat because the biomass, especially shoot biomass, was 3 – 5 fold higher than that of wheat, regardless of the PGPR treatment.

What has to be addressed here is the high salinity (EC_e = 50.4 dS/m) of the soil used in this experiment. In such a highly saline soil, non-halophyte (glycophyte) plants such as oats and wheat are not supposed to produce such high amounts of biomass. Therefore, soil salt leaching was suspected. Since soil samples were discarded before this issue was noticed, the change of soil EC_e could not be measured to verify it. Nevertheless, the germination kinetics supported this assumption. In Figure 3.11, for both oats and wheat, a surge of germination occurred after

about 15 days, regardless of the PGPR treatment. This observation can be explained by salt leaching resulted from excessive watering and large pot bottom holes. Consequently, soil salinity decreased to a level at which seeds were able to germinate and grow. In order to avoid the excess salt leaching, bigger or same size pots with smaller holes were used and watering was controlled in the rest of the greenhouse experiments, where soils of randomly selected pots were analyzed for their $EC_{1:2}$ after each experiment and the decrease in soil salinity ($EC_{1:2}$) was about 10% - 15%.

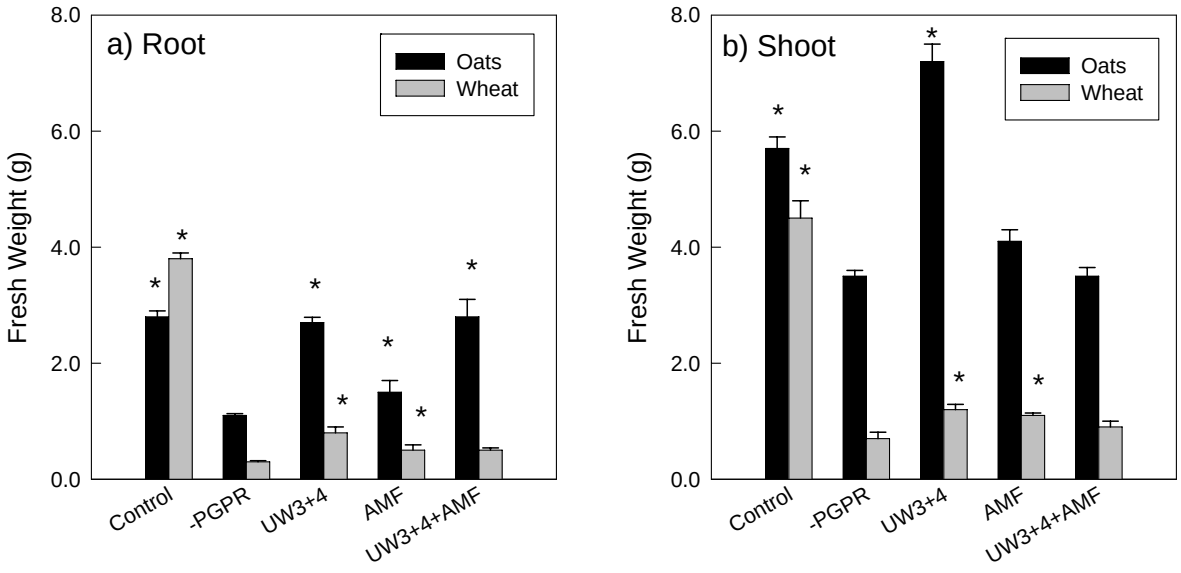
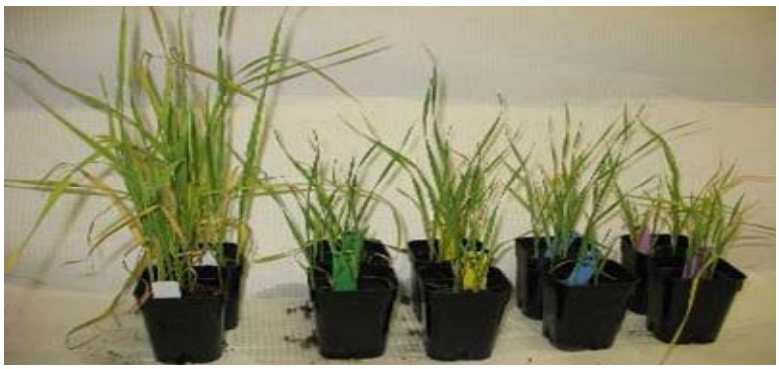


Figure 3.9. The fresh weight of a) roots and b) shoots of oats and wheat grown in saline soil (EC_e = 50.4 dS/m) in 45 days. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR). UW3+4 promoted the root and shoot growth under salt stress. UW3+4 enhanced shoot growth of oats by 100%. Control plants grown in ProMix™ served as a reference.

a) Common oats

Control (Promix) -PGPR UW3+4 AMF UW3+4+AMF

b) Winter wheat



Control (Promix) -PGPR UW3+4 AMF UW3+4+AMF

Figure 3.10. Pictures of a) common oats and b) winter wheat in experiment #2.

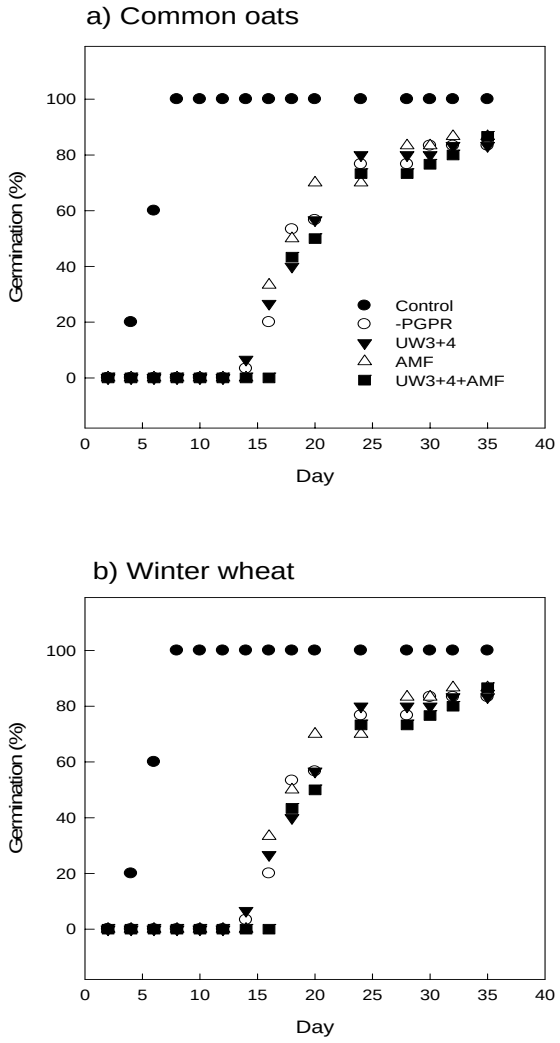


Figure 3.11. Germination of a) oats and b) winter wheat with PGPR and AMF treatments over time. A surge of germination occurred in approximate 15 days for both oats and wheat that grew in saline soils regardless of the PGPR treatment. Control plants grown in ProMix™ served as a reference.

3.7.3. Greenhouse experiment #3: Soil EC_e and plant growth

The purpose of this experiment was to investigate how the increase in salinity affect plant growth. For this experiment, CMH soil (EC_e = 50.4 dS/m, SAR = 12) was diluted with Tri-Mix™ (Kengrove Inc., Ontario) at different ratios to obtain soils of salinity at 12, 17, 25 and 50 dS/m. Ranger barley, Baler oats, Common oats, Inferno tall fescue and Tomcat tall fescue were the plant species tested. No PGPR was inoculated on seeds. The growth period of this experiment was 35 days.

The percent germination rate of all plants fell in the range of 70% – 95% when the EC_e was lower than 25 dS/m. At 50 dS/m, only Baler oats was able to maintain a germination rate of 80%. No germination of Ranger barley was observed (Figure 3.12). This unexpected result is not consistent with the notion that barley is a more salt tolerant crop. Nonetheless, research has shown great variation of salt resistance within plant species such as barley, wheat, rice and oats (Alamgir and Ali, 2006; Dehdari et al., 2005; Farooq and Azam, 2007; Katerji et al., 2005; Katerji et al., 2006; Pandya et al., 2004; Verma and Yadava, 1986). Therefore, some varieties of oats may be more salt tolerant than some barley varieties given the fact that both oats and barley are categorized as crops with high salt tolerance (Table 1.3). The root and shoot dry weight data are shown in Figure 3.13 and Figure 3.14. The result showed that when salinity was under 25 dS/m, the growth of Ranger barley was slightly better than two oats but at 50 dS/m, the growth of Baler oats exceeded two other crops.

Based on these results, Ranger barley and Baler oats were selected as crops planted for the field trial. Other attributes of these two crops also suggested good growth in the field. AC

Ranger barley (also referred to as Ranger barley) has good tolerance to drought and moderate tolerance to soil alkalinity, but poor tolerance to flooding. The forage quality, disease resistance and lodging resistance are very good. Furthermore, its forage maturity is late, i.e. a longer canopy period (Manitoba Forage Council Inc., 2006). Baler oats (also referred to as CDC Baler oats) had high disease resistance and wide large leaves that do not decay after becoming fully mature in the field (CSIDC, 2006). Holding green after maturity is a desirable trait because it implies less return of accumulated salt in shoot tissue into soil before harvesting, resulting in better salt removal efficiency. For the two tall fescues, Inferno generated more shoot and root biomass than Tomcat when salinity is high, so Inferno tall fescue was chosen as one of the grass species for the field test.

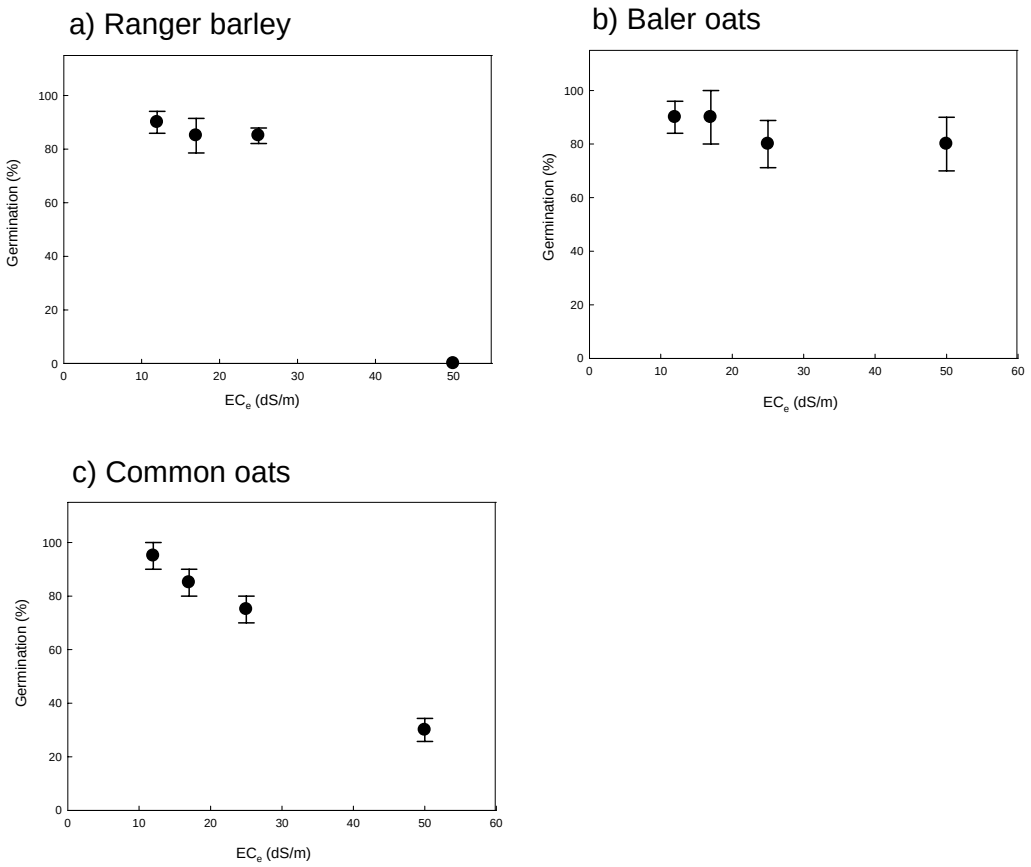


Figure 3.12. Percent germination of plants grown in soils of various salinities in 35 days. The results are expressed as means±1 SE of four replicates. The percent germination rate of three plants fell in the range of 70% – 95% when EC_e was lower than 25 dS/m. At 50 dS/m, only Baler oats was able to maintain a germination rate of 80%.

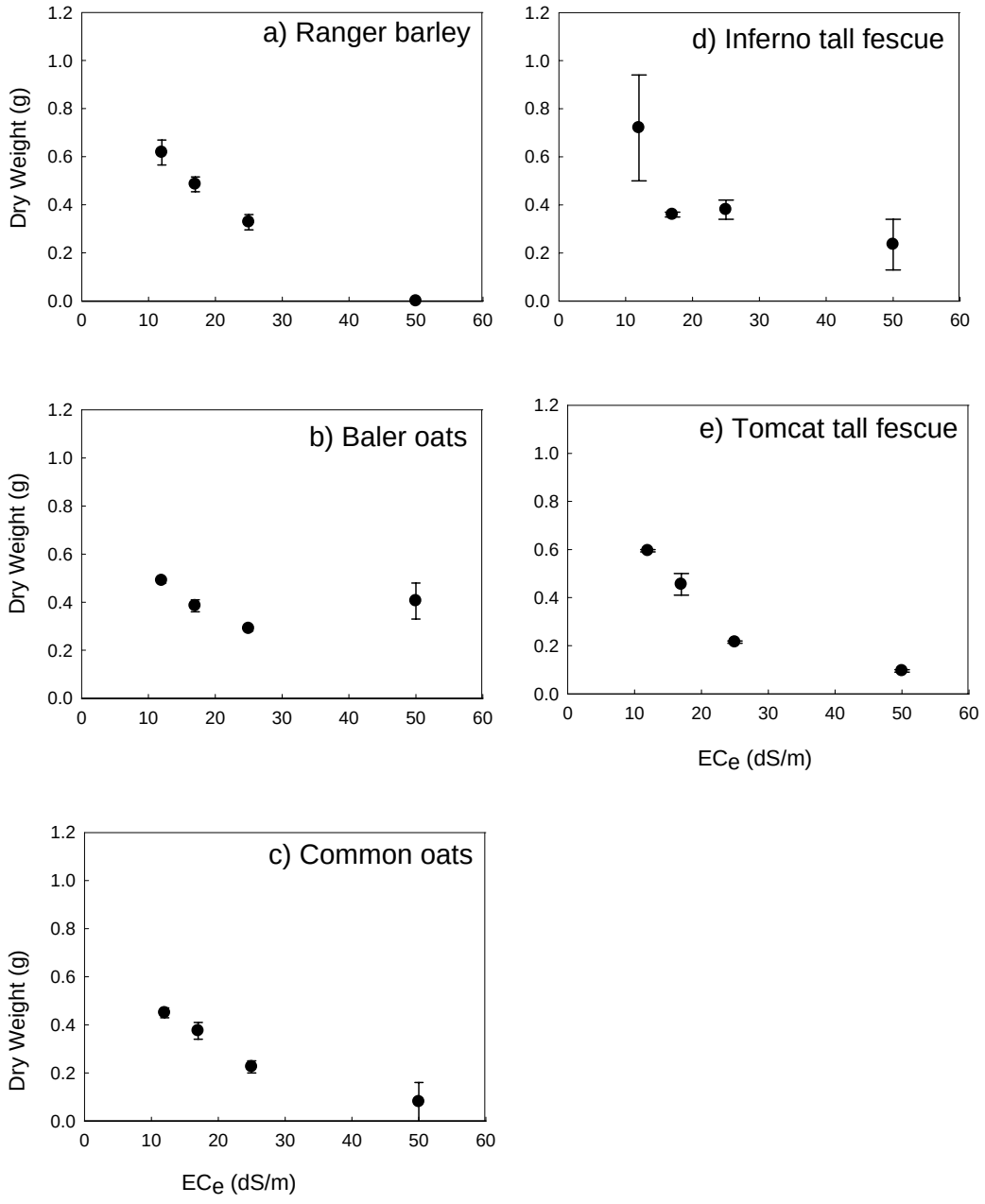


Figure 3.13. Dry weight of roots of plants that grew in soils of various salinities in 35 days. The results are expressed as means±1 SE of four replicates. The root dry weight of grasses approximated that of crops.

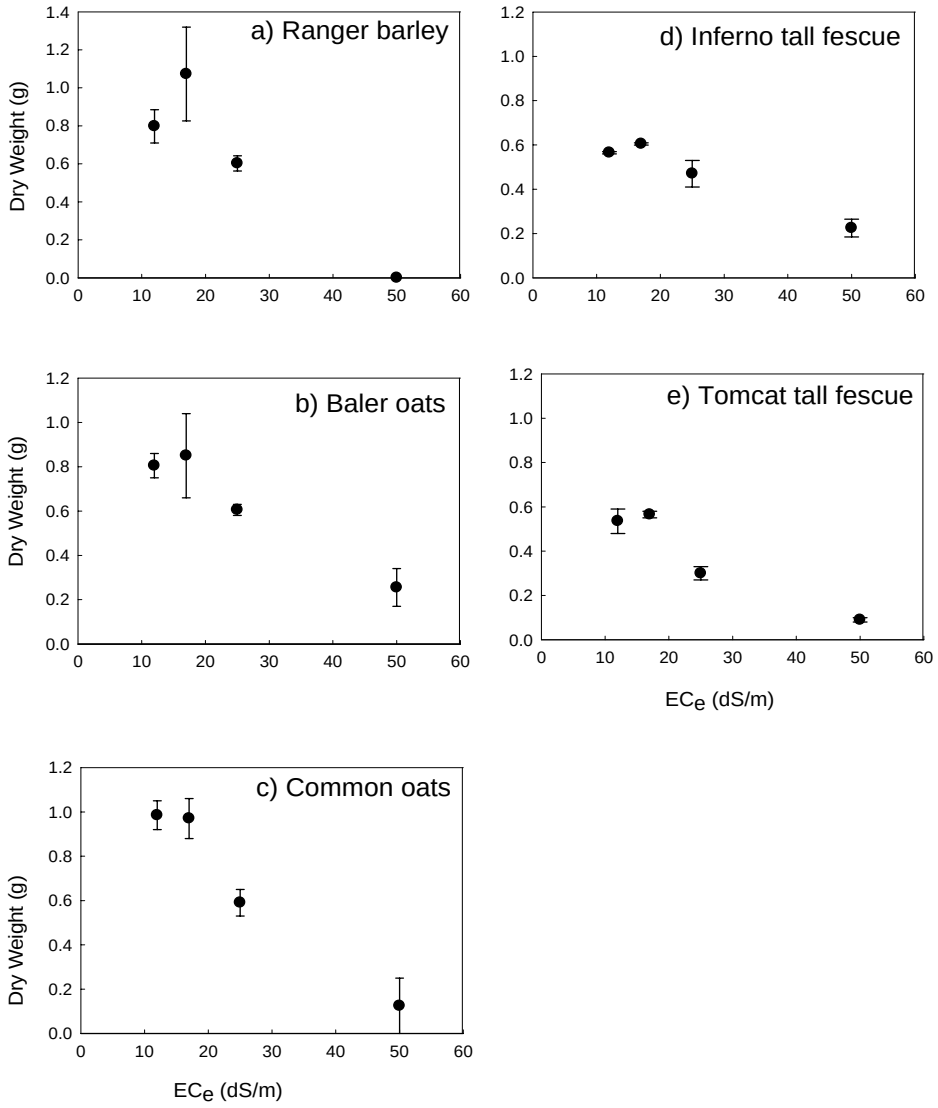


Figure 3.14. Dry weight of shoots of plants grown in soils of various salinities in 35 days. The results are expressed as means±1 SE of four replicates. When salinity was under 25 dS/m, the growth of Ranger barley was slightly better than two oats but at 50 dS/m, the growth of Baler oats exceeded common oats and Ranger barley. The shoot growth of Inferno tall fescue was higher than that of Tomcat tall fescue.

3.7.4. Greenhouse experiment #4: PGPR and AMF effects (Year-2006 isolates)

The purpose of this experiment was to examine the PGPR and AMF effect on plants. The PGPR tested included the newly isolated Year-2006 strains, CMH2 and CMH3. For this experiment, CML soil (EC = 9.4 dS/m, SAR = 11) was used. Common oats, Ranger barley, Excalibur tall fescue and Topgun ryegrass were the plant species tested. PGPR/AMF treatment were UW3+4, AMF, UW3+4+AMF, CMH2 and CMH3. The growth period of this experiment was 45 days.

The effect on plant biomass

The UW3+4 combination again showed the plant growth promotion effect. In Experiment #2, UW3+4 treatment enhanced oats shoot growth by 100% over the -PGPR, while in this experiment UW3+4 enhanced shoot growth of barley and oats by 90%. Additionally, UW3+4 also enhanced the root growth of Ranger barley by 50% (Figure 3.15 and Figure 3.16). The UW3+4 treatment; however, was not the most effective treatment. The indigenous PGPR, CMH2 and CMH3, improved shoot growth of both crops more than UW3+4 (Figure 3.15) possibly because the indigenous isolates were more competitive than the non-indigenous bacteria (Bhattarai and Hess, 1993).

For oats, the shoot and root biomass of plants treated with AMF alone or AMF together with UW3+4 was higher than that of plants treated with UW3+4 alone (Figure 3.15). This result implied that the shoot and root growth of oats treated with UW3+4+AMF were predominantly promoted by AMF rather than UW3+4. The root length colonization data in Table 3.7 indicated that the AMF application method was effective. Both barley and oats that were treated with

AMF alone or together with PGPR had higher root colonization by AMF. Nonetheless, neither the shoot nor root water content was increased by AMF colonization. Therefore, it was concluded that the growth promoted by AMF was not attributed to better water retention but other mechanisms such as production of aquaporin (Diouf et al., 2005; Ouziad et al., 2006), higher antioxidative activities (Waller et al., 2005), lowered Cl^- uptake (Copeman et al., 1996), improved nitrogen or phosphorus uptake (Ruiz-Lozano and Azcon, 2000), electrolytes or soluble sugars (Feng et al., 2002). The arbuscule and hypha of mycorrhizal fungi colonized in roots of oats were shown in Figure 3.19. The roots were sampled from plants that grew in soils sampled from the research site, so the root might have been colonized by other local arbuscular or non-arbuscular mycorrhizal fungi. However, the morphology of the arbuscule and hypha observed resembles *Glomus* spp. (Brundrett et al., 1996).

In conclusion, UW3+4+AMF was most effective for oats, whereas CMH2 was the most effective in promoting barley growth among all microbial treatments. Oats were also tested in Experiment #2 (page 69) with UW3+4 and AMF, so the results from both two experiments were compared to check the consistency of the growth promoting effects of these microbial treatments (Table 3.8). The results showed that the growth promotion effect of UW3+4 on shoots and that of UW3+4+AMF on roots were reproducible with a 100% increase in biomass, while the other treatments performed differently. It must be noted here that the experimental conditions of two experiments were different in soil salinity (50.4 dS/m and 9.4 dS/m in Experiment #2 and #4, respectively), greenhouse temperature and salt leaching. In Experiment #4, the temperature was cooler (in the range of 25 to 35 °C) and watering was controlled.

The growth of Topgun ryegrass and Excalibur tall fescue were promoted by different microbial treatments. For ryegrass, UW3+4+AMF and CMH3+AMF enhanced its shoot and root growth by 300%, whereas CMH2 and its combination with AMF were the most beneficial to the growth of fescue under salt stress (Figure 3.17 and Figure 3.18). In contrast to the PGPR effect on crops, the growth promotion effect of UW3+4 treatment on these two grasses was insignificant. It should be noted that neither UW3+4 nor AMF promoted shoot growth of ryegrass, but the combination (UW3+4+AMF) increased shoot and root biomass of ryegrass by three fold. This synergistic effect, however, did not occur to fescue. Moreover, although AMF treatment alone did not have positively effects on grasses, its combination with UW3+4 and CMH3 were the most effective in enhancing shoot growth of ryegrass, where fescue root growth was promoted the most by CHM3 and AMF in combination (Figure 3.17 and Figure 3.18).

The synergistic effect between PGPR and AMF was reported in Roesti et al. (2006), where a combined bio-inoculation of diacetyl-phloroglucinol producing PGPR strains and AMF synergistically improve the nutritional quality of the grain of spring wheat without negatively affecting mycorrhizal growth. In contrast, adverse effect of PGPR on AMF inoculation in plant roots were reported by Germida and Walley (1996). Five pseudomonad PGPR, including *P. putida*, enhanced spring wheat growth, but some of them adversely affected association between plants and indigenous AMF, resulting in a decrease in root colonization. The author suggested that the inhibition of AMF colonization could be a reason as to why spring wheat growth was not consistently enhanced by PGPR. Moreover, the interaction between a certain PGPR and AMF pair may change because the rhizobacterial community structure is highly dynamic and

influenced by different factors such as the maturity of plants, the fertilizer input and the type of bio-inoculants (Table 3.8) compared the growth results of oats that were treated with the same set of PGPR treatments in Experiment #2 and #4. Regardless of some differences in experimental conditions, such as soil salinity, pot size, watering condition, the growth promotion effect of UW3+4+AMF on roots and UW3+4 on shoots was consistent in both experiments. The effect of UW3+4 and AMF, however, varied.

To summarize, UW3+4+AMF and CMH3+AMF consistently promoted the growth of crops and grasses respectively although other PGPR and/or AMF treatments might exceed the effect of UW3+4+AMF or CMH3+AMF on certain species as discussed above.

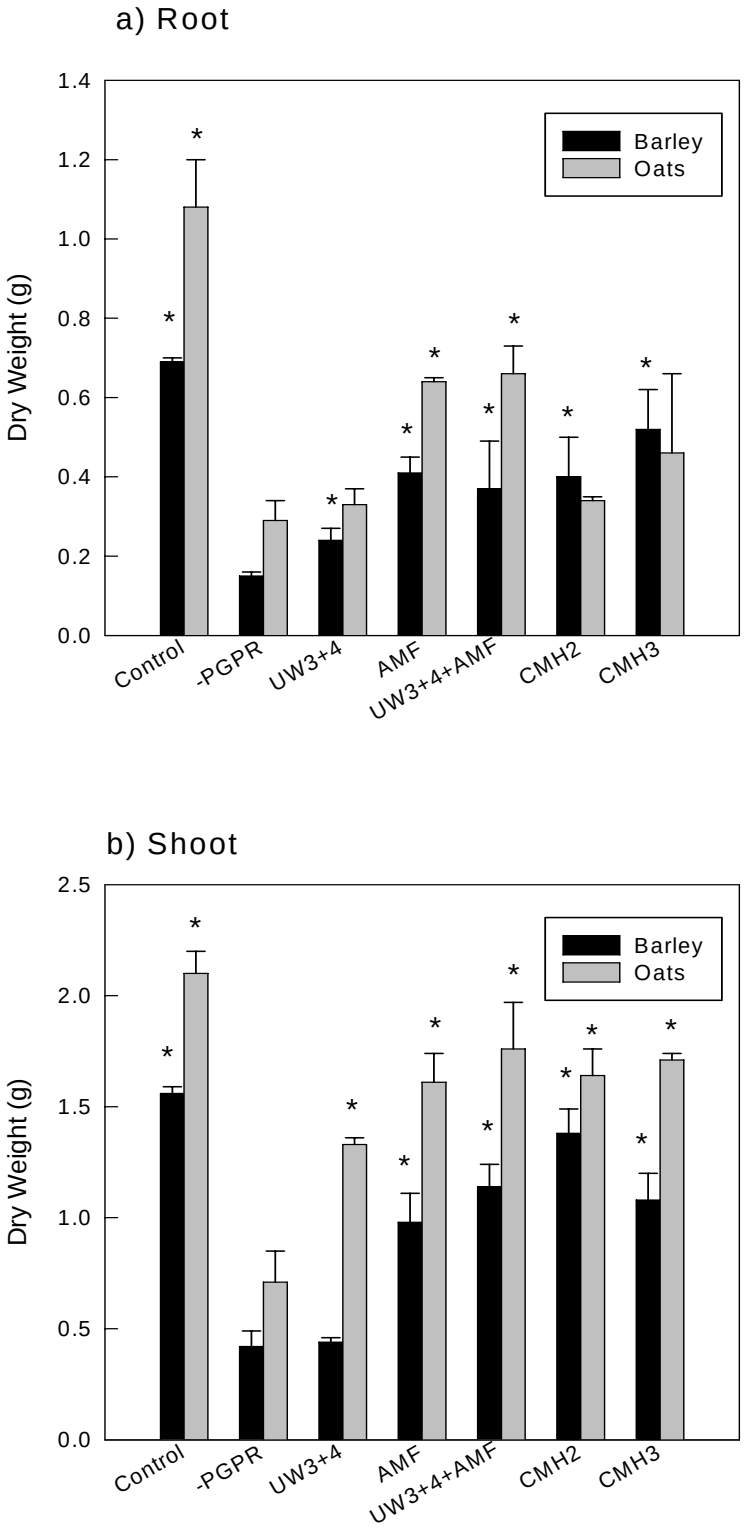
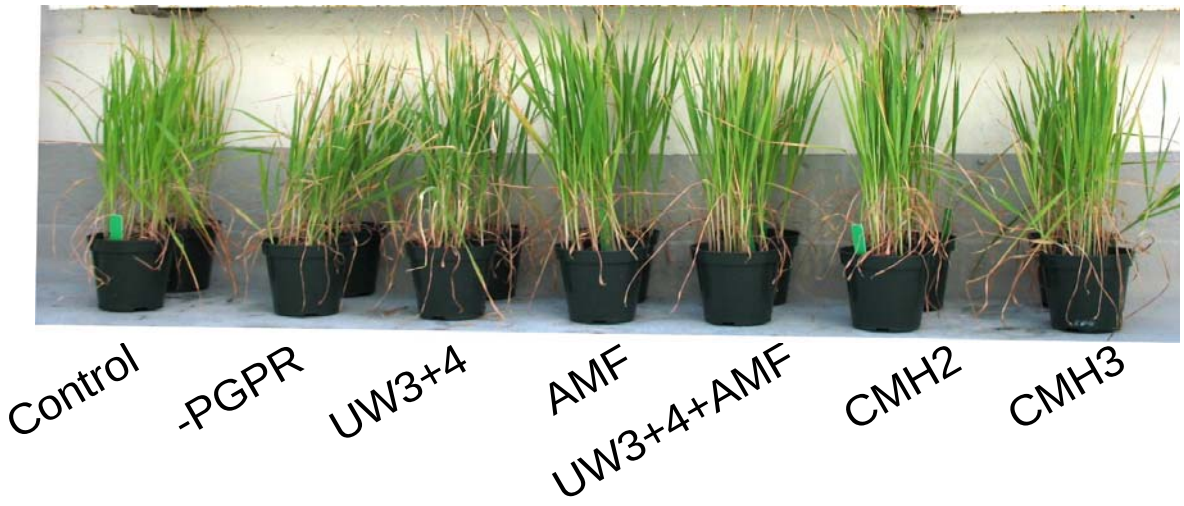


Figure 3.15. Dry weight of a) roots and b) shoots of Ranger barley and common oats in 45 days. The shoot biomass of oats was consistently higher than barley, regardless of the PGPR/AMF treatment. For root and shoot biomass of oats, the AMF and UW3+4+AMF treatments outperformed UW3+4 alone. For barley, all microbial treatments had a positive effect on plant growth except UW3+4. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR of the same plant). Control plants grown in ProMix™ served as a reference.

a) Common oats



b) Ranger barley

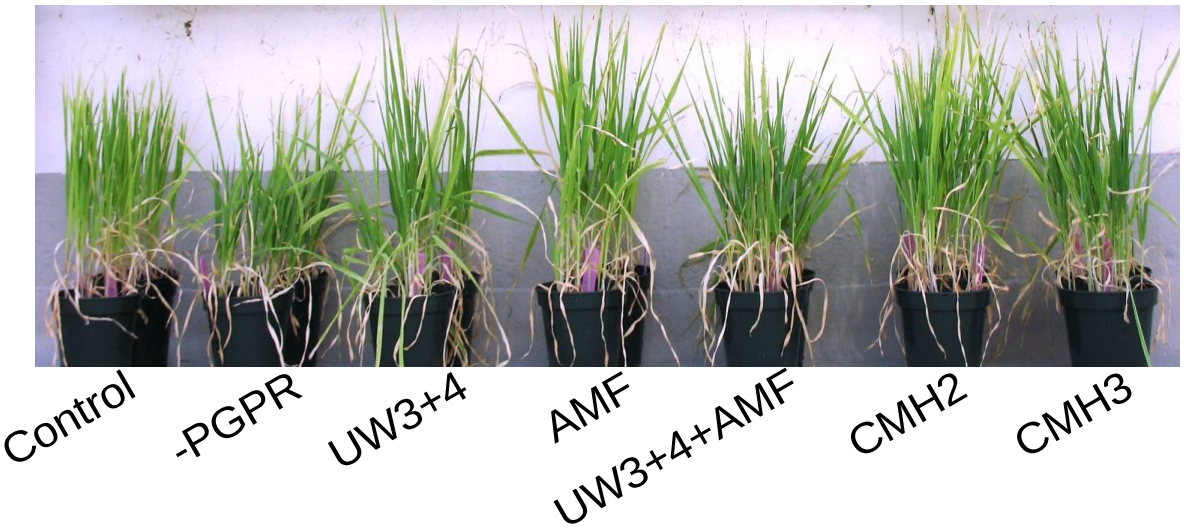


Figure 3.16. Pictures of a) common oats and b) Ranger barley in 45 days.

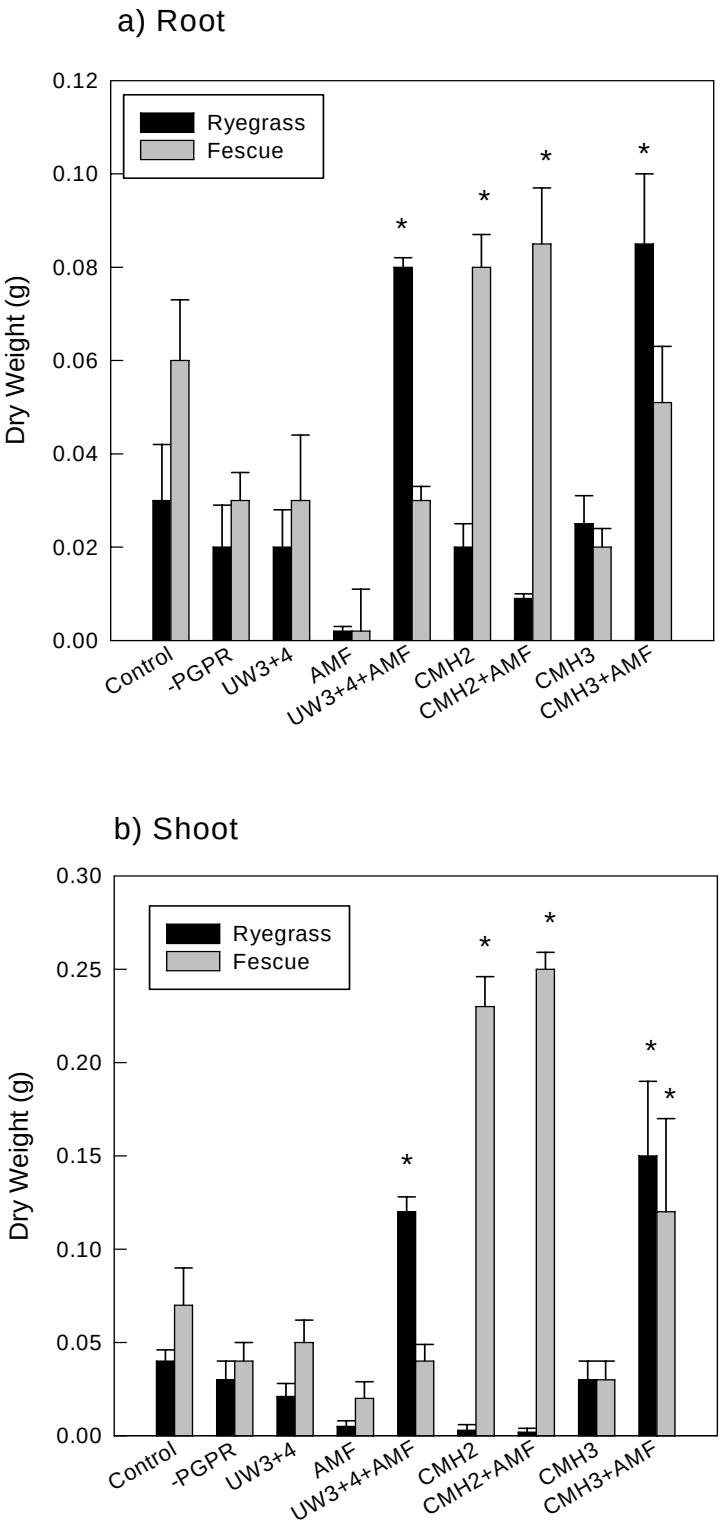
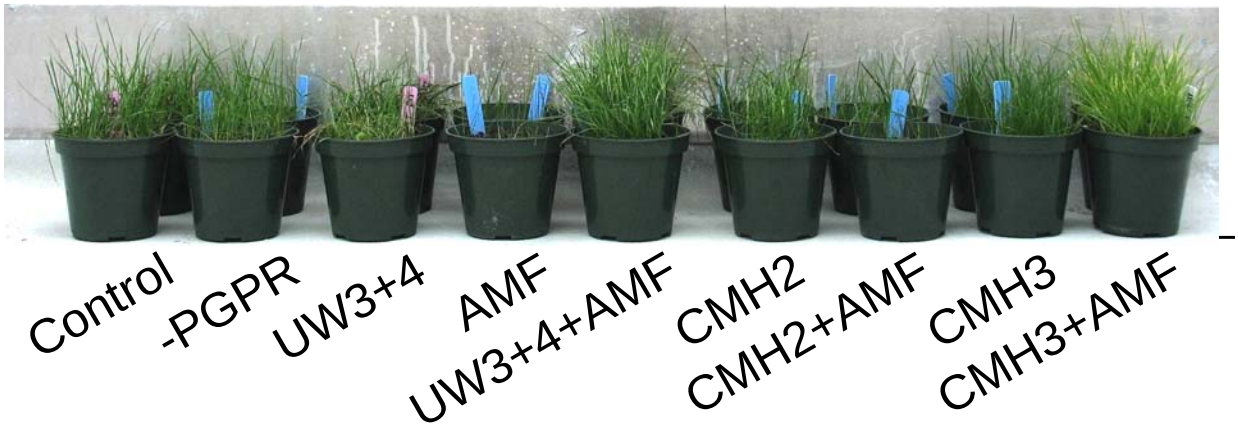


Figure 3.17. Dry weight of a) roots and b) shoots of Topgun ryegrass and Excalibur tall fescue in 60 days. The results are expressed as means ± 1 SE of four replicates. For ryegrass, UW3+4+AMF and CMH3+AMF enhanced shoot and root growth of ryegrass by 300%. CMH2 and its combination with AMF were the most beneficial to the growth of fescue. Control plants grown in ProMix™ served as a reference.

a) Topgun ryegrass



b) Excalibur tall fescue

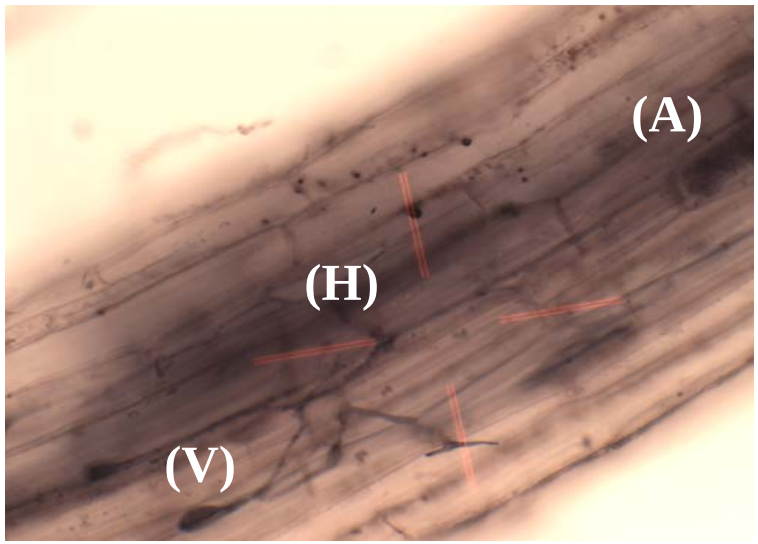
Figure 3.18. Pictures of a) Topgun ryegrass and b) Excalibur tall fescue in 60 days.

Table 3.7. Root length colonized (%), shoot and root water content of common oats and Ranger barley in Experiment #4. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR). Both barley and oats treated with AMF alone or together with PGPR had higher root colonization. The water content did not change upon the application of AMF.

		Root length colonized (%)	Shoot Water content (%)	Root Water content (%)
Oats	-PGPR	10	8	18
	UW3+4	5	16	15
	CMH2	17	7	16
	CMH3	10	10	11
	AMF	77 *	18	16
	UW3+4+AMF	64 *	12	17
Barley	-PGPR	14	7	22
	UW3+4	17	8	20
	CMH2	5	13	18
	CMH3	12	12	17
	AMF	58 *	12	22
	UW3+4+AMF	65 *	11	21

UW3+4: UW3+UW4; UW3+4+AMF: UW3+UW4+AMF

a) Ranger barely



b) Common oats

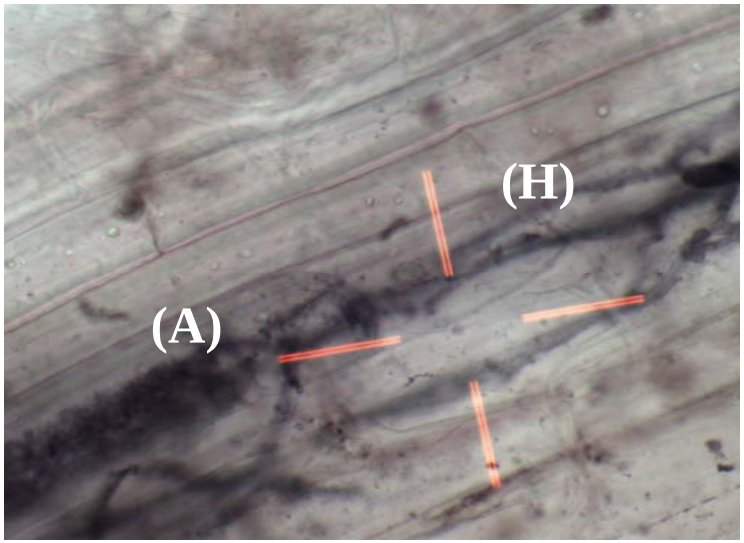


Figure 3.19. Arbuscular mycorrhizal fungi (AMF) colonization in roots of b) barley and b) oats. An arbuscule (A) locates in a root cortex cell with branch intercellular hyphae (H). Vesicules (V) were also observed.

Table 3.8. Comparison of the effects of microbial treatments on shoot biomass of oats in Experiment #2 and Experiment #4. The numbers are percentage increase in biomass when compared with controls. The growth promotion effect of UW3+4 on shoots and UW3+4+AMF on roots were reproducible with about 100% increase in biomass.

	Percent increase in biomass		
	Experiment #2	Experiment #4	Reproducible
Root			
UW3+4	113%	-[a]	No
AMF	13%	106%	No
UW3+4+AMF	120%	110%	Yes
Shoot			
UW3+4	95%	110%	Yes
AMF	-	120%	No
UW3+4+AMF	-	120%	No

[a]: "-" means no significant difference from -PGPR.

The effect on NaCl accumulation

The efficiency of salt removal in the phytoremediation system largely depends on two factors: 1) biomass and 2) concentrations of salt in plant tissue, both of which determine the total amount of salt that can be removed from the contamination site. The results so far have shown that microbial treatments improved plant growth in saline soils; however, only if the salt concentrations in plants remain constant or increase, does the increase of biomass contribute to more efficient salt phytoremediation. Therefore, it is necessary to exam the salt concentrations in the plant tissue, especially the aboveground parts that can be easily harvested.

The concentrations of Na and Cl were listed in Table 3.9 on a mg/kg dry weight basis. Due to costs, four replicates of each treatment were mixed as one composite sample for analysis. Only shoot tissue was analyzed since only the aboveground portion of the plants will be harvested as the salt removal step.

Compared with controls (ProMix™, EC_e = 1.7 dS/m), the salt concentrations of barley and oats grown in the saline soil (EC_e = 9.4 dS/m) without any microbial treatment (-PGPR) were two times higher, and the biomass decreased by 75% (Figure 3.15, b). The PGPR/AMF treatments did not seem to affect salt accumulation though the shoot NaCl concentrations of both oats and barley seemed higher when they were treated with UW3+4 (Table 3.9). However, more replicate samples are required to verify this notion.

When both the biomass and concentration were taken into account, the resultant salt accumulated in the shoot biomass of oats treated with UW3+4 was the highest, being 110 mg NaCl/pot (Figure 3.20). It is because UW3+4 not only increased the biomass but also the salt

concentration. In contrast, the total amount of salt accumulated in barley treated with UW3+4 was not higher than the -PGPR because its shoot biomass (Figure 3.15) was not promoted by the PGPR treatment although its NaCl concentration was 25% higher than the control (Table 3.9). On the other hand, although CMH2 and CMH3 did not promote higher salt concentrations, the amount of salt accumulated was the highest (Figure 3.20) on a mg NaCl/pot basis due to the higher biomass promoted by CMH2 and CMH3. Therefore, UW3+4 was the most effective microbial treatment for oats, while CMH2 or CMH3 was most effective for barley in terms of phytoremediation.

The increase in NaCl concentrations was not as much as shoot biomass. The NaCl concentrations of treated plants were at most 30% higher than those of the -PGPR (Table 3.9), but certain PGPR and/or AMF treatments promoted plant shoot biomass by 100% (Figure 3.15). Moreover, one sample of barley that poorly grew (< 0.1 g dry weight of plants/pot) in highly saline soil (CMM soil, EC_e = 49.5 dS/m with controlled watering) had 19500 mg Na/kg and 52200 mg Cl/kg, similar to the Na and Cl concentrations of barley that grew in much lower salinity (Table 3.9, CML soil, EC_e = 9.4 dS/m). This observation that the NaCl levels in barley tissue did not proportionally increase with the soil salinity was likely due to the salt exclusion (Ashraf, 2004). The poor growth and non-elevated NaCl concentrations of barley in this case further supported the idea that biomass was the more determinative factor for the efficiency of salt phytoremediation using tolerant crops. This constancy of NaCl concentrations of plants grown on sites with different salinity was also observed in the field trials (Section 3.9).

It should be pointed out that AMF seemed to inhibit NaCl uptake. This might result from the retention of sodium and chloride in mycelia of the mycorrhiza. Both oats and barley treated

with UW3+4 had the highest NaCl concentrations, but plants treated with UW3+4 and AMF in combination on average had 20% - 30% lower NaCl concentrations (Table 3.9). In addition, barley treated with only AMF had the lowest NaCl concentrations. This retention of NaCl by AMF was reported in Giri and Mukerji (2004), where Indian sesbania (*Sesbania aegyptiaca* and *S. grandiflora*) treated with *Glomus macrocarpum* had lower Na than the non-AMF plants. Copeman et al. (1996) also found tomato plants inoculated with AMF had lower Cl^- concentrations in roots than the non-AMF plants.

Table 3.9. Concentrations of Na and Cl of shoots of common oats and Ranger barley in Experiment #4. EC_e = 9.4 dS/m. Replicates were collected and mixed as one composite sample for this analysis. The NaCl concentrations of oats and barley were similar, approximating 50000 mg/kg. The Cl/Na ratio molar ratio remained constant among treatments, being around 1.4.

	Cl (mg/kg)	Na (mg/kg)	NaCl (mg/kg)	Cl/Na (molar ratio)	Bioaccumulation factor (BF)	
					Cl	Na
Common oats						
Control (Promix)	20800	5300	26100	-	-	-
-PGPR	44400	18300	62700	1.6	24	7
UW3+4	57000	26100	83100	1.4	30	10
AMF	35400	17700	53100	1.4	21	7
UW3+4+AMF	38900	17400	56300	1.3	19	7
CMH2	38400	17700	56100	1.4	20	7
CMH3	36300	16500	52800	1.4	19	6
Ranger barley						
Control (Promix)	21100	3200	24300	-	-	-
-PGPR	33900	15900	49800	1.4	18	6
UW3+4	41100	21800	62900	1.2	22	8
AMF	29300	14700	44000	1.1	15	6
UW3+4+AMF	29200	17900	47100	1.3	16	7
CMH2	33700	15800	49500	1.4	18	6
CMH3	43200	18400	61600	1.5	23	7

Analyzed by ALS Environmental Inc (Waterloo, ON)

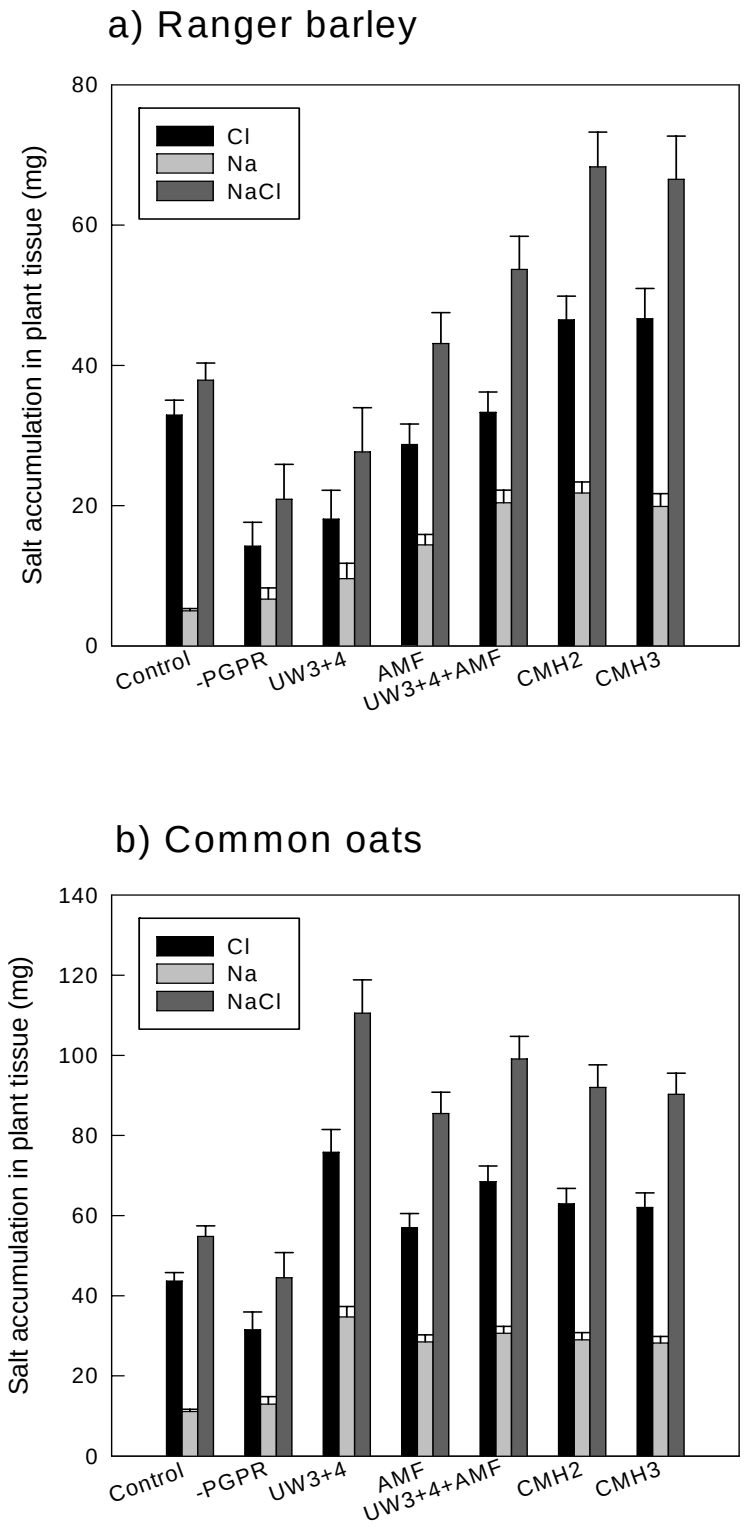


Figure 3.20. The amount of salt accumulation (mg/pot) in plant tissue of a) Ranger barley and b) common oats in Experiment #4. Data were derived from Figure 3.15 and Table 3.9. The results are expressed as means± 1SE of four replicates. The highest amount of salt accumulated in the shoot biomass was 110 mg/pot for oats treated with UW3+4.

3.7.5. Greenhouse experiment #5: PGPR effects on local varieties

The purpose of this experiment was to examine growth promotion effects of PGPR on local plant species or varieties. For this experiment, CMM soil (EC_e = 49.5 dS/m, SAR = 10) was mixed with Tri-Mix™ (Kengrove Inc., ON) to reach salinity at EC_e = 13 dS/m, in which soil, Alfalfa, red spring wheat, Vivar barley and Orchardgrass were tested for the plant growth promotion effect of PGPR treatments UW3+4, CMH2 and CMH3. The growth period of this experiment was 45 days.

Four local species (varieties) were tested for the effect of PGPR on their growth in saline soil. Orchardgrass is a cool season perennial, moderately salt tolerant grass. Red spring wheat is a salt tolerant warm season crop. Vivar barley is a cool season crop that has strong straw that can tolerate intensive management. It has a very high forage yield potential, but does not perform well under drought conditions. (Manitoba Forage Council Inc., 2006). Alfalfa, a N-fixing plant, can increase the fertility of soil.

These plants were tested separately but they can be planted together as a mix that contains salt tolerant crops, a cool season perennial grass and a nitrogen-fixing plant. Crops generally produce more biomass, while grasses have longer growth period. Once the grass establishes and overwinters in the first year, no seeding is required in the next year because new tillers (daughter plants) will start developing from stolons and rhizomes. In addition, grasses usually have extensive and dense root system that can improve soil properties such as draining and aggregation.

PGPR treatment promoted the growth of these local species. CMH3 promoted shoot growth of all four plants. CMH2 and UW3+4 also increased shoot biomass of all species (varieties) except wheat (Figure 3.21). For root biomass, UW3+4 showed growth promotion effect on orchardgrass and alfalfa. The root growth of alfalfa was also enhanced by CMH3 (Figure 3.22). Among four plants, Vivar barley produced the highest shoot biomass when treated with CMH2. Pictures of plant growth at the end of the growth period are shown in Figure 3.22.

A noteworthy result is the effect of PGPR on alfalfa nodulation. The number of nodules of alfalfa treated with UW3+4, CMH2 and CMH3 were significantly higher than that of the untreated (-PGPR). The promotion of nodulation by PGPR was also reported elsewhere. For example, the ACC deaminase-producing *Sinorhizobium meliloti* showed 35 to 40% greater efficiency in nodulating alfalfa, likely by lowering ethylene production in the host plants (Ma *et al.*, 2004). Similarly, *Rhizobium leguminosarum* bv. viciae 128C53K enhanced the nodulation of pea, and the minus ACC deaminse mutants showed lower nodulation efficiency (Ma et al., 2003a). Belimov et al. (2001) suggested that ACC deaminase-producing PGPR are present in various soils and have potential as a bacterial inoculum for improving nodulation and plant growth, particularly under unfavorable environmental conditions. Arshad et al. (2007) also reviewed how inoculation with ACC deaminase-producing bacteria may promote phytoremediation for metals and organic contaminants.

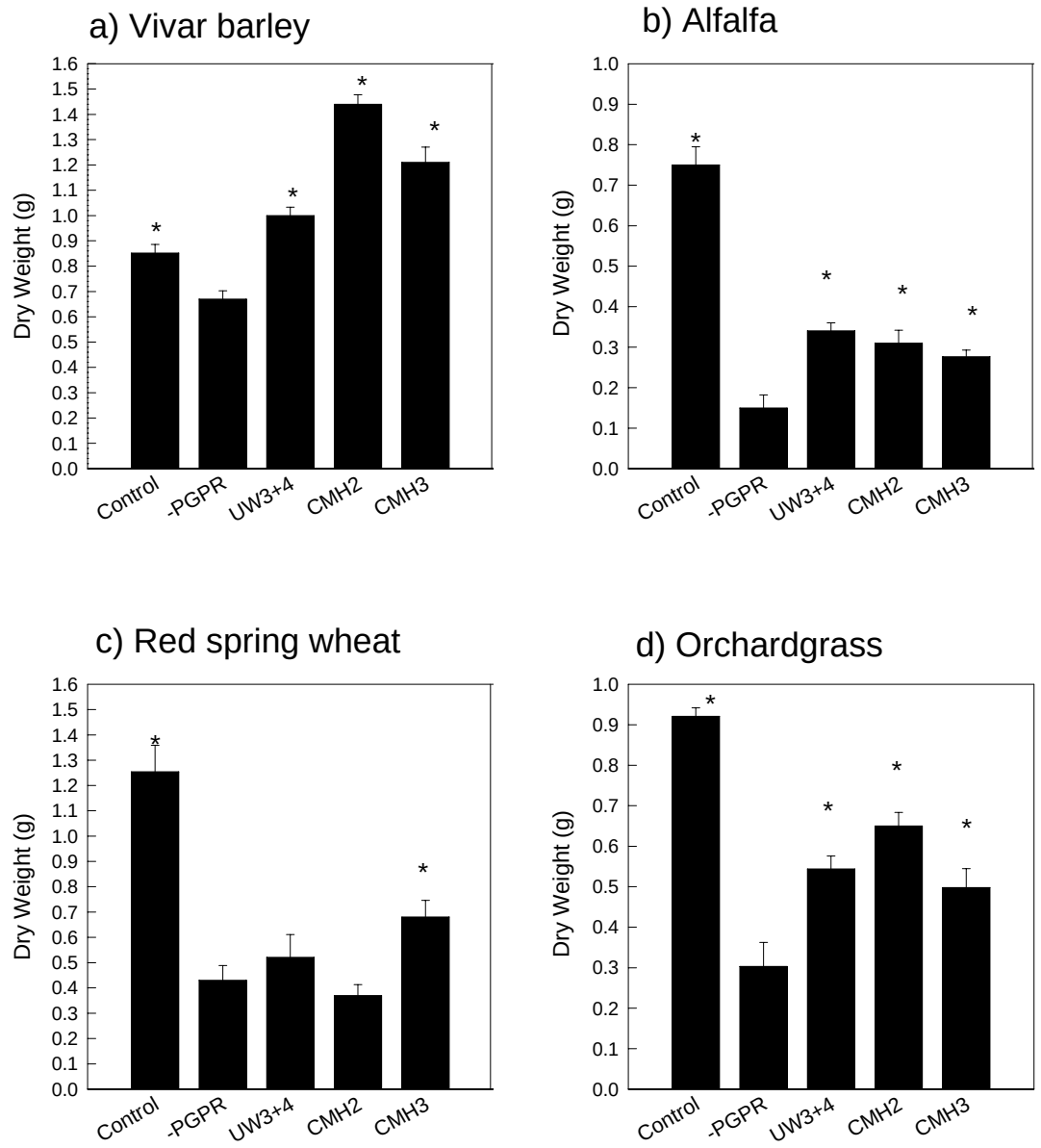


Figure 3.21. Shoot dry weight of a) Vivar barley, b) alfalfa, c) Red spring wheat and d) Orchardgrass in 30 days. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR). CMH3 promoted shoot growth of all four plants. Vivar barley produced the highest shoot biomass when treated with CMH2.

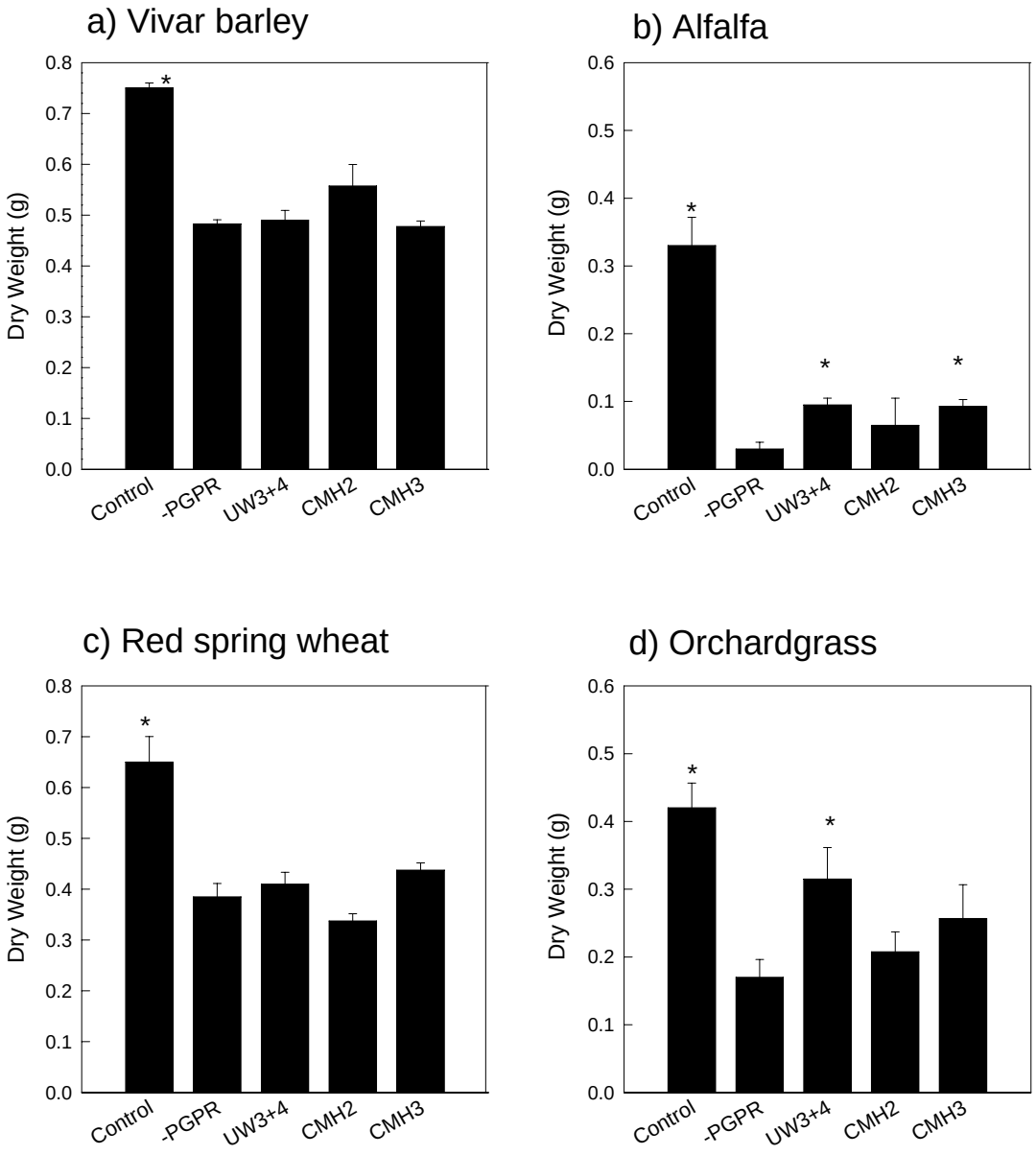


Figure 3.22. Root dry weight of a) Vivar barley, b) alfalfa, c) red spring wheat and d) Orchardgrass in 30 days. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR). UW3+4 showed growth promotion effect on orchardgrass and alfalfa. The root growth of alfalfa was also enhanced by CMH3.

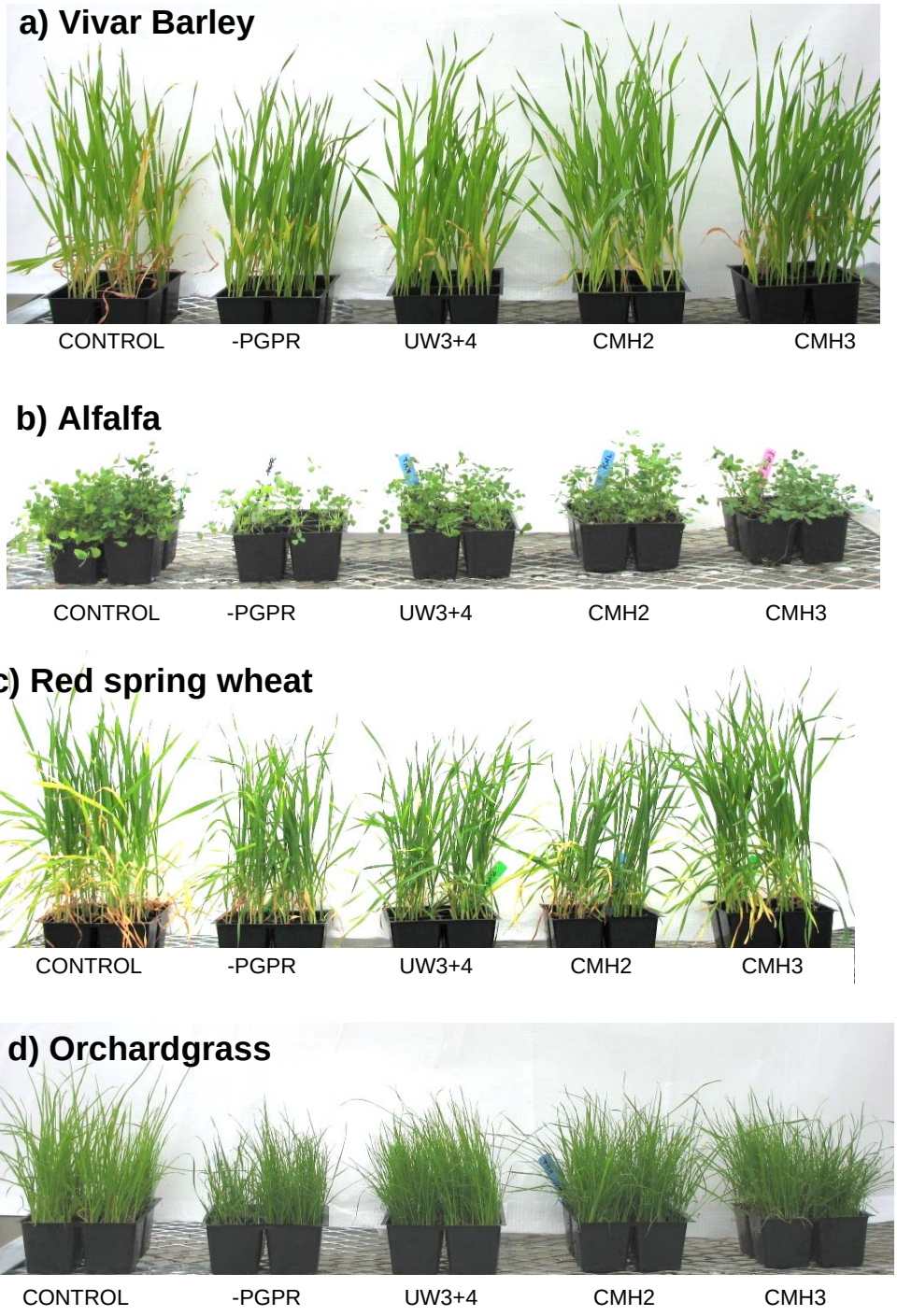


Figure 3.23. Pictures of a) Vivar barley, b) alfalfa, c) Red spring wheat and d) Orchardgrass in 30 days.

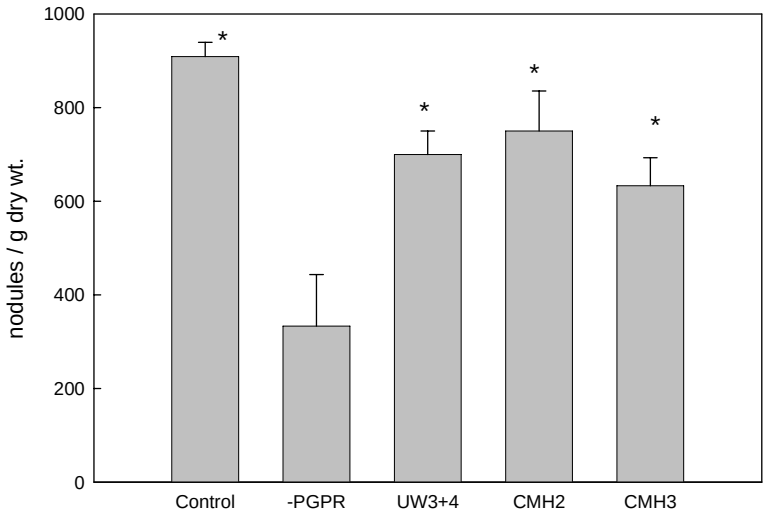


Figure 3.24. Nodulation of alfalfa roots treated with PGPR. The results are expressed as means±1 SE of four replicates. Data were analyzed by one-way analysis of variance (ANOVA) and the Dunnet test (* $P < 0.01$ versus -PGPR). All PGPR tested promoted root nodulation of alfalfa.

3.7.6. Greenhouse experiment #6: PGPR effects of Year-2007 isolates

The purpose of the experiment was to test the newly isolated indigenous PGPR in soils sampled from the field in 2007. The soils used were AL-2007 (EC_e = 14.3 dS/m, SAR = 7) and CMS-2007 (EC_e = 8.6 dS/m, SAR = 10). Plants tested were Baler oats and Ranger barley. The PGPR tested were UW3+4, CMH3, ALR1, ALR2, ALR7, CMR6, CMR7 and CMR8. Plants were grown in two-inch square pots with controlled watering (no excess salt leaching) for 30 days.

Year-2007 isolates were tested for potential field trials in year 2008. ALR1, ALR2, ALR7, CMR6, CMR7 and CMR8 were isolated from soil of the AL and CMS sites after the sites were amended with compost in May 2007. The PGPR effect varied with soils and plant species. In general, the most effective PGPR were UW3+4, CMH3, ALR1 and CMR6 for AL-2007 soil, and UW3+4, CMH3, and ALR2 for CMS-2007 soil. UW3+4 and CMH3 were consistently effective in promoting plant growth regardless of soils and plant species. The growth promotion effect by UW3+4 and CMH3 were again observed in this experiment. The magnitude of promotion, being 50% - 100%, was slightly lower or similar to that in Experiment #4 (CML soil, EC_e = 9.4 dS/m). The results also showed that not all ACC deaminase-producing rhizobacteria promoted plant growth. For instance, plants treated with CMR7 and CMR8 had less biomass than the untreated plants grown in CMS-2007 soil (Figure 3.25, B).

Based on the biomass results, CMR6, ALR1 and ALR2 were new isolates recommended for further testing as candidates for the year 2008 field trial. Additionally, it would be worthwhile to search for PGPR that are less susceptible to environmental changes.

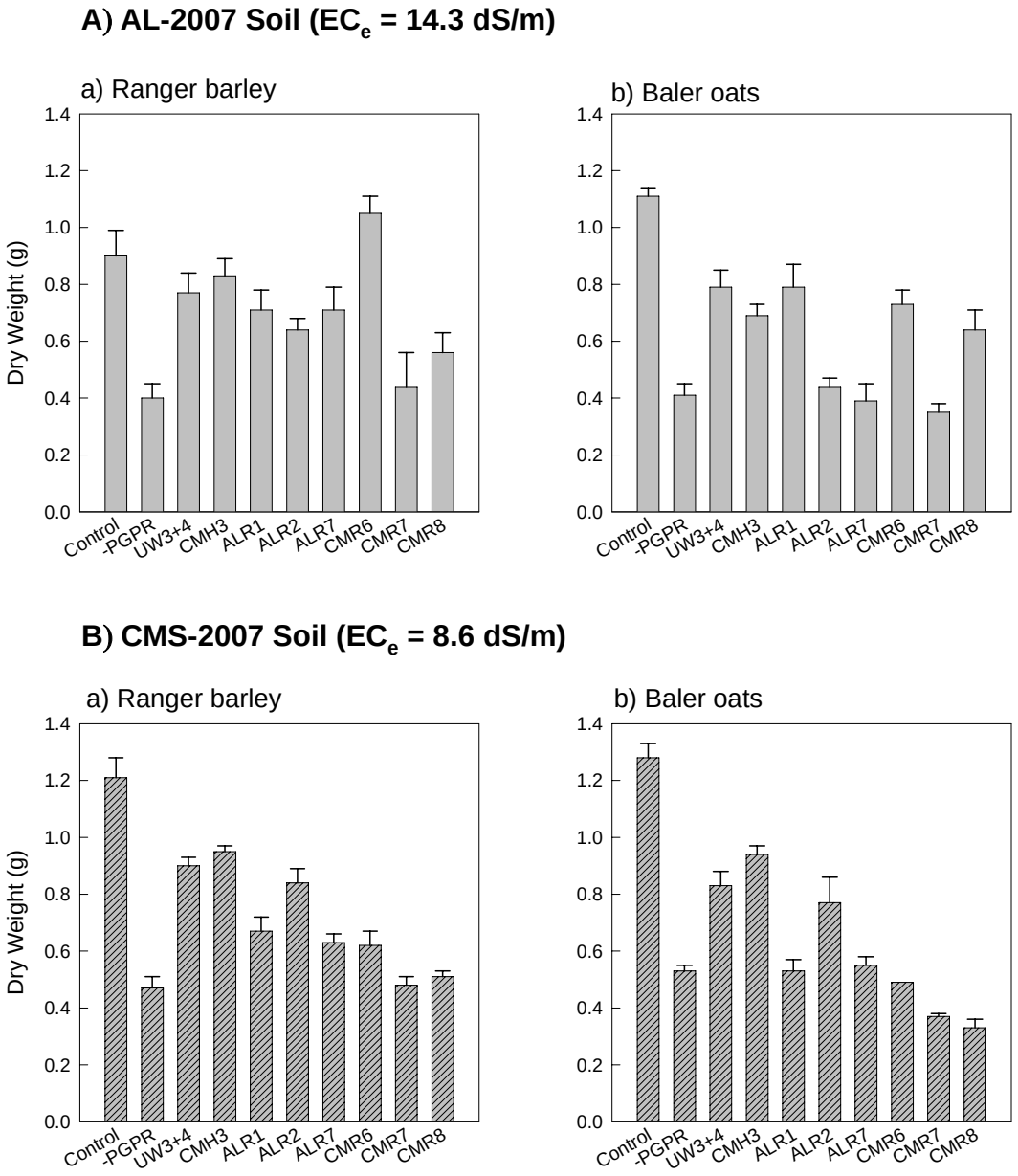


Figure 3.25. Shoot dry weight of 30-day old Ranger barley and Baler oats treated with various indigenous PGPR in AL-2007 (EC_e = 14.3 dS/m) and CMS-2007 (EC_e = 8.6 dS/m) soils. In AL-2007 soil, the most effective PGPR was CMR6 for barley, while CMH3 and UW3+4 were the most effective in promoting shoot growth of both barley and oats in the CMS-2007 soil. The results are expressed as means±1 SE of four replicates.

3.8. Identifications of PGPR isolates

In order to be able to test the Year-2006 indigenous PGPR CMH2 and CMH3 in the year 2007 field trials, the identification of these two indigenous ACC deaminase-producing isolates was necessary. Species of the isolated indigenous PGPR were identified primarily by the genetic (16s rRNA gene) method and complemented by the carbon metabolism method (BioLog MicroPlate™).

3.8.1. 16s rRNA gene sequencing

Based on the 16s rRNA gene sequences (Figure 3.26 and Figure 3.27), CMH2 was identified as *Acinetobacter* sp. (100% identity) and CMH3 as *Pseudomonas* sp. (100% identity). The BioLog MicroPlate™ assay was then conducted to further resolve the strains among the candidate species (> 97%).

3.8.2. The BioLog MicroPlate™ assay

The metabolism pattern of wells was analysed by the Biolog MicroLog3 (release 4.0). CMH2 was identified with 100% probability as the Gram-negative oxidase-negative *Acinetobacter haemolyticus*/genospecies 4. CMH3 was identified as Gram-negative oxidase-positive *Pseudomonas corrugata* with 100% probability. The identification generated by BioLog MicroPlate™ matched with the result of sequencing.

P. corrugata was reported as a strong antagonist of certain phytopathogenic bacteria and a broad spectrum of phytopathogenic fungi (Chun, 2000; Pandey and Palni, 1998) although it also caused pith necrosis disease of tomatoes and a few cultivars of pepper. Chun (2000) used *P. corrugata* as a bio-control agent and increased potatoes yield by 17%. It is categorized to be in the biosafety level 1 group (http://www.atcc.org/). The biosafety 1 organisms are the well-

characterized agents not known to consistently cause disease in healthy adult humans and of minimal potential hazard to laboratory personnel and the environment.

A. haemolyticus was occasionally found in clinical samples (Pantophlet *et al.*, 1999) though *Acinetobacter* species are generally considered nonpathogenic to healthy individuals. Therefore, based on the precautionary principle, CMH2 would not be applied in the field trial due to its potential hazard to the environment (biosafety level 2) despite the high ACC deaminase activity.

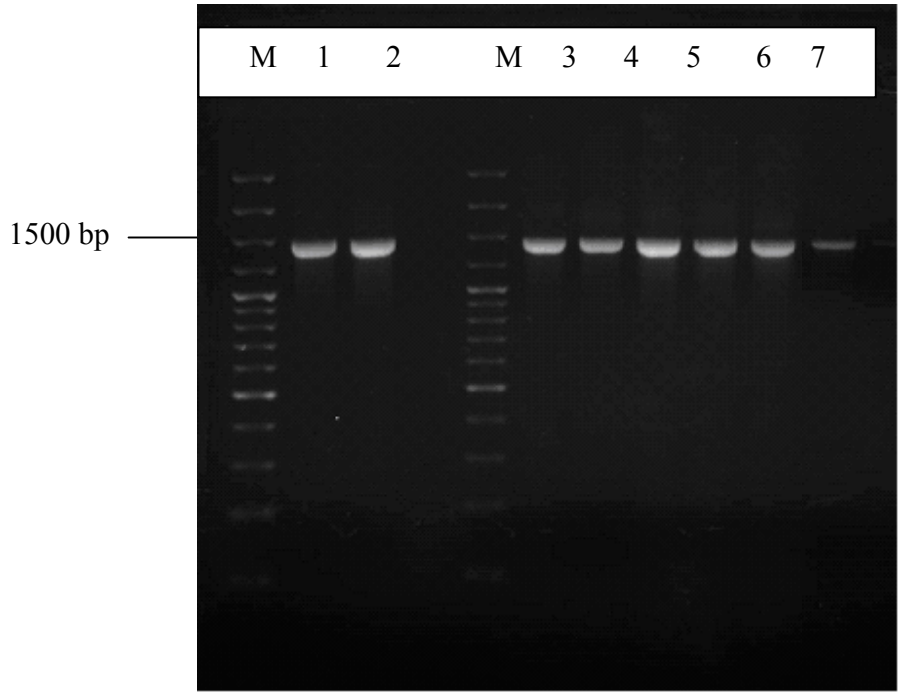


Figure 3.26. Bands of 16s rRNA gene of isolates. M: marker (GeneRuler™ 100bp Plus, Fermentas). Lane 1 to 8 are CMH2, CMH3, CMR6, CMR7, CMR8, ALR1, ALR2 and ALR7. The bands were then cut out and the DNA was extracted for sequencing.

a) CMH2 (1343 bp)

TTCCGAAGGGATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGNATCACTTGTGACCTTGCGCTAATAGATGAGCCTA
AGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCG
CGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTCTGGTTAATACCCAGAGATAGTGGACGT
TACTCGCAGAATAAGCACCGGCTAACTCTGTKCCAGCRGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGC
GTAAAGCGTGCGTAGGTGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTA
GAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCC
ATCTGGCCTAATACTGACACTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGA
TGTCTACTAGCCGTTGGGGTCTTTGAGACTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGA
CTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTG
GGCCTTGACATAGTAGAAACTTTCCAGAGATGGATTGGTGCCTTCGGGAATCTACATACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGAWKTTGGGTTAAGTCCCGCMACGAGCGCAACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAG
GATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTA
CAATGGTCGGTACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAAC
TCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGGGAGTTTGTTGCACCAGAAGTAGCTAGCCTAACTGCAAAGAGGGCGGTTACCACGGTGTGGCCGATGACTGGG
TGAA

b) CMH3 (1245 bp)

CTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACA
CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAA
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TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTG
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CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGT
CGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
CCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATCATGACTGGGG

Figure 3.27. The 16s rRNA gene sequences of a) CMH2 (*Acinetobacter haemolyticus*) and b) CMH3 (*Pseudomonas corrugata*).

3.9. Field tests on salt contaminated sites

The description of the three research sites were in Section 2.1. Soil properties of CMS, CMN and AL sites are listed in Table 3.2. Based on the results of greenhouse tests, UW3+4 and CMH3 were selected for their more reproducible growth promotion effect throughout the greenhouse experiments. Plants, including Ranger barley, Baler oats, Inferno tall fescue and Topgun ryegrass, were treated with PGPR approximately two weeks prior to planting and shipped to the research sites.

3.9.1. Soil salinity

The soil salinity (EC_e) in dS/m of each subsection was shown in Figure 3.28, Figure 3.31 and Figure 3.33. The CMS and AL site were highly saline, with an average EC_e of 18 and 23 dS/m, respectively. In contrast, the EC_e values of the CMN site were low, mostly under 10 dS/m, with some areas where EC_e rose above 20 dS/m. It should be noted here that soil flooding and cracking occurred on sites of high salinity (Figure 3.30), especially CMS, and likely inhibited seed germination. On the CMS site (SAR = 10), areas with severe cracking had less or none vegetation coverage (Figure 3.29 and Figure 3.30). Soil with higher salinity and SAR (saline-sodic soil) tend to have flooding problems due to the dispersion of clay particles into pore space that is otherwise available for drainage. As soil dries, soil crusting or cracking might occur (Richards, 1954).

3.9.2. Plant growth and the PGPR effect

The plants and PGPR combination for each site were listed in Table 3.10. The percent vegetation coverage was determined by visual estimation of the percentage of the subsection covered with plant establishment. Areas covered with weeds were not included. In order to

distinguish the PGPR effect, plant growth on areas of similar plant coverage (> 80%) was compared.

In general, the plant coverage increased with the decrease in soil salinity. The sites with high salinity (CMS and AL) had uneven and low vegetation cover, while CMN had almost 100% coverage throughout the entire site. The percent vegetation coverage of CMS and AL site after a two-month long growing period was shown in Figure 3.29 and Figure 3.34. According to Figure 3.12 (Greenhouse Experiment #3), 80% germination rate of oats was expected at areas with salinity less than 25 dS/m, but the vegetation coverage and plant growth was poor on CMS and AL site (Figure 3.35, a and b), likely due to flooding and soil cracking. For instance, areas with severe cracking of the CMS site generally had poor or no plant coverage (Figure 3.29). In addition to germination, soil salinity also affected the following plant growth. In general, the crop grew taller (30 - 40 cm) and went fully mature on the CMN site, while the plants were shorter (< 20 cm) and withered before becoming mature on the CMS and AL site.

When fescue and oats were planted as a mix on sites where the plant vegetation was patchy, tall fescue generally covered the same or relatively more areas than did oats (Figure 3.29 and Figure 3.34). At areas where both the crop and grass grew, the crop overshadowed the grass at first but the grass was able to continue growing after the crop withered in five months (Yu, 2007). Because both ryegrass and tall fescues are cool-season perennial species, planting the mixture of a crop and a grass resulted in prolonged plant growth (Figure 3.32, c) and hence more uptake of salt by plants. For example, the co-growth of ryegrass with barley on the CMN site enhanced the amount of salt accumulated in plant biomass by 15% due to the longer growth period of the grass (see the detailed calculation on page 111).

At plots of the CMN site where ryegrass was planted alone, weeds overshadowed the ryegrass at some spots, leading us to conclude that ryegrass alone might not be an optimal candidate for sites of lower salinity (EC_e < 10 dS/m) because the local plants may be more competitive (Figure 3.32, b). Nonetheless, these weeds could be good candidates for salt remediation if they accumulate high levels of salt and generate sufficient biomass.

After a five-month growth period, plant shoot biomass on areas (50 cm × 50 cm) (Figure 3.36) with similar vegetation coverage (> 80%) and soil salinity (EC_e) were compared to determine the PGPR effect. The PGPR treated plants were taller and had longer roots than the untreated plants (Yu, 2007) in the field. In fact, the shoot biomass of plants treated with PGPR was 30% - 175% higher than the untreated ones (Table 3.11 and Figure 3.37) across the three sites. For example, UW3+4 treatment increased the shoot biomass of barley on CMN site by 153%, from 300 to 760 g/m^2. On the same site, the shoot biomass of ryegrass was enhanced by UW3+4 by 67%, from 180 to 300 g/m^2. For the CMS site, the indigenous PGPR CMH3 increased the shoot biomass of oats by 28% (Wu, 2007). An increase in shoot dry weight was also observed on the AL site.

3.9.3. NaCl accumulation in plant shoot tissues

The NaCl concentrations of the leaves, straws and spikes varied. As shown in Table 3.12, for the untreated barley most of the Na and Cl were accumulated in leaves and straws, while the Na and Cl concentrations in the spikes were only 15% of the concentrations in the straws or leaves. This result suggests that not only is the total shoot biomass critical to the salt phytoremediation efficiency, but also the parts of plant tissue that contribute to the total mass. Therefore, the

factors including fertilizer, microorganisms, plant species or harvest time should be carefully chosen in order to maximize the salt removal efficiency. In addition to the lower concentrations of NaCl, the grains can contribute to more than 50% of the total dry weight of mature plant samples (Table 3.12), and hence the NaCl concentration analysis can be largely skewed by the various amounts of grains of each sample. Therefore, for the rest of the samples, only the straws and leaves were collected for analysis of NaCl concentrations (Table 3.13).

The higher NaCl concentrations in leaves and straws have been discussed elsewhere. Both Na^+ and Cl^- were taken up by plants predominantly through passive symplastic pathways, driven by gradients and respiration fluxes (Tester and Davenport, 2003; White and Broadley, 2001). Na^+ and Cl^- translocate mainly upwards in xylem and accumulate in shoots or leaves although a small portion of them are in phloem and can travel downward to roots (Smith et al., 1980). As a consequence, leaves or shoots accumulate Na to higher concentrations than roots (Tester and Davenport, 2003). Flora tissues generally had lower Cl^- than other shoot parts. Moreover, tissues that are fed predominantly through the phloem, e.g. fruits and seeds, tend to have the lowest Cl^- .

The Na, Cl, Ca, K and Mg concentrations of shoot tissues of PGPR treated samples were listed in Table 3.13. NaCl concentrations in barley and oats were similar. However, oats had higher concentrations of Na, Cl as well as K and Mg than Inferno tall fescue on both CMS and AL site. The NaCl concentrations in plants ranged from about 30000 to 70000 mg/kg, which was also the range of NaCl concentrations of plants grown in the sampled soil in the greenhouse (Table 3.9 and Table 3.6).

There was no significant difference of NaCl accumulation in shoot biomass among PGPR treatments. In addition, the salt accumulation in shoot tissue (Table 3.13) did not differ much among three sites although the soil salinity levels varied greatly among them. Similar result was also reported by Cheng et al.(2007a), where Na concentrations of canola plants remained to be around 60 mg/g despite the increase in NaCl input into soil.

3.9.4. Estimation of time required for salt remediation of the CMN site

A rough estimation of the time required to remove 50% of the salt from the top 50cm soil of the CMN (about 0.38 hectare in size) site is approximately seven years when barley and ryegrass are planted as a mix and treated with UW3+4. The calculation is as follows. Approximate numbers are used for clear demonstration, and all concentrations used here are on a dry weight basis.

The volume of the top 50 cm of soil of CMN site is 1900 m^3 (3800 $m^2 \times 0.5$ m). The soil was assumed to have a density of 1.5 g/cm^3 and water content of 20%. With these assumptions, the dry weight of this amount of soil is 2.3×10^6 kg (1900 $m^3 \times (1.5 \times 10^3$ $kg/m^3) \times 80\%$). The average salinity of the CMN site is around 7 dS/m (Figure 3.31) and the NaCl concentration of soil with this level of salinity is 3.5 g/kg (Table 3.2). Thus, the total amount of salt of the top 50 cm of soil of the CMN site is 8050 kg (2.3×10^6 kg × 3.5 g/kg).

The amount of forage yield of barley that was treated with PGPR was 1.4 kg dry mass/m^2 (Figure 3.10), equal to 14000 kg/hectare. According to the growth on the site in 2007, it is feasible to mow barley twice per season, so the yield is 28000 kg/hectare per year. Based on Table 3.13, the NaCl concentration of barley plants is about 50 g/kg. Therefore, barley treated

with PGPR can take up 1400 kg NaCl/hectare every season (14000 kg/hectare × 2 mowings × 50 g/kg). Based on the same calculation, barley not treated with PGPR can take up 600 kg NaCl/hectare every season.

The amount of forage yield of ryegrass is estimated as 0.3 kg dry mass/m^2 (Table 3.11), equal to 3000 kg/hectare. Based on the growth in 2007, it is possible to mow the ryegrass twice per season; the first mowing can take place when the barley matures and the grass continues to grow for another two months for the second mowing. Thus, the annual yield is 6000 kg/hectare. Based on Table 3.13, the NaCl concentration of ryegrass is estimated to be 30 g/kg. Therefore, ryegrass treated with PGPR can take up 180 kg NaCl/hectare every season (3000 kg/hectare × 2 mowings × 30 g/kg). Based on the same calculation, ryegrass not treated with PGPR can take up 108 kg NaCl/hectare every season.

To sum up, the time to remove 50% of the salt (4025 kg) of the top 50 cm soil of the CMN site (0.38 hectare) is estimated to be 6.7 years (4025/[(1400+180)*0.38)]) when PGPR is applied. In comparison, it takes 15 years (4025/[(600+108)*0.38)]) to do so if plants are not treated with PGPR.

3.9.5. Conclusions and suggestions for field trials

The PGPR UW3+4 and CMH3 treatment promoted plant growth of both grasses and crops. The salt removal efficiency is the highest when the mix of a crop and a grass was used. The co-growth of ryegrass increased the amount of salt accumulated in plants by 15% due to its longer growth period. Salt removal by phytoextraction was most effective for sites with moderate salinity, such as the CMN site, where plants can produce satisfactory amount of biomass. For

AL and CMS, the NaCl concentrations of shoots were similar to those grown in the CMN site but the biomass was only 10% of the CMN site. Thus, it will take much longer time to lower the salinity to an acceptable level. For AL site, N and P fertilizer is recommended based on the results of Section 3.7.1, where the application of fertilizer enhanced shoot biomass of barley and oats by up to 300% without lowering the NaCl concentrations in plant tissue. For CMS site, the flooding problem should be solved first. Surface or tile drainage is recommended. For CMN site, the growth of barley was satisfactory. However, other cool season grasses that can produce more biomass than ryegrass are recommended. One suggestion is wheatgrass, which has been used extensively in reclamation of moderately-alkaline soils with a pH as high as 10 (Richards, 1954).

Table 3.10. The planting plan of the CMS, CMN and AL site. The plant species, PGPR, and plot numbers for each site are listed. “Mix” is a mixture of the crop and grass at 1:1 ratio (v/v).

Site	PGPR	Plant		
CMS		Baler oats	Inferno tall fescue	Mix (oats + tall fescue)
	-PGPR	1, 12	7, 16	9
	UW3+UW4	2	5, 14	8, 11
	CMH3	4, 13	3, 6, 15	10
CMN		Ranger barley	Topgun ryegrass	Mix (barley + ryegrass)
	-PGPR	2, 8	4, 10	6, 13
	UW3+UW4	1, 9	3, 11	5, 7, 12, 14
AL		Baler oats	Inferno tall fescue	Mix (oats + tall fescue)
	-PGPR	2, 8	4, 10	6, 12
	UW3+UW4	1, 7	3, 9	5, 11

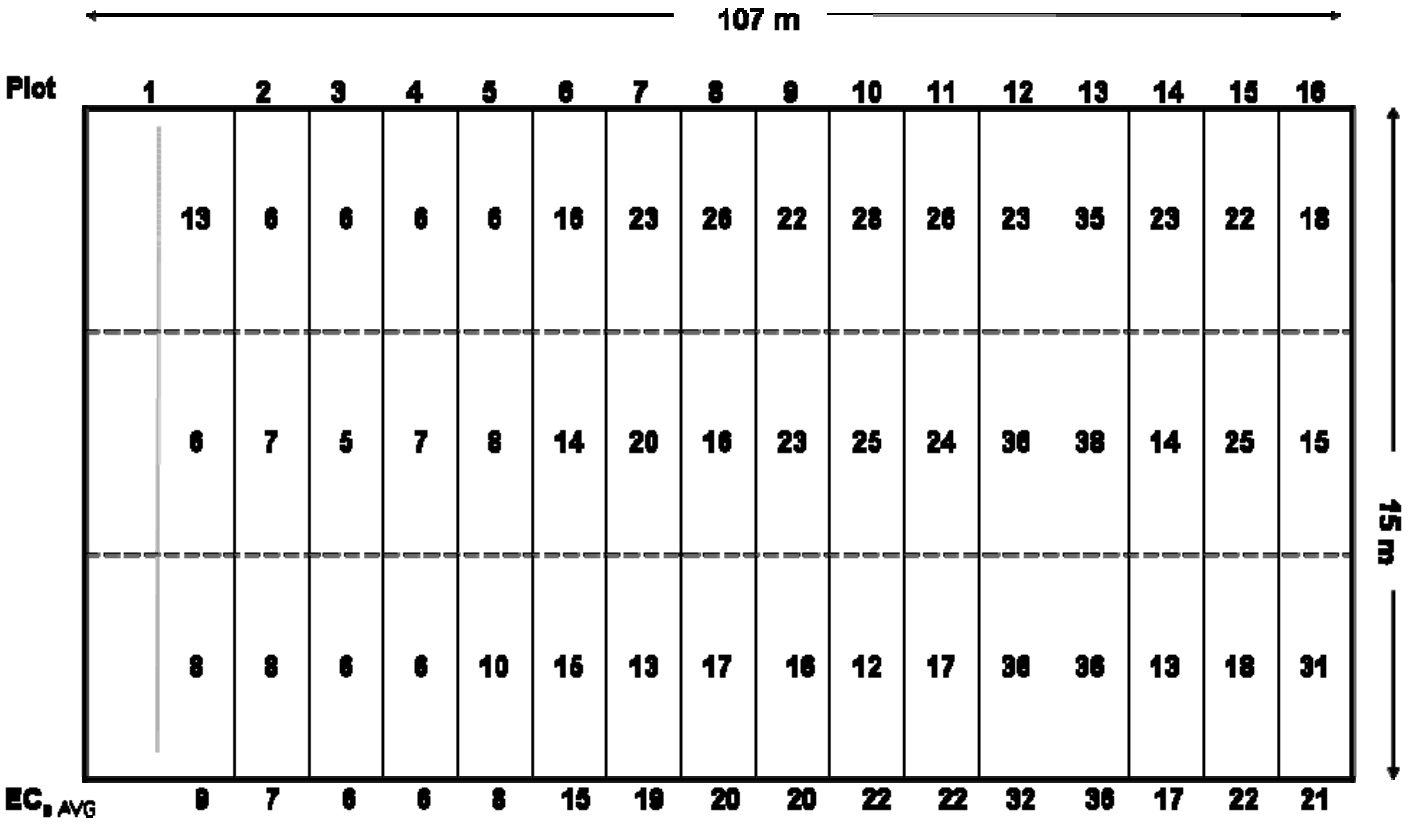

Figure 3.28. The soil salinity (EC_e in dS/m) of the Cannington Manor South (CMS) site. The $EC_{e\ AVG}$ is the average EC_e of each plot. The EC_e values of the site ranged from 5 to 38, with an average of 18 dS/m. The soil of Plot 1-6 was less saline.

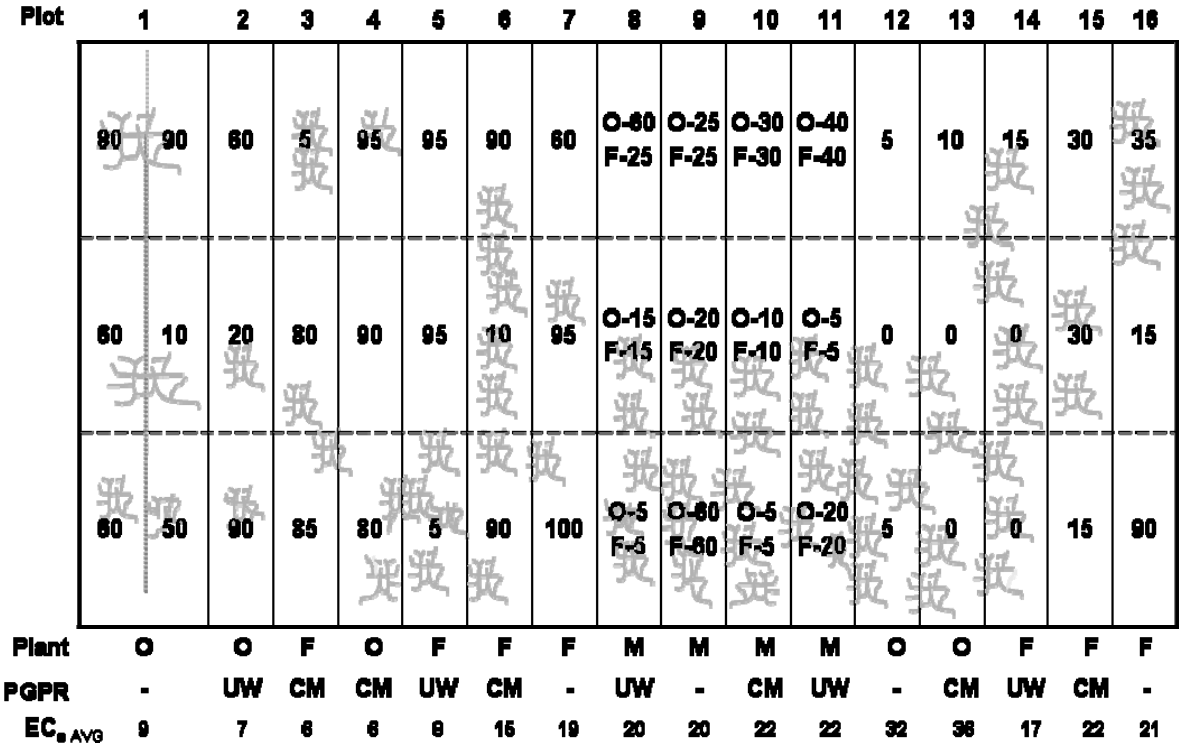

Figure 3.29. The percent (%) coverage by Baler oats and Inferno tall fescue of each subsection of the Cannington Manor South (CMS) site. At pots where oats (O) and fescue (F) were planted as a mix (M), the coverage of both plants was indicated. For example, "F-15" means Inferno tall fescue covered 15% of the subsection. The average EC_e of each plot was listed as $EC_{e\ AVG}$. Soil cracking is indicated as 𝌆. The plant coverage on the areas with severe cracking was generally low. UW: UW3+UW4; CM: CMH3.

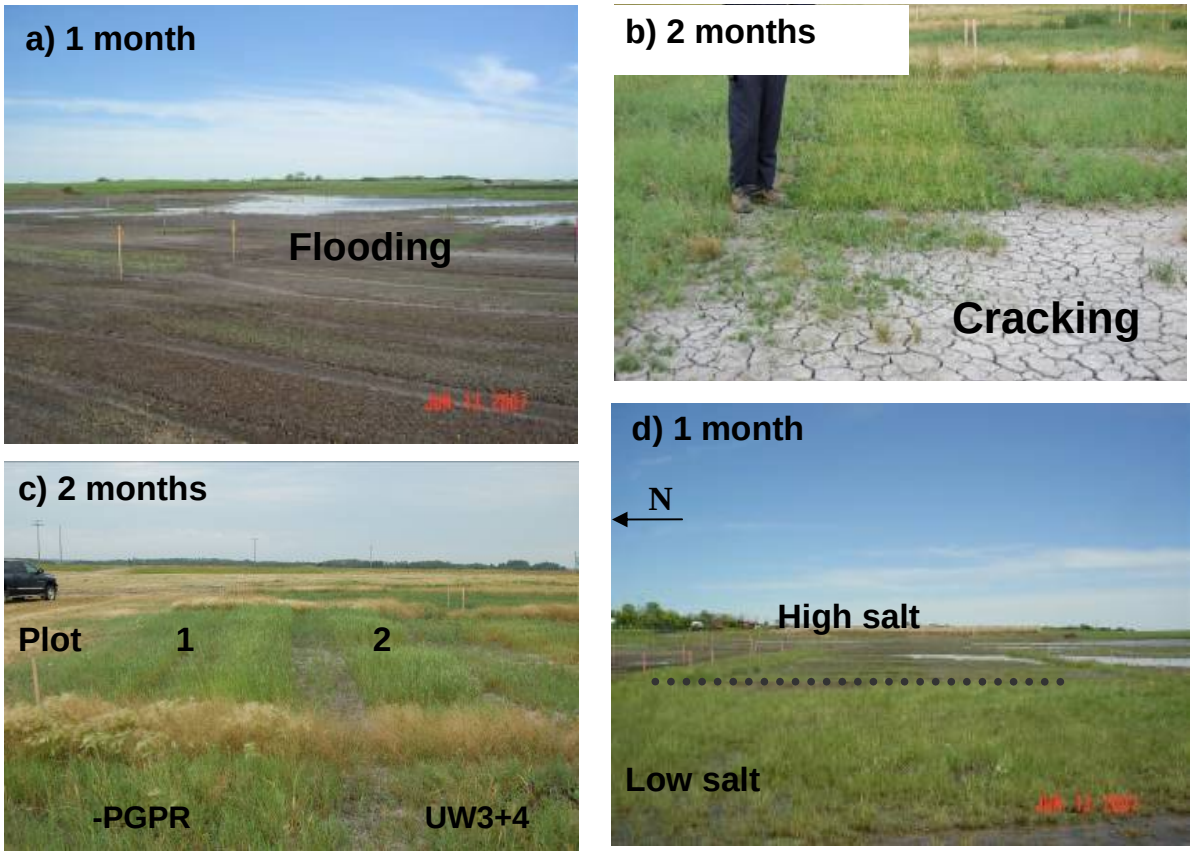

Figure 3.30. Plant growth at the Cannington Manor South (CMS) site. Flooding (a) and soil cracking (b) occurred. The PGPR effect on oats was observed after 2 months (c). The plant growth was poorer (d) in soil with higher salinity (Figure 3.8).

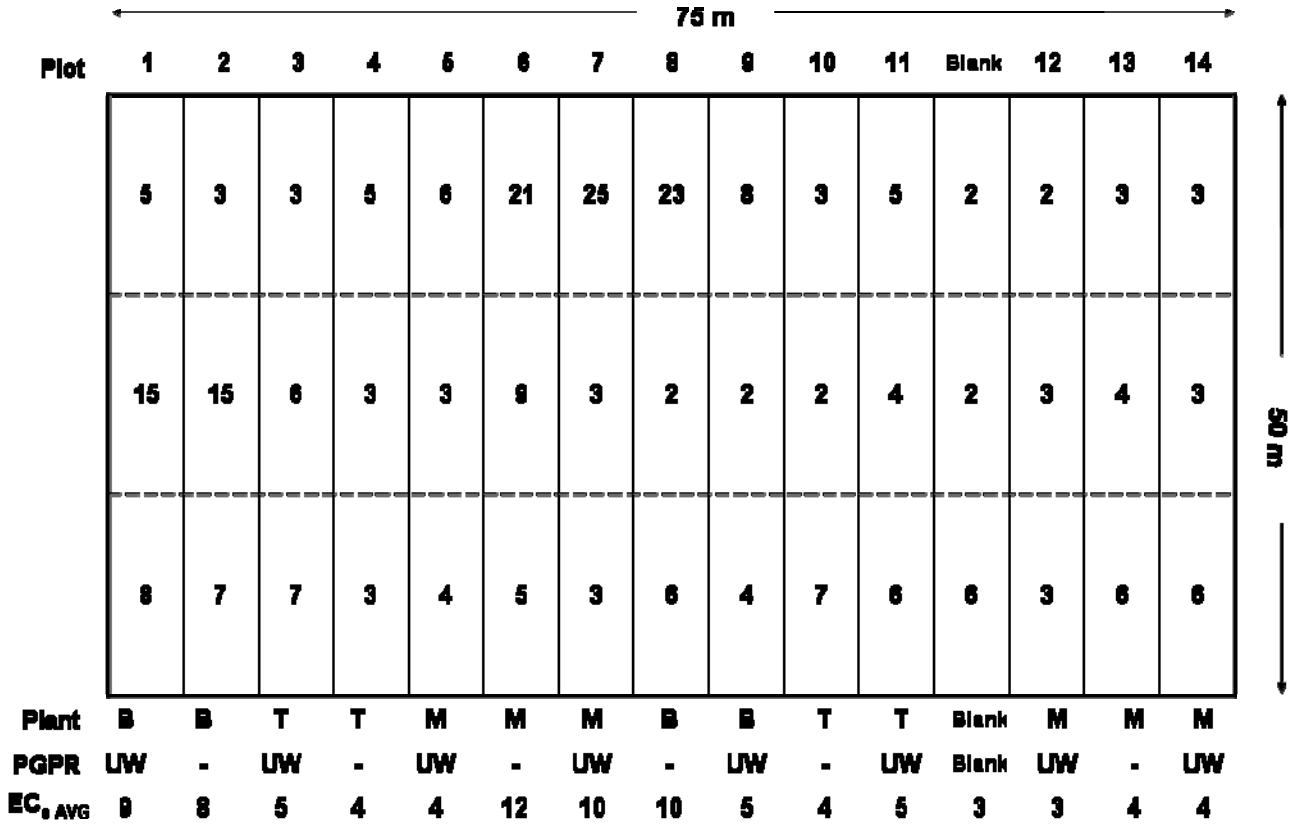

Figure 3.31. The soil salinity (ECe in dS/m) and plant-PGPR combinations of the Cannington Manor North site (CMN). The EC_e values of the CMN site were mostly under 10 dS/m, with a small area of high salinity (> 20 dS/m). B: Ranger barley; T: Topgun ryegrass; M: mix of barley and ryegrass; UW: UW3+UW4.

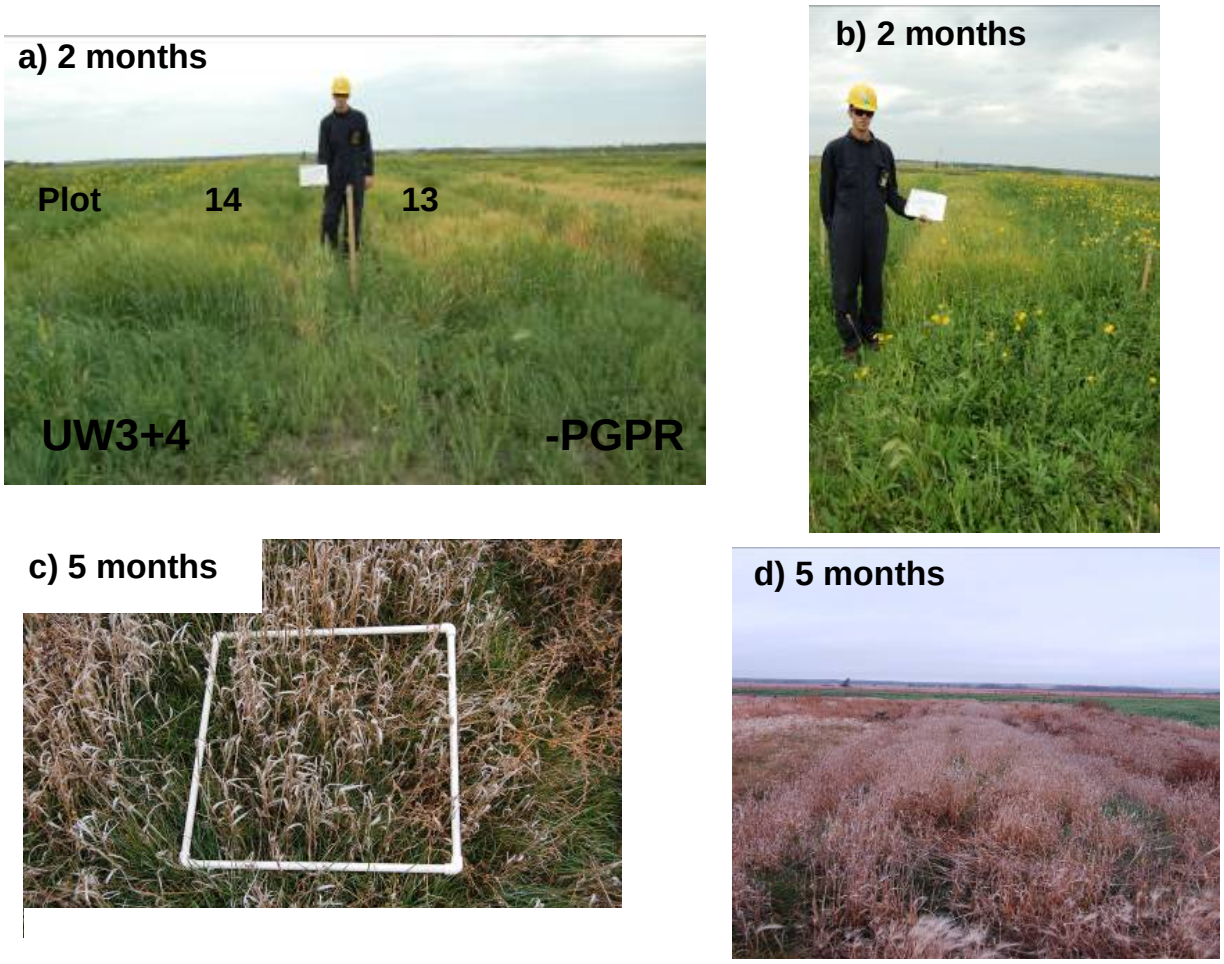

Figure 3.32. Plant growth at the Cannington Manor North (CMN) site. The PGPR effect was observed after 2 months (a). Weeds (with yellow flower) also established in some areas (b). Where ryegrass and barley as a mix, the ryegrass continued growing after the co-planted barley turned fully mature and withered (c). The plant that remained green is ryegrass (d).

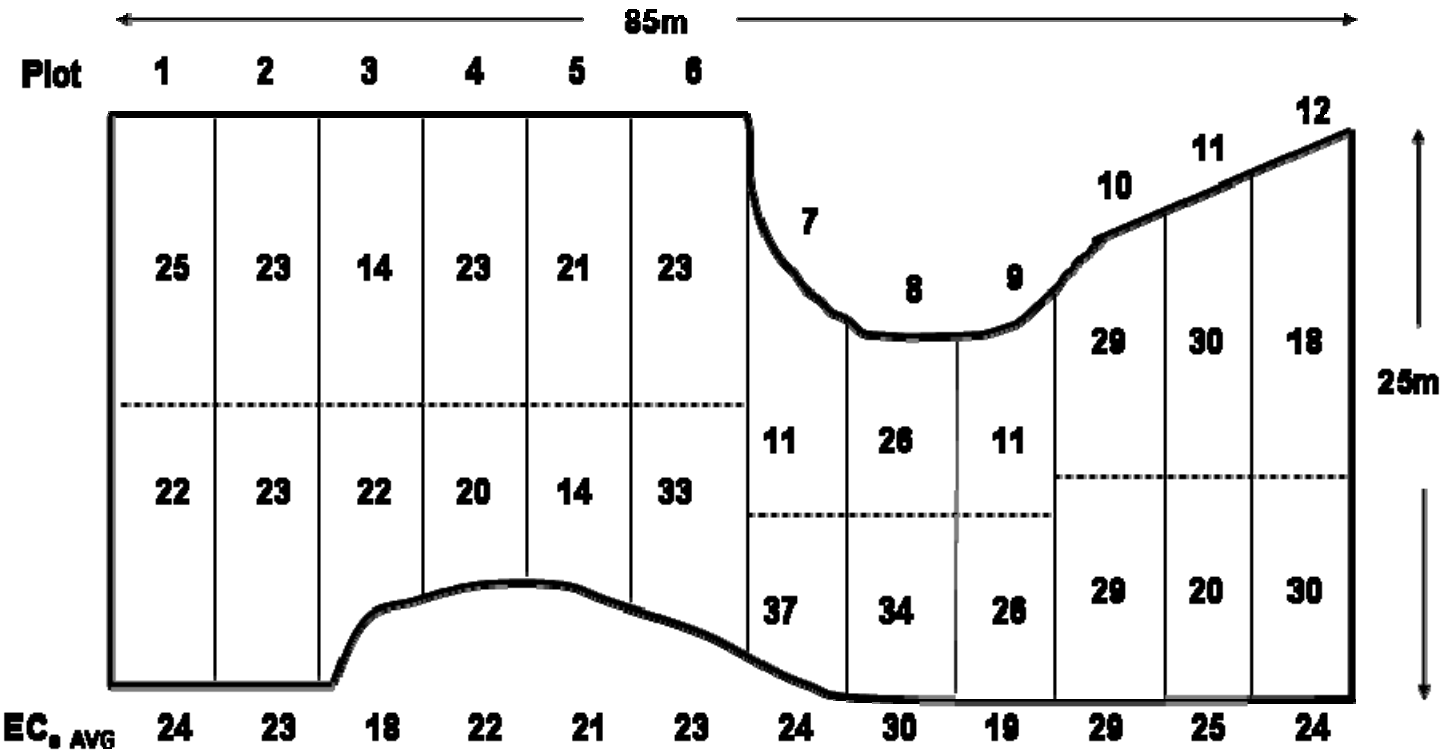

Figure 3.33. The soil salinity (ECe in dS/m) of the Alameda (AL) site. The average EC_e of each plot was listed as $EC_{e\ AVG}$. The EC_e values of the site ranges from 11 to 34, with an average of 23 dS/m.

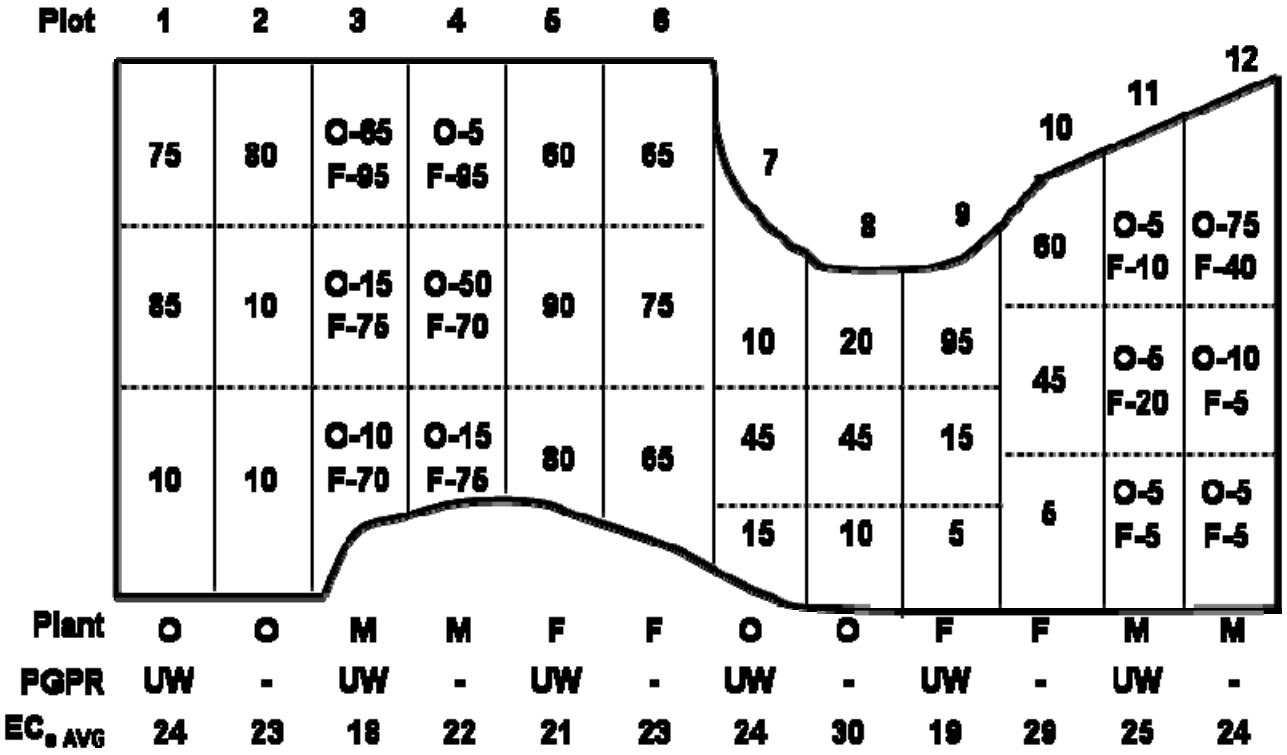

Figure 3.34. The percent (%) coverage by Baler oats and Inferno tall fescue of each subsection of the Alameda (AL) site. At pots where oats (O) and fescue (F) were planted as a mix (M), the coverage of both plants was indicated. For example, "O-5" means oats covered 5% of the entire subsection. The average EC_e of each plot was listed as EC_{eAVG}. UW: UW3+UW4.

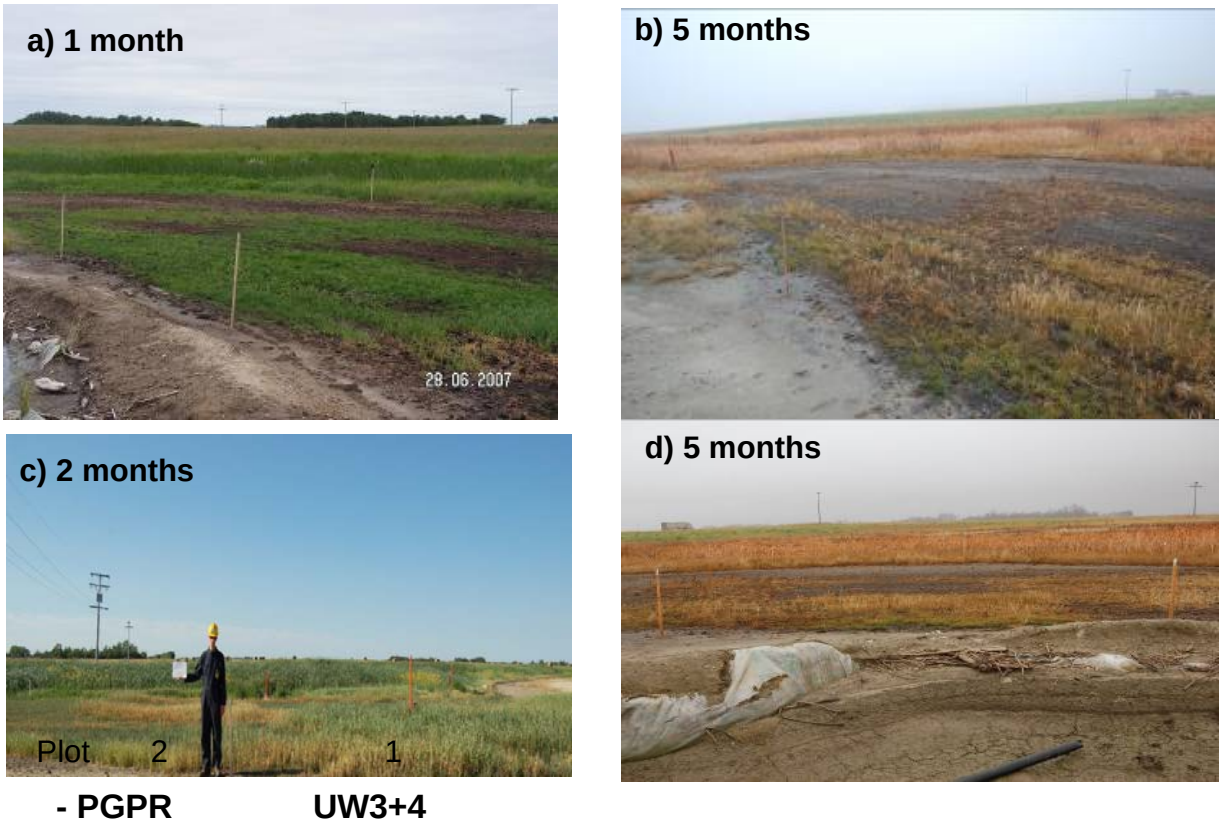

Figure 3.35. Plant growth at the Alameda (AL) site. Plant germination and growth was uneven (a). The plant coverage pattern did not change greatly after 1.5 month. Areas without plant growth remained unvegetated until harvest (b). The PGPR effect was observed after 2 months (c). Plant withered after 5 months (d).

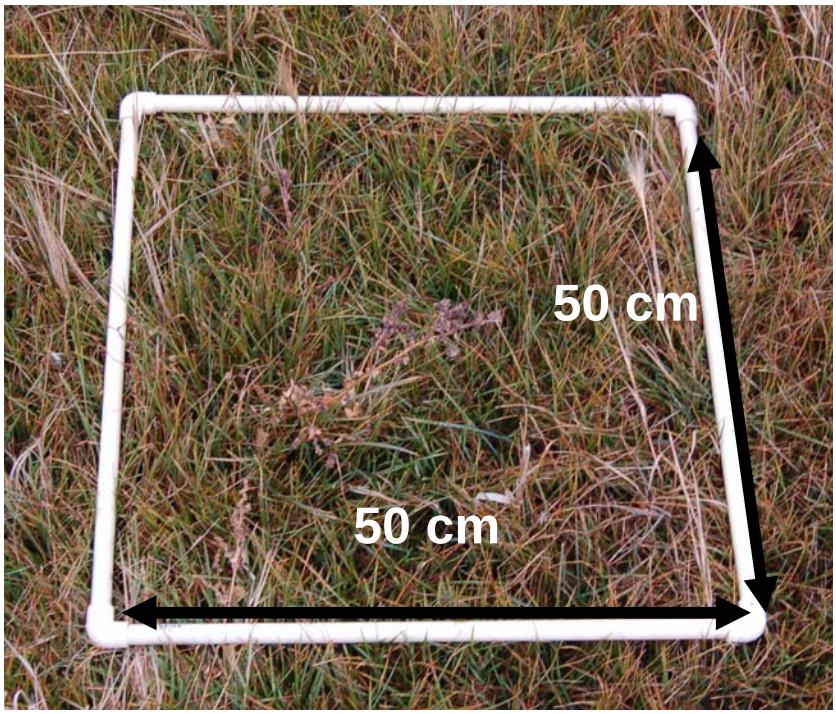

Figure 3.36. The frame used for sampling plant biomass in the field.

Table 3.11. The dry weight of aboveground tissue of 5-month old plants treated with and without PGPR in the field. Dry biomass of plants in an area (50 cm × 50 cm) with similar vegetation coverage (> 80%) and soil salinity was collected. PGPR treatment promoted plant growth by 28% - 175%.

Site	Plant	EC_e (dS/m)	PGPR	Dry weight (g) per m^2	Increase
CMS	Baler oats	6	- PGPR	360	
		6	CMH3	460	28%
CMN	Ranger barley	4	-PGPR	600	
		4	UW3+4	1400	133%
	Topgun ryegrass	3	-PGPR	180	
		4	UW3+4	300	67%
	Mix	3	-PGPR	300	
		4	UW3+4	760	153%
AL	Baler oats	23	-PGPR	160	
		24	UW3+4	440	175%
	Inferno tall fescue	23	-PGPR	80	
		21	UW3+4	120	150%

Mix: mixture of barley and Topgun ryegrass at 1:1 ratio (v:v)

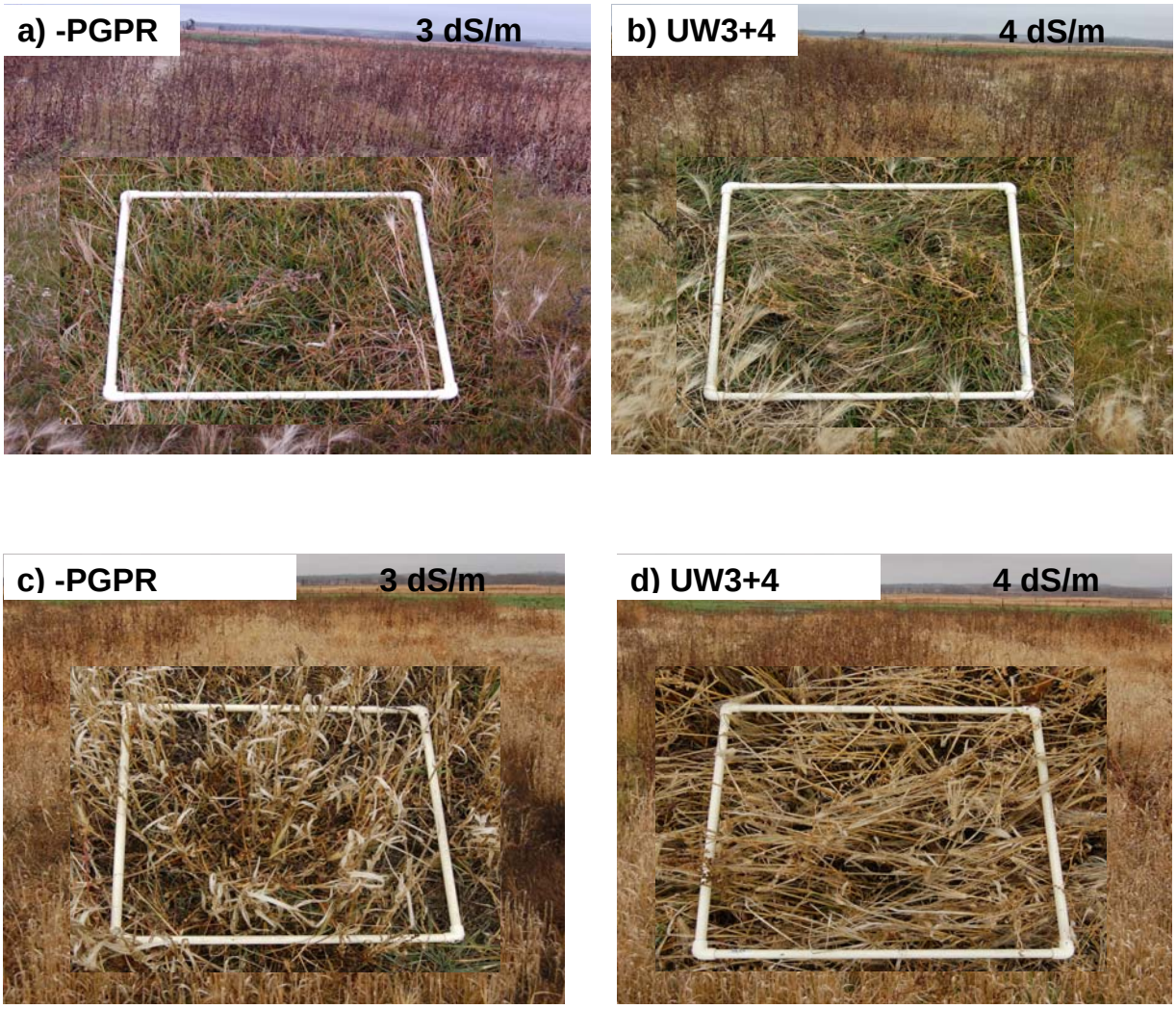

Figure 3.37. Ryegrass (a and b) and barley (c, d and e) sampled for biomass measurement (Table 3.11) at the Cannington Manor North site after 5 months of growth. The growth promotion effect was observed.

Table 3.12. Distribution of Na, Cl, B, Ca, K and Mg of an untreated (-PGPR) barley sample taken from the CMN site. Unite for element concentrations: mg/kg.

	Dry weight (g)	Na	Cl	B	Ca	K	Mg
Leaf	1.5	10300	27000	14	5060	28600	2560
Straw	4.8	21500	39600	2	860	22000	1170
Spike (grain)	8.2	1100	3270	2	680	7630	1530

Table 3.13. Concentrations of Na, Cl, B, Ca, K and Mg in plant tissues sampled from the 2007 field trials. (Unit: mg/kg)

Site	EC_e (dS/m)	Plot	PGPR	Plant	Na	Cl	NaCl	Cl/Na	B	Ca	K	Mg
CMS	19	7	-PGPR	INF	5660	27600	33260	3.2	26	4920	35800	2790
	20	8	UW3+4	INF	5860	32400	38260	3.6	22	6980	36600	3740
	15	6	CMH3	INF	4820	26400	31220	3.6	28	4180	42600	2980
	20	9	-PGPR	OT	13900	43500	57400	2.0	12	6000	25000	4390
	22	11	UW3+4	OT	17300	50000	67300	1.9	11	6820	27500	4980
	22	10	CMH3	OT	13000	35900	48900	1.8	25	7260	23400	4880
		Average					46057					
CMN	9	6-2	-PGPR	BL	18801	36564	55365	1.3	5	1872	23590	1505
	3	7-2	UW3+4	BL	18100	32200	50300	1.2	5	2550	28600	2270
	21	6-1	-PGPR	BL	8530	22600	31130	1.7	8	2760	21900	2450
	25	7-1	UW3+4	BL	17700	55900	73600	2.0	5	3350	39900	3460
		Average					50892					
AL	22	4	-PGPR	INF	4120	31900	36020	5.0	9	2870	51400	2390
	18	3	UW3+4	INF	2430	35400	37830	9.4	8	2900	60600	2570
	22	4	-PGPR	OT	18000	78700	96700	2.8	9	9200	65900	4980
	18	3	UW3+4	OT	11000	50600	61600	3.0	7	2590	64800	1820
		Average					59440					

INF: Inferno tall fescue; OT: Baler oats; BL: Ranger barley
Cl/Na: molar ratio

3.10. Conclusions

Several ACC (1-amicocyclopropane-1-carboxylate) deaminase-producing PGPR and the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* improved plant growth in saline soils. This promotion effect, however, varied with plant species, soil salinity and soil fertility. The indigenous PGPR CMH3 and the combination of UW3 and UW4 (UW3+4) consistently promoted shoot growth of both barley and oats grown in soils by approximately 100%. Oats and barley treated with AMF had higher root length colonized (RLC) and biomass than the non-AMF plants in soils. For grasses, AMF+CMH3 and AMF+CMH2 were the most effective for Topgun ryegrass and Inferno tall fescue, respectively. The concentrations of NaCl in the plants grown in salt-impacted soils ranged from 24300 – 83100 mg/kg. For Year-2007 isolates, CM6, ALR2 and ALR1 were effective in promoting plant growth. For alfalfa, PGPR UW3+4, CMH2 and CMH3 not only enhanced shoot biomass but also increased its root nodulation. Results demonstrated that fertilizer effectively increased biomass, and more importantly the biomass of PGPR treated plants that were supplied with fertilizer was approximately 20% higher than that of plants treated with fertilizer alone. Therefore, research sites were amended with compost before planting of the 2007 field trial. Liquid fertilizer was not used due to its high cost and constraints in the field.

For field trials, germination was poor and uneven on the highly saline sites, CMS and AL (EC_e > 20 dS/m). In contrast, the plant coverage was dense on the CMN site, where salinity was mostly under 10 dS/m. After five months, shoot biomass on a 0.25 m^2 area with similar soil salinity and plant coverage (> 80%) was collected from one treated (+PGPR) and one

untreated (-PGPP) plot to determine the PGPR effect. The results showed that PGPR promoted shoot dry weight by 30% - 175% across the three sites. The NaCl concentrations of barley, oats and tall fescue averaged 53 g/kg, 66 g/kg and 35 g/kg, respectively. The sodium content of barley and oats were similar but significantly higher than that of Inferno tall fescue regardless of soil salinity. The salt removal of the CMN site was the most effective among three sites due to the large amount of shoot biomass produced. The amount of salt accumulated in the shoots on the CMN site was estimated to be 1580 kg per hectare per year when both barley and ryegrass were planted together as a mix and mowed twice. The time required to remove 50% salt in the top 50cm soil of this moderately saline site was therefore estimated to be seven years. In conclusion, PGPR-promoted phytoremediation was proven to be a feasible and effective remediation technique for soils with moderate salinity.

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APPENDIX

1. DF salt minimal medium (based on Dworkin and Foster, 1958)

Trace-1 stock

H_3BO_3	10mg	
$MnSO_4 \cdot H_2O$	11.2mg	
$ZnSO_4 \cdot 7H_2O$	124.6mg	Dissolve one by one slowly in 100mL sterile
$CuSO_4 \cdot 5H_2O$	78.2mg	MiniQ water. The solution can be fridged up to
MoO_3	10mg	7 months.

Trace-Fe stock

$FeSO_4 \cdot 7H_2O$	100mg	Dissolve in 10mL sterile MiniQ.

ACC stock

ACC 0.5M	5.055g (0.05mole)	Dissolve in 100mL, filtered through 0.2µm filter, aliquot 300µl in centrifuge tubes and freeze at -20C.

2. DF minimum salt medium [+$(NH_4)_2SO_4$, +glucose] (per liter)

KH_2PO_4	4.0g	
$Na_2HPO_4 \cdot 7H_2O$	11.3g	
$MgSO_4 \cdot 7H_2O$	0.2g	
Gluconic acid	2.0g	
Citric acid$\cdot 2H_2O$	2.3g	Dissolved in 800mL sterile MiniQ
$(NH_4)_2SO_4$	2.0g	(nitrogen source)
Glucose	2.0g	(carbon source)
Trace-1 stock	0.1mL	add
Trace-Fe stock	0.1mL	add
		Bring the volume to 1 liter
		Autoclave for less than 20min

3. DF minimum salt medium [-$(NH_4)_2SO_4$, +glucose] with ACC (per liter)

KH_2PO_4	4.0g	
$Na_2HPO_4 \cdot 7H_2O$	11.3g	
$MgSO_4 \cdot 7H_2O$	0.2g	
Gluconic acid	2.0g	
Citric acid$\cdot 2H_2O$	2.3g	Dissolved in 800mL sterile MiniQ
Glucose	2.0g	(carbon source)
Trace-1 stock	0.1mL	add

Trace-Fe stock	0.1mL	add
		Bring the volume to 1 liter
		Autoclave for less than 20min,
ACC 0.5M stock	6.0mLl	Thaw, pour in when still cold (nitrogen source)

4. **DF minimum salt medium [-$(NH_4)_2SO_4$, +glucose] with ACC (5 agar plates)**

DF [-$(NH_4)_2SO_4$, +glucose]	100 mL	
Bacto-Agar	1g	add
		Autoclave for 20min, cool to 45℃, pour plates (20mL/each plate)
ACC 0.5M stock	60µl/plate	Thawed, spread on plates

5. Salkowski's reagent (based on Khalid et al., 2004)

Concentrated sulphuric acid	150 mL	
Distilled H_2O	250mL	
0.5M $FeCl_3 \cdot 6H_2O$	7.5mL	(1.35 g/10mL H_2O)

6. 2 mg/mL L-trp

L-trp	0.1g	Dissolve in 50mL warm H_2O
		Filter through 0.2µm membrane.

7. 0.2% 2,4-DNP

Concentrated HCl	17mL	
Distilled H_2O	100mL	
2,4-DNP	0.2g	
		Add HCl into H_2O in a dark brown bottle.
		Dissolve 2,4-DNP and store at 4℃

8. 0.1M Tris-HCl

pH 7.6

Trizma HCl	6.06g
Trizma base	1.39g
	Dissolve in 500mL distilled H_2O

pH 8.0

Trizma HCl	4.44g
Trizma base	2.65g
	Dissolve in 500mL distilled H_2O