Plant-Growth Promoting Rhizobacteria Enhanced Phytoremediation of Saline Soils and Salt Uptake into Plant Biomass

by

Gregory MacNeill

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Author's Declaration

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

Abstract

Soil salinity affects an estimated one billion hectares worldwide. Excess salinity inhibits plant growth, limiting crop production. This is caused by osmotic stress in saline soil, nutrient imbalance and specific ion toxicity. There have been many methods of remediation investigated, including excavation, soil washing and phytoremediation.

Phytoremediation involves the growth of plants on impacted soils to degrade or sequester contaminants. The remediation of salts relies on the uptake of ions into plant biomass where the salt is sequestered and the biomass can then be harvested. This method removes the salt from the site and leaves the top soil in place, which aids in revegetation after site remediation is completed. Plant-growth promoting rhizobacteria (PGPR) improves plant growth by lowering the levels of stress ethylene within the plant, thereby increasing the biomass available to sequester ions.

The objectives of this research were to investigate the efficiency of phytoremediation of salt impacted soils in field remediation sites. Previously isolated strains of PGPR (UW3, *Pseudomonas putida*; UW4, *Pseudomonas putida*; and CMH3, *Pseudomonas corrugata*) were used in field trials involving the planting of oats (*Avena sativa*), annual ryegrass (*Lolium multiflorum*), tall wheatgrass (*Agropyron elongatum*) and tall fescue (*Festuca arundinacea* C.V. Inferno). The salt tolerance of various switchgrass (*Panicum virgatum* L.) cultivars (Cave-In-Rock, Southlow, Forestburg, and common) was compared to tall wheatgrass and Inferno tall fescue to investigate the potential of switchgrass for phytoremediation. Improvement of seed germination under salt stress by H_2O_2 pre-treatment was investigated both as an individual treatment and in combination with CMH3 treatment. The ion uptake into plant biomass was

compared to the change in salinity, to determine how much of the decrease in site salinity is accounted for by uptake of salt by plants.

H_2O_2 pretreatment resulted in a 50% increase in root and shoot emergence of tall wheatgrass under 75 mM NaCl stress compared to control treatments, which matched the germination improvement observed with PGPR treatment. The combination of H_2O_2 and CMH3 showed a similar improvement to root emergence under stress, but had no observable effect on shoot emergence when compared to the no-H_2O_2-no-PGPR control. Switchgrass cultivars showed a lower germination rate than tall wheatgrass at salt levels from 0 mM to 150 mM NaCl. The measured uptake of Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl^- into plant biomass during a phytoremediation field trial was able to account for approximately 70% of the observed change in salinity in 2008. In 2009 the uptake of Na^+ and Cl^- into *Kochia scoparia*, a weed species that invaded the field site after a hard frost, was able to account for 36% of the observed change in salinity.

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1 Introduction

Excess salinity is one of the most widespread types of soil contamination. It is estimated to affect 20% of all arable land, with a total global impact of approximately 1 billion hectares (Ghassemi, Jakeman, & Nix, 1995). Elevated salt levels in soil affect land usability and lowers crop yield, with high levels rendering land unusable (Howes Keiffer & Ungar, 2002; Wong et al., 2005). Human activities such as upstream oil and gas production (eg. brine water spills), salting of road ways in winter, and irrigation of agricultural land can all result in a build up of salt.

Various methods of soil remediation and land reclamation have been used at salt impacted sites. Physical removal and sequestration of contaminated soil, flushing through soil with fresh water, addition of gypsum to alleviate sodium specific stress, dilution of salt concentration by addition of fresh soil or organic material, and phytoremediation have all been examined as potential methods of salt soil cleanup (Howes Keiffer & Ungar, 2002; Qadir & Oster, 2002; Qadir, Steffens, Yan, & Schubert, 2003). Currently, the most common method of salt removal is leeching through the root zone carrying ions deeper into the soil, below the root zone (Qadir et al., 2003). This method does not remove salt contaminants from the soil, but only moves them deeper into soils and nearer to ground water. Phytoremediation has the potential to remove these contaminants quickly and inexpensively.

1.1 Soil salinity and impacts of salt on soil quality

Soil salinity is cause for concern due to its impact on plant growth. It can be expressed as the electrical conductivity (EC) of a soil solution extract, which is a measure of total free ion charge concentration and is reported in units of deciSiemens/meter (dS/m) (Environmental Sciences Division, 2001). One way salinity can be reported as the EC of a solution in a saturated

paste (EC_e), defined as a soil sample with deionised water added just to the saturation point. Formation of a saturated paste involves a subjective determination of the volume of water required to saturate a soil sample. This subjectivity in sample preparation can lead to error prone results when working with large numbers of samples. Thus, instead a set ratio of soil to water is often used, which has been shown to correlate well with the EC_e (conductivity of a saturated paste) (Sonmez, Buyuktas, Okturen, & Citak, 2008). These ratios are reported as $EC_{x:y}$ where x is the mass of soil and y is the volume of water used. An example would be mixing 15 g of soil and 30 mL of H_2O to measure an $EC_{1:2}$. Equation 1 is used to convert between $EC_{x:y}$ and EC_e.

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

K is an empirically determined conversion factor found by comparison of measured EC_e and $EC_{x:y}$ values, averaged over several samples for a given soil type. A less common measurement of total salinity is total dissolved solids (TDS), and reports the amount of dissolved ions in a solution with the units ppm. TDS, which is measured by weighing precipitated minerals of filtered water that has been thoroughly dried from a known volume of sample, takes into account all dissolved material. The heating and drying process can result in loss of solutes by processes such as decomposition of bicarbonate (Walton, 1989). Conversion from TDS to EC is done with Equation 2

$$TDS = k \times EC \qquad \text{Equation 2}$$

The k value for this equation is typically between 0.5 and 0.75, and must be determined based on the ionic content, as ion species of differing charge will affect the ionic strength of a solution, and therefore affect the salinity in a non-linear relationship (Alva, Sumner, & Miller, 1991).

Brine affected soils, which can occur in areas of upstream oil and gas production in Western Canada, typically contain high levels of Na^+ and Cl^-. They also typically have higher than normal levels of Ca^{2+}, Mg^{2+}, K^+, and SO_4^{2-} (Howes Keiffer & Ungar, 2002). The concentrations of these ions are used to classify types of salt impacted soils, and are outlined in Table 1.1. Brine affected soils are usually classified as saline and sodic due to high EC_e and high sodium concentration. Sodium is of particular concern for soil quality due to its impact on packing of charged soil particles. Negatively charged soil particles are typically matched with divalent cations such as calcium and magnesium in soil, which connects adjacent clay particles into larger flocs. These larger flocs cannot pack tightly which allows space for air, water and root penetration in soil. Addition of excess monovalent cations, such as sodium, results in cation exchange between divalent and monovalent cations at negative charges on the soil particles. This exchange disrupts the flocculation of soil, causing the particles in the flocs to disperse, and allowing soil particles to pack more tightly (Bohn, McNeal, & O'Connor, 1985). The risk of damage to soil structure by sodium can be determined by using the Sodium Adsorption Ratio (SAR) shown in Equation 3. This value is a comparison of the concentration of sodium to calcium and magnesium, which typically act as divalent counter ions in soil flocculation (Ghassemi et al., 1995).

$$SAR = \frac{[Na^+]}{\sqrt{\frac{([Ca^{2+}] + [Mg^{2+}])}{2}}} \qquad \text{Equation 3}$$

In addition to impacting the quality of soil structure, addition of salt has toxic effects on plant growth, health and metabolism which will be discussed later in this chapter.

Table 1.1 Types of salt affected soils, grouped by contributing ions (Szabolcs, 1989).

Ions Causing Salinity	Classification of Soil	Main Mode of Impact on Biomass Production	Main Reclamation Method Used
Na^+, Cl^- and SO_4^{2-}	Saline Soil	High osmotic pressure, ion specific toxicity, soil structure impacts	Removal of ions by leaching
Na^+ and alkali hydrolysis (i.e. $NaCO_3$)	Alkali Soil	Alkali pH, soil structure impacts	Chemical amendments to lower pH
Mg^{2+}	Magnesium Soil	High osmotic pressure, ion specific effects	Chemical amendments
Ca^{2+}	Gypsiferous Soil	Acidic pH, ion specific effects	Chemical amendments to increase pH

The EC_e and SAR are used as guidelines to determine soil quality for land use, and are typically considered separately. In Canada, each province sets guidelines for salt levels in soil, although many guidelines are very similar between jurisdictions. Guidelines for Alberta are shown in Table 1.2, and Saskatchewan remediation guidelines are shown in Table 1.3. These regulations show that the general requirement for salinity is below a value of 2 dS/m and the requirement for sodicity as measured by the SAR is below 4 or 5, depending on which province the soil is located in. The effect of salt on sensitive plant species at 2 dS/m is negligible compared to control samples, and soil is considered impacted above 4 dS/m (Howes Keiffer & Ungar, 2002). These are reflected in the guideline values used in both Alberta and Saskatchewan, shown in Table 1.2 and Table 1.3 respectively.

1.2 Impact of soil salinity on plant growth

The main concern regarding saline soil is the impact it has on plant growth. Salinity is one of the most severe environmental stresses on plants (Tester & Davenport, 2003). This can result in lowered crop yield, and eventually result in completely unproductive soil. At this point water and wind degradation of top soil can result in permanent loss of productivity, and eventually lead to desertification of previously productive land (Ghassemi et al., 1995). Plant stress may be a result of changes to osmotic pressure in the rhizosphere, ion specific damage to plants after salt uptake, or inhibition of nutrient uptake (Bhandal & Malin, 1988; Blaha et al., 2000; Tester & Davenport, 2003).

Table 1.2 Soil Quality guidelines for Alberta Unrestricted Land Usage (Environmental Sciences Division, 2001)

Soil Parameters		Category			
		Good	Fair	Poor	Unsuitable
Topsoil (0-60 cm)	EC (dS/m)	<2	2-4	4-8	>8
	SAR	<4	4-8	8-12	>12
Subsoil (60-120 cm)	EC (dS/m)	<3	3-5	5-10	>10
	SAR	<4	4-8	8-12	>12

Table 1.3 Classification of Soils by EC and SAR for Saskatchewan Upstream Petroleum Site Remediation Criteria (Saskatchewan Petroleum Industry/Government Environmental Committee, 2009)

Criteria	Unconditional Use	Moderately Saline	Saline	Highly Saline
EC (dS/m)	<2	3-5	6-8	>8
SAR	<5	6-8	9-12	>12

An increase in salt concentration in the soil immediately surrounding the root, known as the rhizosphere, increases the water potential in roots above the water potential of soil. This change in water potential lowers the movement of water from soil into roots, mimicking drought stress, limiting water and nutrient uptake (Bhandal & Malin, 1988; Blaha et al., 2000). In response to saline conditions, plants will translocate a higher concentration of ions into their shoots and leaves in comparison to their roots. This movement of ions results in uptake of additional ions into the roots due to differences in concentration between the roots and soil, and movement of water into the plant in response to changes in osmotic potential (Tester & Davenport, 2003). The mimicking of drought stress results in plant responses to limit water loss such as closure of stomata. This prevents the loss of water vapour, but also prevents gas exchange between the leaf and the atmosphere. As photosynthesis continues and CO_2 is depleted within the leaf, excess solar energy results in reduction of O_2 and formation of reactive oxygen species, which can cause cell damage (I. Cakmak, 2005).

Once inside the plant, ion specific effects can have negative impacts on plant health. Sodium, occurring in high concentrations in brine contaminated soils, has many modes of action against plants. In addition to changing osmotic potential between the soil and root, sodium has numerous physiological effects related to competition for binding sites with other ions. The ionic radius of Na^+ is similar to the ionic radius of K^+, which allows competition between these two ions for uptake channels into plant roots. This competition results in an overabundance of Na^+ in plant tissues compared to K^+ when Na^+ levels are elevated in soils. This difference in concentration within the plant results in various physiological and biochemical changes within the cell. For instance, under normal conditions, tRNA is coordinated with K^+ during normal protein synthesis. Under conditions of elevated Na^+ relative to K^+, Na^+ can displace K^+ on tRNA

resulting in inhibited protein synthesis (Bhandal & Malin, 1988). This competitive mechanism of damage suggests that instead of requiring low Na^+ in soils for plant growth, a high K^+:Na^+ ratio is needed; meaning addition of potassium to sodic soils may promote plant growth (I. Cakmak, 2005; Cuin, Miller, Laurie, & Leigh, 2003). The same competitive uptake mechanism can result in a decrease in Ca^{2+} and Mg^{2+} uptake due to high soil Na^+. Displacement of calcium ions, normally found bound to membrane to maintain stability, can affect membrane permiablity, and can have an impact on secondary messaging within plant cells (Li, Shi, Fukuda, & Yang, 2010). Lowered calcium concentration within the plant has also been linked to impaired gas exchange rates, affecting photosynthetic capability (Tzortzakis, 2010). A deficiency in Mg^{2+} is known to inhibit chlorophyll synthesis and function, further lowering photosynthetic rates in plants (Li et al., 2010).

1.3 Mechanisms of salt tolerance in plants

Plants can be divided into two groups based on their ability to cope with salt stress. Halophytes are well adapted to growth when exposed to salt water, while glycophytes are less salt tolerant (Howes Keiffer & Ungar, 2002; Kachout et al., 2009; R. Munns, 2002). Halophytes are capable of unimpeded growth at salinities that would completely inhibit growth in glycophytes. The difference between these groups is not in the stability of their enzymes. Enzymes and physiological processes in halophytes are inhibited by the same concentration of salt as enzymes from salt sensitive glycophytes (R. Munns, 2002). The tolerance mechanisms of halophytes can be grouped into avoidance, and acclimation or adaptation as species evolve to survive in different climates.

Avoidance mechanisms involve growing only in favourable conditions. Seeds will not germinate in environments that are too saline for plant growth, and plants will limit the extent of

their root growth through the soil if saline soils are encountered (Breckle, 1990). Acclimation mechanisms are more varied, more complex, and allow for plant growth at higher salt concentrations instead of finding areas of lower contamination. These mechanisms involve limiting salt access to the plant, minimizing the internal concentration of salts within plant tissue, keeping salt away from physiological targets to protect their function, and production of enzymes and metabolites that protect cell structures and repair damage (Breckle, 1990).

As stated previously, enzymes in salt tolerant plants are inactivated by high salt concentrations within the cytoplasm. To avoid metabolic inhibition ions must be kept separate from enzymes, and vacuoles provide an area for sequestration. Vacuoles, comprising the bulk of a cell's volume, have membrane bound Na^+/H^+ antiporters (Parida & Das, 2005). The H^+ gradient is formed by ATPase and pyrophosphatase transporters, which are expressed at a higher level in more tolerant species and induced by presence of salt stress (Parida & Das, 2005; Tester & Davenport, 2003).

Selective ion uptake into roots limits Na^+ access while allowing K^+ to enter the plant (Parida & Das, 2005). More water is taken up by a plant than is used, with the difference accounted for by transpiration. Solutes in the rhizosphere will either be excluded or taken up with the water. In some tolerant plants, the exclusion of sodium is in the range of 97% (R. Munns, Cramer, & Ball, 1999). The exclusion of Na^+ varies with the species of plant. The mode of action can either be through the use of selective ion channels to restrict uptake into the roots, or ATP-linked sodium pumps to prevent transport of Na^+ further into the plant after the sodium moves passively down its concentration gradient into the root (Hopkins, 1995). The selective uptake of ions helps plants ensure they maintain an appropriate ratio of potassium and sodium ions for their health. Among glycophytes, monocotyledon species require a higher K^+:Na^+ ratio

and have a lower storage capacity than dicotyledon species, and therefore must be more selective in their ion uptake than dicotyledon (Flowers & Yeo, 1988; Glenn, Brown, & Blumwald, 1999). As with glycophytes, halophytes have different K^+:Na^+ requirements for growth if the plant is a monocotyledon or a dicotyledon. Monocotyledonous plants tend to have less water content and smaller vacuoles, and so require less sodium ion to balance osmotic potential for water uptake as dicotyledonous halophytes (Glenn et al., 1999).

Exclusion of ions is not the only tolerance mechanism used for salt tolerance in plants. The difference in osmotic pressure between the rhizosphere and within the plant cells lowers the availability of water to the plant. To overcome this, the plant must use mechanisms to adjust its own osmotic potential. Glycophytes do this through the production of compatible solutes, highly soluble organic molecules that do not interfere with normal metabolism. These molecules, including glycine betaine, polyols, proline and various secondary metabolites, make water uptake more favourable by adjusting osmotic pressures, but also serve to protect the structure of proteins and ribosomes from elevated Na^+ (Tester & Davenport, 2003). In halophytes the osmotic potential is either entirely, or almost entirely, due to the presence of Na^+, K^+, and Cl^-. Halophytes balance exclusion of salt ions at the root to prevent damage and accumulation of salts in tissue to toxic levels with the requirement of salt ions to balance osmotic potential to continue water uptake (Glenn et al., 1999). The cells protect enzyme function by sequestration of ions in vacuoles and maintaining a Na^+:K^+ ratio that is favourable for normal cell function (Flowers & Yeo, 1988). Succulence in leaves is observed in many halophytes and results in more vacuole volume for ion sequestration. This adaptation allows plants to tolerate higher levels of salt uptake by providing more space for sequestration, and still protects enzymes from salt concentration that would lead to impaired function (Parida & Das, 2005; Tester & Davenport, 2003). Salt

concentrations within the plant can also be lowered by excretion of salts on to the leaf surface as the uptake of additional salt ions continues with water uptake. The exudation of salt results in salt crystal formation outside of the plant where the salt can fall away and does not impact plant growth (Tester & Davenport, 2003), but does return to the soil, replacing previously removed ions.

The various mechanisms of coping with salt stress all center around maintenance of water flow and protection of enzymatic function. The energy from ATP required for active transport of overabundant salt ions into vacuoles for sequestration or exudation from roots is an energy dependant system, lowering growth rates for affected plants. Excess salt can also result in increased productions of reactive oxygen species and oxidative damage. Production of ROS scavengers in response to oxidative stress and repairing damage caused by oxidation of cellular structures by ROS also have an impact on growth by diverting energy to repair that would normally be available for biomass production.

1.4 Remediation techniques for salt impacted soils

There are various methods available for remediation of contaminated soils, and the method used depends on the type of contaminant found in the soil. Organic contaminant levels can be lowered through volatilization or degradation (Gerhardt, Huang, Glick, & Greenberg, 2009), but these options are not available when dealing with ionic contaminants. Saline soils must be remediated by physical removal of ions from the soil. Methods that are available to remediate salt contaminated soils include excavation, leaching and recovery, electrokinetic restoration, and phytoremediation.

Excavation is the simplest method, as it involves removal of all contaminated soil from a site, storage in a landfill, and replacement of removed soil with clean soil from another location (Sorvari, Antikainen, Kosola, Hokkanen, & Haavisto, 2009; Wirthensohn, Schoeberl, Ghosh, & Fuchs, 2009). This method is expensive, as it involves heavy equipment and transportation, treatment and disposal of contaminated soil (Wirthensohn et al., 2009). The reason it is commonly used is because of the short time requirement, the complete removal of contaminants, and the elimination of future legal liability due to exposure (Sorvari et al., 2009).

Leaching of salts out of soil by flushing with fresh water can be done *in situ* (on site) or *ex situ* (off site). After removal of salt ions, the soil can be replaced if excavation is required. The process of *ex situ* leaching involves many of the same steps of excavation and land filling (Sorvari et al., 2009), with the additional cost of soil cleaning and transportation cost to replace the soil back on site (Menzies, Fulton, Kopittke, & Kopittke, 2009). Leachate from the soil flushing will contain all removed contaminants, and therefore must be recovered to prevent contamination of another previously unimpacted site. This imposes further costs of removal and storage of leachate (Lemming, Friis-Hansen, & Bjerg, 2010; Menzies et al., 2009; Sorvari et al., 2009) to ensure that contaminating ions are removed, and further environmental damage does not occur. It should be apparent that as the area of a remediation project that uses leaching increases, the cost and complexity can make this method of remediation no longer feasible.

Another method of remediation of salts is an electrokinetic process. This takes advantage of the electrochemical properties of ions, drawing them through a soil/water matrix towards an anode or cathode, depending on the charge of the ion (J. Cho, Kim, Chung, Hyun, & Baek, 2009). In studies involving removal of ions from over fertilization of greenhouse soils, up to 99% of certain ions were removed, and the EC was lowered by 60% in a 96h period, and

quantitative removal of sodium ions was achieved in ten days (J. Cho et al., 2009; J. Cho, Park, & Baek, 2010a; Kim, Cho, Baek, Yang, & Ko, 2010). These studies, however, involved small volumes of soil in a laboratory setting, and few field trials involving this technology have been performed. In addition, removal of sodium is slower than removal of potassium from soil (J. Cho, Park, & Baek, 2010b). This difference in removal rate is counterproductive, as competition between sodium and potassium for binding sites within plants is one of the causes of damage to plant health. Additionally, chloride ions are converted to chlorine gas by this process, forming a highly oxidizing compound which damages electrodes used in the removal process. The lack of information on successful field scale studies, equipment requirements, creation of harmful by-products, and removal of required ions such as potassium can make use of electrokinetic remediation a less desirable method.

Phytoremediation uses the ability of plants to take up ions into their biomass. Here, the ions are sequestered, the biomass can be harvested, and the contaminants removed from sites (Cheng, Park, & Glick, 2007; Huang, El-Alawi, Gurska, Glick, & Greenberg, 2005). The accumulation of contaminants in biomass is termed phytoextraction, and can be used for undegradable organic and inorganic compounds (Rock, 1997). Phytoextraction is entirely dependent on the ability of plants to produce extensive root systems to reach contaminants in soil and to produce high levels of above ground biomass in which to sequester contaminants (Glick, 2003). This method of remediation does not require transport of soil or the use of expensive equipment, which lowers remediation costs in comparison to the remediation methods that were discussed above. Phytoremediation is a passive method, requiring little in the way of site disturbance. As mentioned previously, however, plant growth is impeded by the presence of salt in soils. This means that as the contaminant concentration increases, the efficacy of

phytoremediation will decrease unless some method of improving growth conditions is employed. Addition of potassium containing fertilizer to compensate for the high Na^+/K^+ ratio (Cuin et al., 2003), dilution of salt impacted soil with the addition of clean soil and organic material to lower the EC of soil in the rooting zone of plants, and using salt tolerant plant species for use in remediation can all improve plant growth during phytoremediation. Addition of gypsum ($CaSO_4$) increases the amount of calcium available in soil, lowering the SAR and making the soil more suitable for plant growth.

1.5 Effect of plant-growth-promoting-rhizobacteria (PGPR) on plant growth

Phytoremediation is entirely dependent on the ability of plants to grow in contaminated areas. However, contaminants can lower rates of germination and biomass production, lowering the effectiveness of the remediation process. One way to improve plant growth is to lower the amount of ethylene, a stress hormone, in plant tissue. This can be done by the action of plant growth promoting rhizobacteria (PGPR) (Glick, 1995). PGPR are naturally occurring bacteria which can improve plant growth through a symbiotic relationship with the plants whose rhizosphere they inhabit (Glick, 1995; Glick, 2003). These microbes can assist plant growth through indirect (e.g. competition with infectious organisms) or direct methods. Direct methods include the production of siderophores which improve plant nutrient uptake, the production of auxins to stimulate plant root growth, the fixation of nitrogen to improve nutrient availability, and the lowering of the precursor of stress ethylene concentrations through the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Chang, 2008; Hong, Pasternak, & Glick, 1991; Huang et al., 2005; Patten & Glick, 1996; Penrose & Glick, 2003). PGPR strains that produce the auxin indole-3-acetic acid (IAA) and consume ACC through high ACC deaminase activity to lower stress response in plants have been used to remediate petroleum (Huang, El-

Alawi, Penrose, Glick, & Greenberg, 2004; Huang et al., 2005) and salt (Chang, 2008; Wu, 2009) impacted soils.

ACC deaminase activity improves plant growth by lowering the available ACC in the ethylene biosynthesis pathway (Glick, Penrose, & Li, 1998). ACC produced by this pathway is secreted by the plant into the rhizosphere, where ACC deaminase producing PGPR can consume ACC as a source of fixed nitrogen. This promotes the growth of PGPR in the rhizosphere, causing more ACC to be consumed, and therefore, more ACC to be secreted into the rhizosphere. As more ACC is secreted by the plant, less is available for ethylene biosynthesis and stress signalling, and less plant growth inhibition is observed (Glick et al., 1998). PGPR that produce IAA secrete the auxin into the rhizosphere, where the plant is able to take up the hormone, resulting in improved cell growth. This increase in IAA concentration leads to an upregulation of ACC synthase production and activity, and results in higher levels of ACC formation (Kende, 1993). The proposed pathway of IAA and ACC deaminase activity in plant growth promotion is shown in Figure 1.1.

1.6 Effect of reactive oxygen species on seed germination rates

One of the effects of osmotic stress on plants is closure of stomata during the day to limit water loss due to evaporation. The closure of stomata is successful in preventing water loss in an already water limited environment, but also halts gas exchange between the plant and the atmosphere. A lack of gas exchange results in an increase in the concentration of O_2 within the leaf in relation to the concentration of CO_2 (Lechno, Zamski, & Tel-Or, 1997). The result of this change in concentrations is less available CO_2 to accept electrons from photosystem I and II compared to the level of available O_2. Figure 1.2 shows the path of electron movement in cases of open stomata (A) and closed stomata (B). If O_2 functions as a terminal electron acceptor, the

result is elevated production of reactive oxygen species (ROS), which cause cellular damage (I. Cakmak, 2005). During photosynthesis under non-stressed conditions, 20-25% of electrons will be diverted to the formation of ROS (I. Cakmak, 2000; Robinson, 1988). This percentage increases as stress conditions limit CO_2 availability resulting in an increase in the $[O_2]$:$[CO_2]$ ratio within plant cells (Biehler & Fock, 1996). It follows that plants with increased antioxidant expression would be able to cope with the higher level of ROS production caused by stomatal closure. Experiments involving exogenously applying H_2O_2 to seeds and seedlings have shown activation of antioxidant systems, giving better tolerance to abiotic stresses (Wahid, Perveen, Gelani, & Basra, 2007; Wang, Li, Wang, & Li, 2010). In addition to improving coping mechanisms for ROS damage caused by abiotic stresses, application of H_2O_2 to seeds has been shown to improve the rate of germination, possibly due to oxidation of germination inhibitors (Ogawa & Iwabuchi, 2001). These combined effects may help improve yields of plants in a phytoremediation trial by increasing the percentage of seeds that germinate and improving plant health by pre-inducing ROS scavenging enzymes before salt stresses are faced.

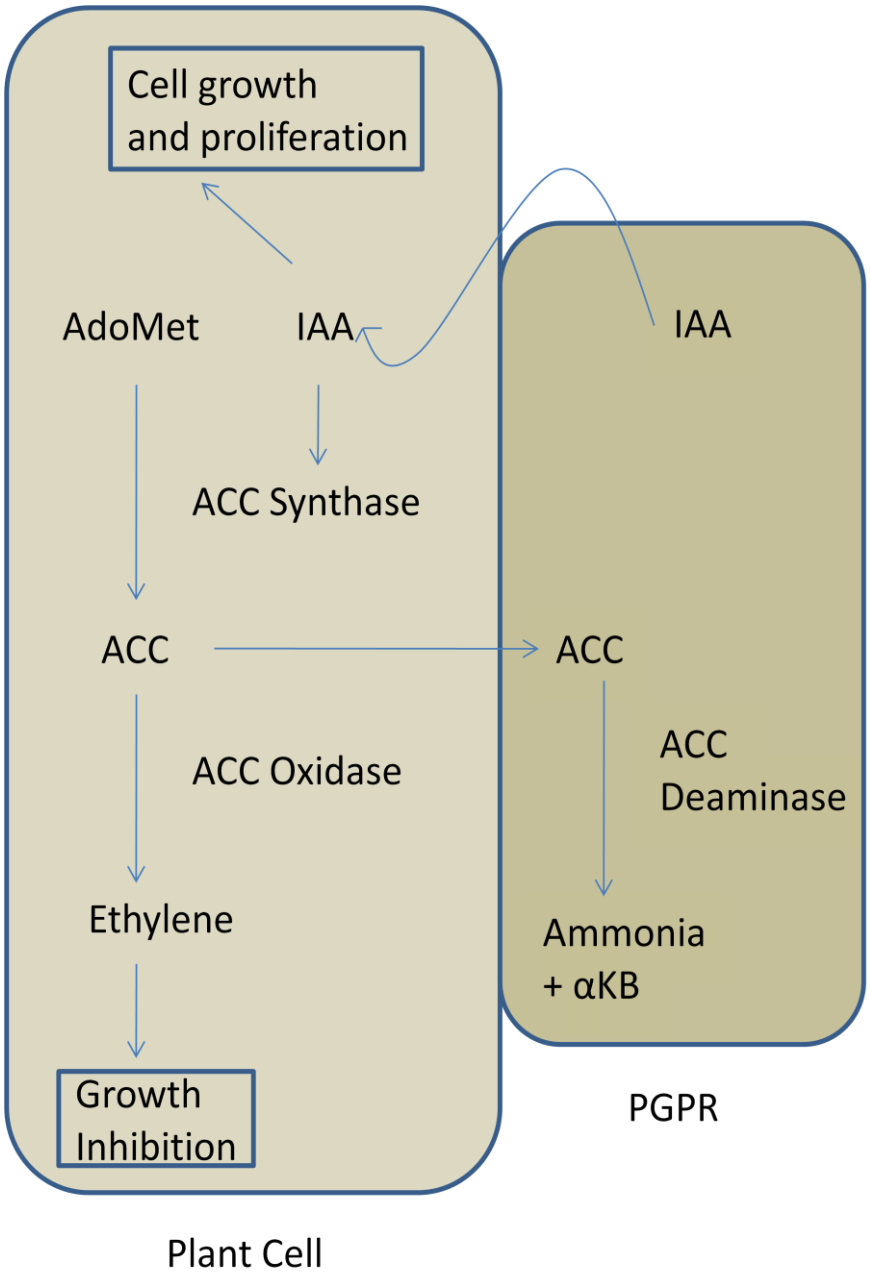
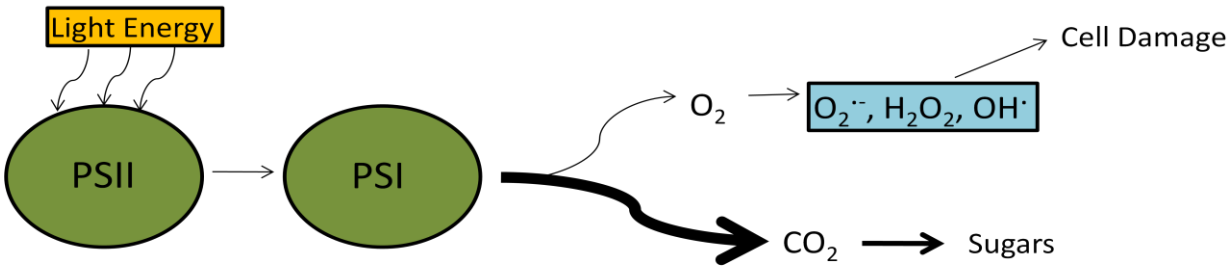


Figure 1.1: Plant Growth Promoting Rhizobacteria (PGPR) schematic. PGPR consumes the ethylene precursor ACC, lowering the stress response within plants by lowering the stress signal. IAA is produced within the plant, stimulating both plant cell growth and upregulation of ACC synthase. This upregulation of ACC synthase causes more ACC to be produced, and increases the available fixed nitrogen for PGPR to consume in the rhizosphere (Glick et al., 1998).

A



B

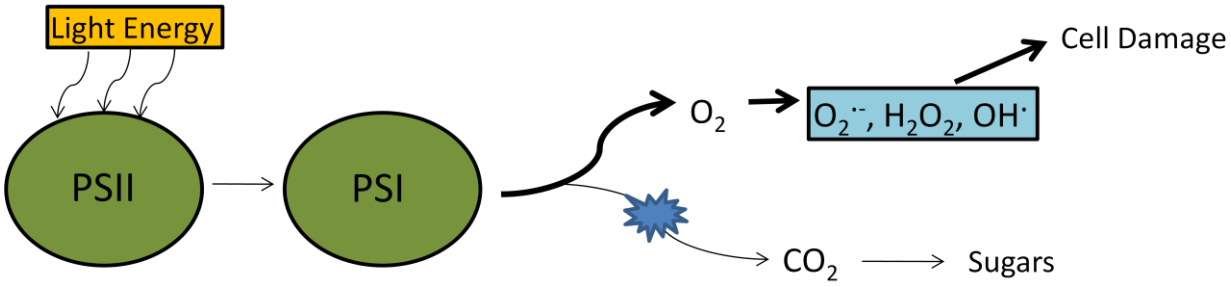


Figure 1.2: Transfer of light energy through photosystems in plants. A shows the normal movement of electrons, resulting in CO_2 acting as terminal electron acceptor and fixation of carbon into sugars. B shows the result of exposure to osmotic stress resulting in closure of stomata and cessation of gas exchange, resulting in oxygen concentrations to increase and oxygen to become the terminal electron acceptor. In this scenario, reactive oxygen species are produced and cellular damage occurs. (I. Cakmak, 2005)

1.7 Research objectives

Phytoremediation enhanced with PGPR has been shown to increase the rate of remediation when used in petroleum and salt contaminated soils (Chang, 2008; Huang et al., 2004; Huang et al., 2005; Wu, 2009). Improved biomass production and increased soil infiltration of root systems in PGPR treated plants has been credited with improved remediation rates in phytoremediation trials. This is done by increasing plant biomass to sequester non-biodegradable contaminants and making biodegradable contaminants available to rhizosphere bacteria for consumption.

Wahid *et al*. (2007 and 2008) have shown that sunflower seeds imbibed with H_2O_2 had increased rates of germination compared to control seeds under unstressed conditions. They also showed improved wheat plant growth under saline conditions. Improvements of germination rates in flowers and cereal crops have also been found following H_2O_2 treatment (Naredo, Juliano, De Guzman, & Jackson, 1998; Ogawa & Iwabuchi, 2001). According to the mechanism proposed by Ogawa and Iwabuchi (2001), H_2O_2 improved germination by oxidizing germination inhibitors.

As outlined by Glick (1998), PGPR improve plant growth under stressful conditions by lowering the concentration of the stress hormone ethylene in plants, resulting in less growth inhibition. Therefore, PGPR and H_2O_2 pretreatment may be combined to work in tandem, resulting in further improvement to growth under saline conditions. One of the objectives of this thesis was to test these methods of improving plant performance under saline conditions and determine if their combination is more effective than individual treatments. This was tested through germination assays, greenhouse trails, and preliminary field trials.

Chang (2007) showed correlation between increased salt uptake into plant biomass when compared to plants grown on uncontaminated soil, and a decrease in soil salinity during phytoremediation field trials. Regions of the fields that experienced flooding during the growth season and had no plant growth showed an increase in EC_e over the course of the year. This increase in salinity may be due to movement of soluble salts with the bulk water flow into low lying areas of the field. At the time, no work was done to test the effect of water flow on salt movement in soils being remediated using PGPR enhanced phytoremediation. It was proposed that heavy rainfall may move salts deeper into the soil horizon and past the rooting zone, effectively flushing the soil and decreasing the EC_e values of the surface soils. To test this, soil samples were taken at 3 depths down to 1 meter, and the EC of these samples were determined for the field site.

The ionic content of plant material grown during field seasons was measured and compared to the change in salinity observed during that field season. The amount of sodium, calcium, potassium, magnesium and chloride was used to calculate the amount of salt removed, and the theoretical change in EC based on equations that relate ionic strength and conductivity. This was used to carry out a mass-balance calculation to determine how much of the observed changes in salinity during the field season.

The objectives for this research are:

1. Investigate various plant species for their ability to produce biomass on saline soils and sequester salt, leading to improved rates of phytoremediation.
2. Determine the effect of H_2O_2 seed imbibition on rates of germination under saline conditions, both alone and in combination with PGPR treatment.

3. Monitor vertical movement of salt in soils of a field trial to determine net direction of salt movement during phytoremediation.
4. Carry out mass balance measurements and calculations of ion content of plant tissue and relate it to observed salinity changes during field seasons.

2 Materials and Methods

2.1 Growth of PGPR cultures

Bacterial stocks of previously isolated ACC deaminase producing strains of UW3 (*Pseudomonas putida*, (Glick, 1995)), UW4 (*Pseudomonas putida*, (Glick, 1995)) and CMH3 (*Pseudomonas corrugata*, (Chang, 2008)) were inoculated into 100 mL sterile Tryptic Soy Broth (TSB) (BD Biosciences, Mississauga, ON) in a 250 mL Erlenmeyer flask, and grown at 23 ± 1ºC on a rotary shaker at 80 rpm. After 24 hours, cultures were transferred aseptically to Falcon tubes, which were centrifuged at 2000 rpm for 15 minutes. The supernatant was removed, and autoclave sterilized reverse osmosis filtered water (18Ω) (ddH_2O) was used to resuspend the pellet of cultured bacteria. The resuspended pellets were then centrifuged again, and resuspended in ddH_2O.

2.2 Seed treatment with PGPR

Bacterial cultures grown as per Section 2.1 were grown in 1 litre of TSB for 24 hours on a rotary shaker at 80 rpm. The bacterial culture was transferred to centrifuge tubes, and centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed with ddH_2O, and centrifuged again. The supernatant was discarded, the pellet was resuspended in ddH_2O and the optical density at 600 nm (OD_{600}) was measured using a spectrophotometer. The OD_{600} was then adjusted to 2.0 using ddH_2O. Methylcellulose polymer (Sigma Aldrich, Oakville, ON) was prepared at 1.5% w/v by mixing on a stir plate for 1 hour until all clumps had broken apart, and then autoclaved for 40 minutes. The polymer forms a white gel, which undergoes reverse gelatinization upon cooling and becomes clear. This clear polymer was combined with bacterial solution at a ratio of 200 mL methylcellulose to 1 L

bacterial solution. A commercial seed colourant (Color Coat Blue, Becker Underwood, Saskatchewan) was added in a ratio of 17.5 mL to 1 L bacteria suspension and methylcellulose polymer slurry. This coloured slurry was stirred by magnetic stirrer for the duration of seed treatment. Seeds were treated in a HEGE 11 seed treater (Wintergsteiger Inc., Austria) for 2 minutes in batches of 2.5 L of seed. Coloured slurry was added at a ratio of 10 mL per 2.5 L of cereal seeds and 20 mL per 2.5 L of grass seed. Seeds were planted within 1 month of bacterial application.

2.3 H_2O_2 imbibing of seeds

Seeds were soaked in a sodium hypochlorite (bleach) solution (1% v/v) in a 1.5:1 bleach solution to seed volume ratio for 10 minutes to surface sterilize the seed, followed by three washings with RO water (18 Ω). Seeds were soaked in solutions of H_2O_2 solution (H_2O_2 treatment) for 3 hours or in sterile RO water (control treatment) for 3 hours, after which seeds were washed 3 times with sterile RO water and allowed to dry in a laminar flow hood. Seeds were used within 3 days of H_2O_2 imbibing.

2.4 Colourimetric assay of antioxidant presence in peroxide treated seed

Presence of antioxidants in seeds treated with H_2O_2 was determined by measurement of absorbance of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich, Oakville, ON) to determine quenching of the stable radical by antioxidants (Brand-Williams, Cuvelier, & Berset, 1995; Wierenga, 2005). The absorbance at 520 nm is used to determine the change in concentration of the stable DPPH radical to the protonated form, as shown in Figure 2.1. Seeds treated with H_2O_2 using the method in section 2.3 (0 μM, 30 μM, 60 μM, 90 μM, and 120 μM H_2O_2) were ground in a Waring blender, and 0.02 g of powered seed was weighed into

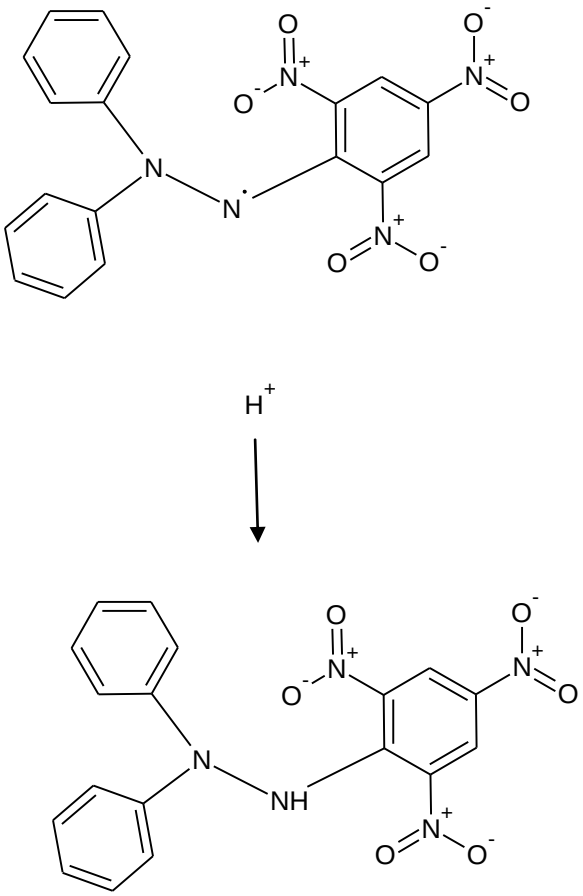


Figure 2.1 Diagram of the protonation reaction of DPPH. The reaction causes the radical to change structure from the purple radical to the yellow non-radical (Wierenga, 2005).

microfuge tubes. To the tube, 1 mL 80% ethanol was added, and tubes were shaken for 24 hours at 80 rpm. The tubes were then centrifuged at 8000 g for 10 minutes, the supernatant was collected and stored at 4ºC until measurement. DPPH was dissolved in 80% ethanol to a concentration of 200 μM, and stored at 4ºC for up to 2 weeks. In a cuvette 500 μL DPPH, 300 μL 80% ethanol and 200 μL sample supernatant was added, inverted to mix, and the absorbance read at 520 nm. A negative control was read with 500 μL DPPH and 500 μL 80% ethanol, and a positive control with 500 μL DPPH, 20 μL 14 mM ascorbic acid and 480 μL 80% ethanol were read at 520 nm. The absorbance of samples with supernatant was compared with control treatment (0 μM H_2O_2) absorbance. Samples were tested in triplicate, and average absorbencies were compared by t-test at 80% confidence.

2.5 Seed germination assay

Whatman filter paper (#1) was cut to fit into the bottom of a 3 inch petri plate. The filter paper was soaked with 2.0 mL of solution being used for germination. The solution was NaCl in ddH_2O for treatments or ddH_2O for control treatments. Twenty seeds per plate were placed in four rows in a 4 seed: 6 seed: 6 seed: 4 seed pattern. Seeds were watered twice daily with 1.0 mL, and lids were left slightly ajar to prevent water logging. Petri plates were covered with paper towel to minimize air movement and prevent light exposure. Emergence of root and shoot radicals were monitored and recorded daily. Emergence was considered a radical growing 1 mm past the seed coat.

2.6 Greenhouse plant germination and growth assay

Soils collected from field sites inside the impacted zone and outside of the impacted zone were used for contaminated and control soils respectively. Soils were dried and sieved prior to use to remove large gravel and ensure similar soil texture between control and contaminated

soils. Square pots (2”x2”) were filled up to approximately ½” from the top pot with soil, and 16 seeds were planted in a 4x4 grid and then covered with a thin layer of soil. The soil was wetted with ddH_2O by spray bottle until all the soil was moist and then the trays of soil were moved to a greenhouse where they were watered daily with 15 mL ddH_2O per pot, under natural sunlight and greenhouse lights, with temperatures ranging from 25-35ºC during the day and 20-27ºC during the night. Germination of plants was monitored daily, and determined by counting shoots that had broken the soil surface. If above ground biomass production was to be determined, the plants were removed from the greenhouse and the soil was allowed to dry on a bench top. The dried soil was pressed to cause it to crumble from the root system, and the root and shoots were separated. Plant biomass was then dried and weighed.

2.7 Research field site information

For this research, salt impacted sites in Western Canada were used. Each field was used in a phytoremediation field trial over two growing seasons from May 2008 October 2008 and from June 2009 to October 2009.

Soil samples of surface soils were taken by Dutch auger (3” diameter) to 30 cm, and were made as a composite of three random bores within 1 m of the sample points shown in Figure 2.2 and Figure 2.3. Soil samples of depths below the top 30 cm at the Kindersley site were taken by truck mounted drill with an 8 inch diameter soil auger to a depth of 1 m. The soil was separated into buckets for 0-30 cm, 30-60 cm and 60-100 cm, and the soil of two bore holes was combined for each sample point. Soils in the buckets were mixed and taken for samples, with the extra soil being put back into the bore holes according to the depth it was taken from. Soil samples at depths below 30 cm on the Brazeau site were taken by repeated augering with the Dutch auger

until the desired depth was reached, and composites were taken using the same method at the surface samples.

Plant tissue samples were taken by assignment of randomly generated coordinates on a grid placed over the plot to be sampled, and a 0.5 m x 0.5 m square was used to determine the area in which plant tissue would be taken for each sample point. The plant tissue was stored in a plastic bag in a cooler with ice until they could be moved to a refrigerator or were dried for determination of dry mass.

2.7.1 Kindersley, Saskatchewan field site

The Kindersley field site (51°21'40"N, 109°48'51"W) is located near Alsask, Saskatchewan, approximately 60 km west-south-west of Kindersley, Saskatchewan. The reported cause of salinity on this site was breakage of a brine water pipeline north of site, resulting in a spill zone (Figure 2.4) extending southward. Planting maps for 2008 and 2009 are shown in Figure 2.2and Figure 2.3. The Kindersley field site was 1680 m^2 in 2008 and expanded to 2240 m^2 in 2009. The average EC_e at the start of the field season in 2008 was 5.6 dS/m, with a range of 0.96 to 16.09 dS/m. The site was prepared for planting by rototilling the soil to a depth of 2 inches. Seeds were planted at a rate of 100 lbs/acre for tall wheatgrass (*Agropyron elongatum*, Cribit Seeds, Winterbourne, ON), oats (*Avena sativa*, Cribit Seeds, Winterbourne, ON), annual ryegrass (*Lolium multiflorum*, Ontario Seed Company, Waterloo, ON) and tall fescue (*Festuca arundinacea* C.V. Inferno, Ontario Seed Company, Waterloo, ON). After planting, 11-52-0 (N:P:K) slow release fertilizer was applied at a rate of 35 lbs per acre. In 2009, switchgrass (*Panicum virgatum* L., Ontario Seed Company, Waterloo, ON) was planted by broadcast seeder at a rate of 22 lbs/acre.

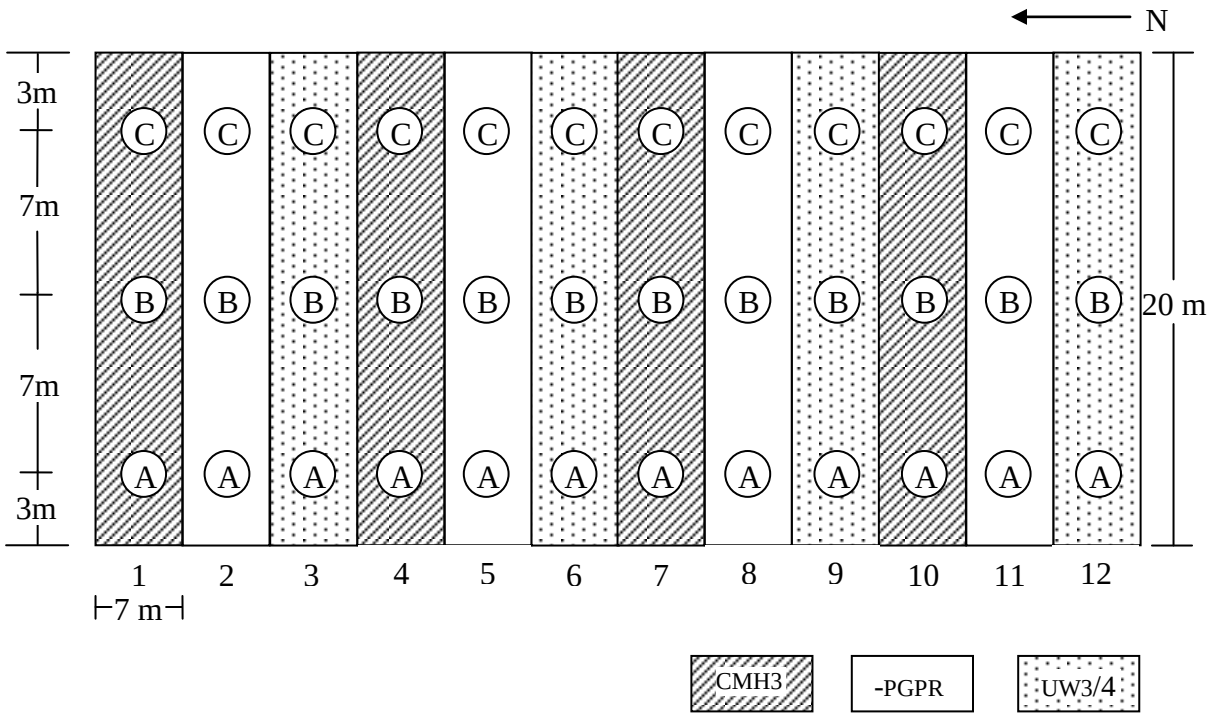


Figure 2.2: Kindersley 2008 planting map. All plots were seeded with tall wheatgrass, Inferno tall fescue, annual rye grass and common oats. The seeding rate for each species was 100 lbs/acre, and 35 lbs/acre fertilizer (11-52-0) was applied after planting. The site was prepared for planting by tilling to a depth of 2 inches by rototiller, and soil samples were taken after tilling, but prior to planting and fertilization.

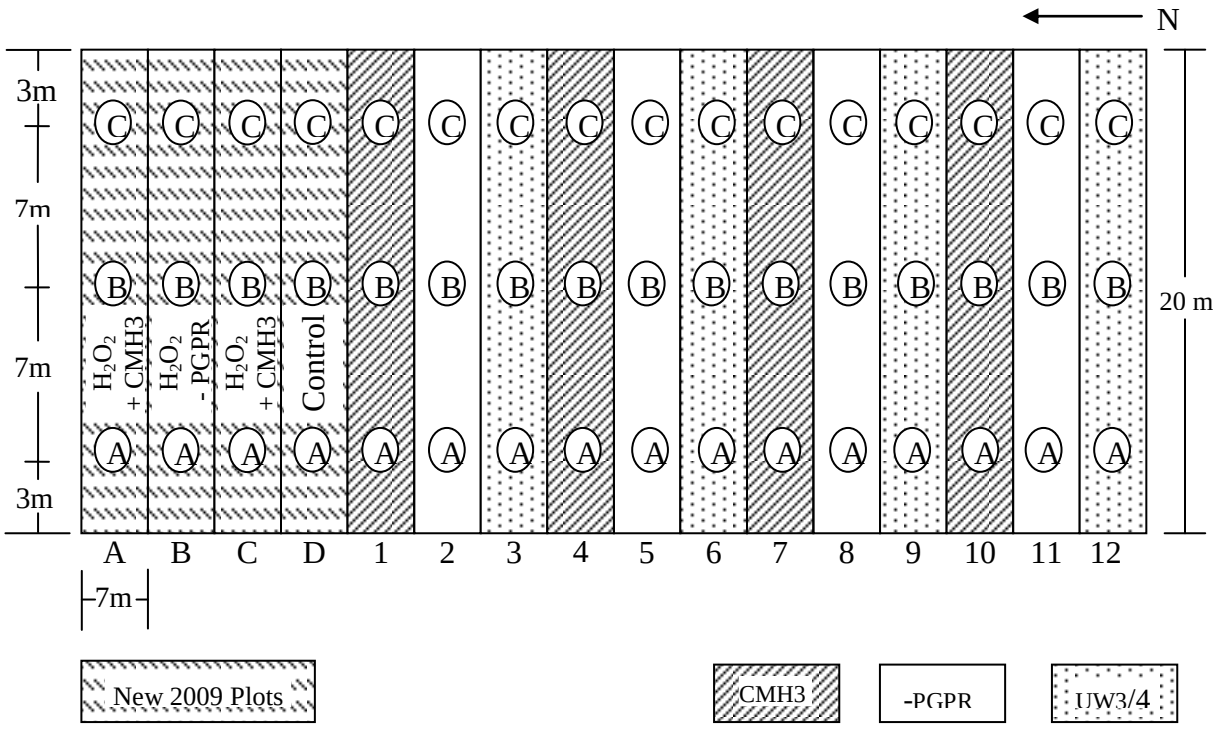


Figure 2.3: Kindersley 2009 planting map. Plots 1-6, and 10-12 were tilled to 2 inches with a rototiller prior to sampling and planting were seeded with tall wheatgrass, Inferno tall fescue, annual rye grass and common oats. Plots 7-9 were not tilled or planted. Any plants remaining or seeds that had fallen in 2008 were allowed to regrow. Plots 10-12 were planted with common switchgrass. Plots A-D were rototilled and planted with Tall wheatgrass to test field viability of hydrogen peroxide treatment for seed germination.

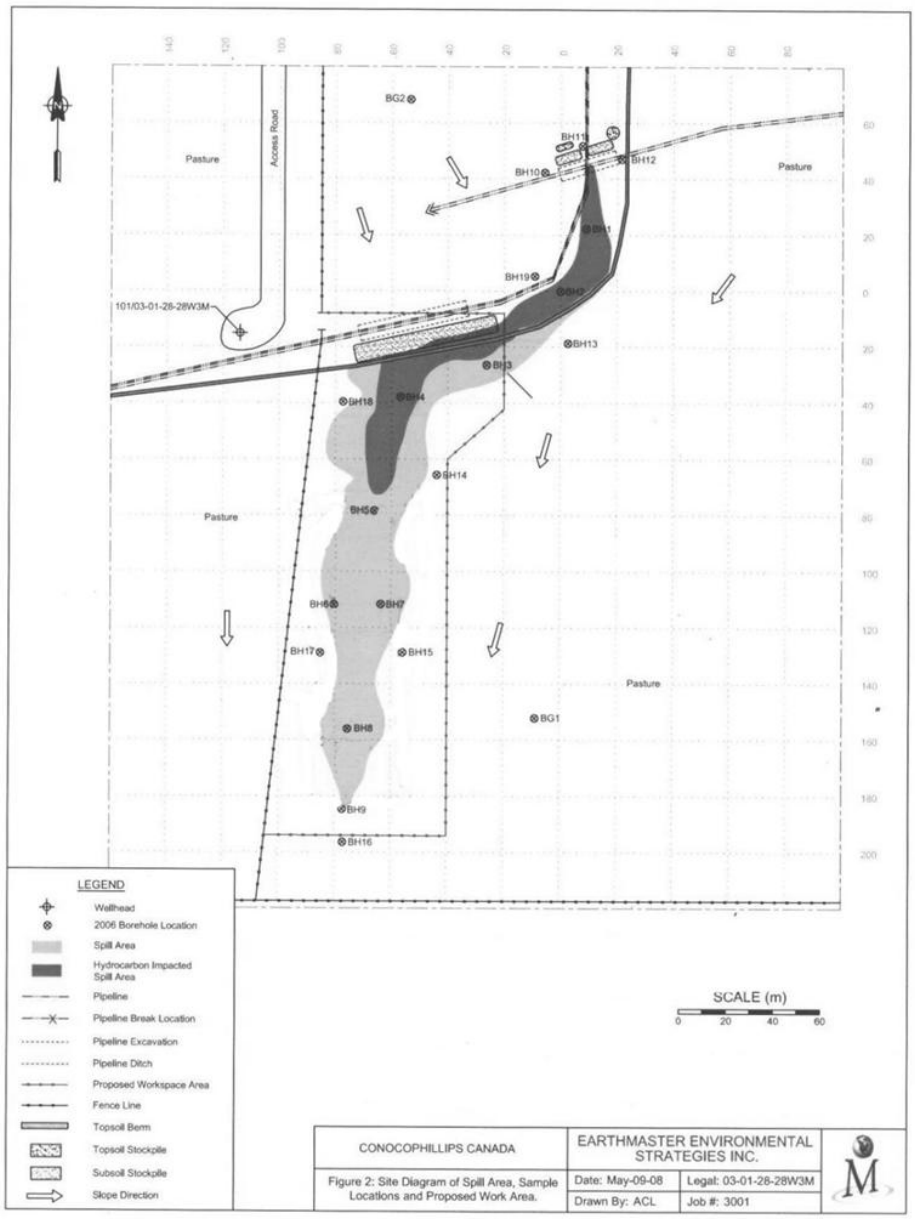


Figure 2.4: Spill map of the Kindersley field site. Dark grey shows brine and petroleum contamination areas, and lighter grey shows brine contamination areas. The proposed field site was on the south end, including only brine impacted soils.

2.7.2 Brazeau, Alberta field site

The Brazeau field site (53° 9' N, 115°46' W) was located west of Drayton Valley, Alberta. The site was comprised of excavated soil from an offsite sump which had been transported for treatment. The planting area was 150 m x 40 m, divided into 6 evenly sized plots. The planting maps for 2008 and 2009 are shown in Figure 2.5 and Figure 2.6. In 2008 all plots were tilled and planted with tall wheatgrass (52.88 lbs/acre), Inferno tall fescue (64.37 lb/acre), annual rye grass (77.99 lbs/acre) and common oats (111.02 lbs/acre) using a Brillian seeder. In 2009, plots 1-4 were planted with tall wheatgrass, annual rye grass, and Inferno tall fescue at a rate of 100 lbs/acre and common oats at a rate of 200 lbs/acre using a Brillian seeder. In 2009, plots 5-6 were planted with switchgrass at rate of 22 lbs/acre by broadcast spreader, and packed with the roller of the Brillian seeder used in plots 1-4. In 2009, fertilizer (20-20-20) was applied at a rate of 50 lbs/acre.

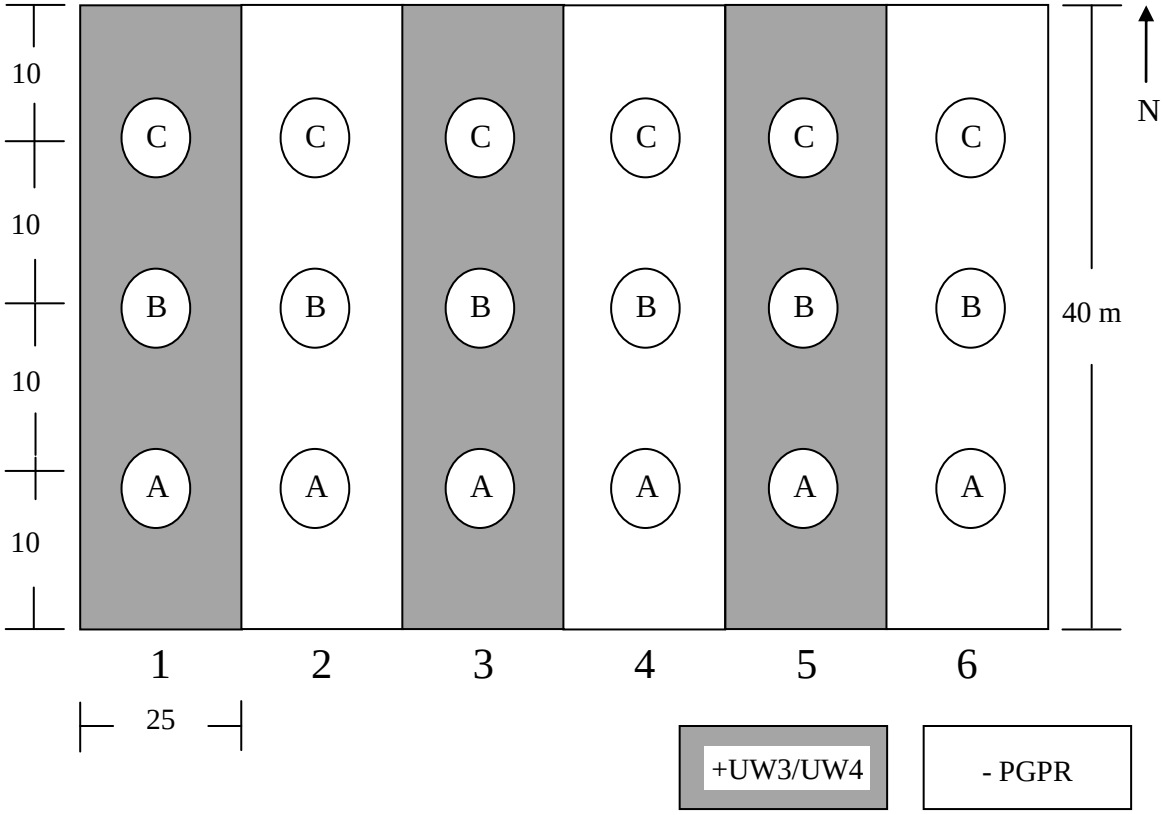


Figure 2.5: Planting and sampling map for 2008 field season on the Brazeau, AB field site. All plots were planted with tall wheatgrass, Inferno tall fescue, annual rye grass and common oats.

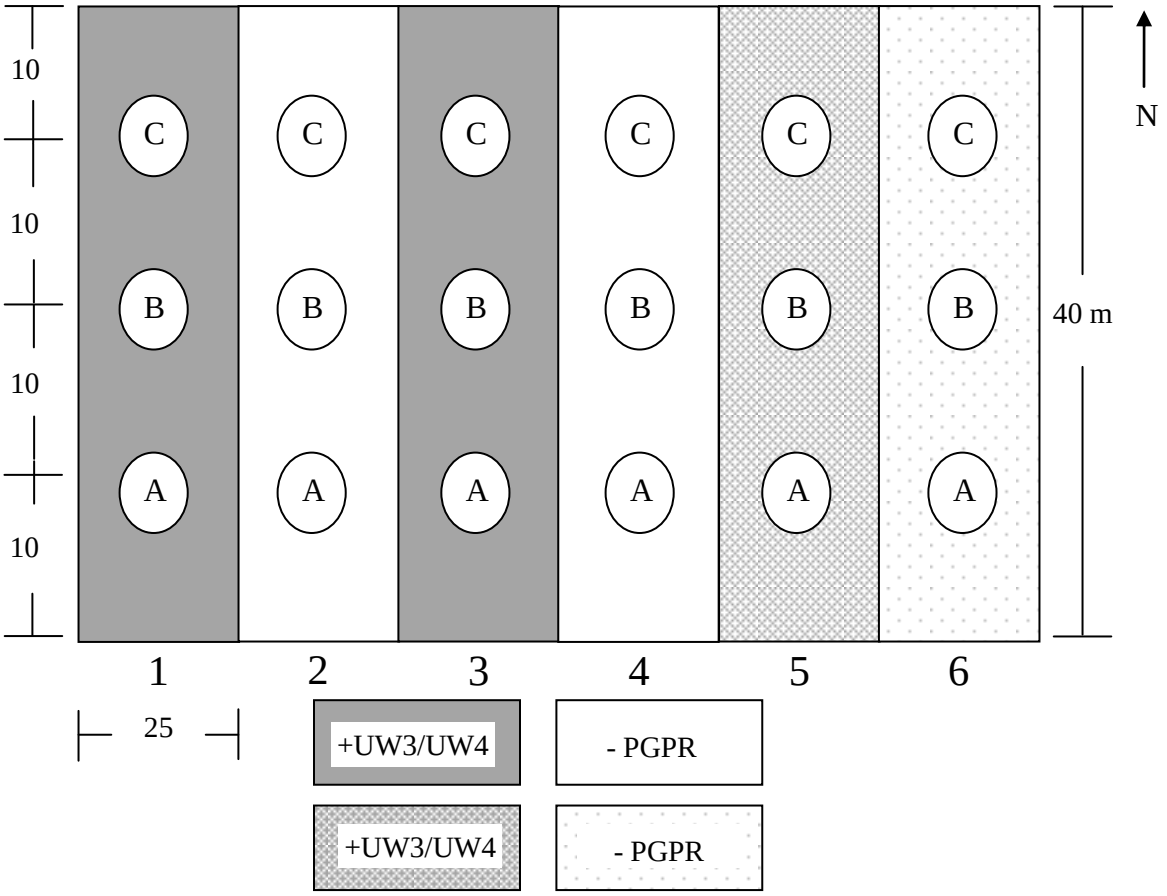


Figure 2.6: Planting and sampling map for 2009 field season on the Brazeau, AB field site. Plots 1-4 were planted with tall wheatgrass, Inferno tall fescue, annual rye grass and common oats, and plots 5 and 6 were planted with switchgrass.

2.8 Determination of soil salinity

Salinity of soil samples was determined by measurement of $EC_{1:2}$ values for all samples and conversion to EC_e by calculation with an empirically determined K value (Equation 1, Section 1.1). Measurements were carried out using air dried soil samples, ground with mortar and pestle and filtered through a 4 mm particle size wire sieve. For measurement of $EC_{1:2,}$ 15 g of soil was mixed with 30.0 mL of ddH_2O (18 Ω) in a 50 ml falcon tube. Tubes were mixed on a shaker plate at 80 rpm for 30 minutes to fully mix, and then allowed to settle overnight. The supernatant was measured using an electrical conductivity meter (EC meter) (Oakton Instruments, Vernon Hills, Illinois, USA). All samples were measured in triplicate, and were considered accurate if all replicates are within 10% of the average $EC_{1:2}$.

To determine the K value for a site, EC_e measurements are made directly by testing the conductivity of solute in a saturated paste. Soil and ddH_2O were mixed in a 50 mL Falcon tube to form a saturated paste. Formation of a saturated paste was determined by a shiny surface appearance of the paste, slight flowing when the surface is disturbed, the paste sliding cleanly from an aluminum spatula, and an absence of pooling water. Soil and ddH_2O were added to achieve this saturated paste criteria. The tube with saturated paste was then centrifuged at 2000 rpm for 10 minutes, and the electrical conductivity of the supernatant was measured using an EC meter. Comparison of EC_e and $EC_{1:2}$ values for a field sample with Equation 1 gives a K value. Multiple K values were determined for a field to determine an average K value to estimate EC_e values from $EC_{1:2}$ measurements for the remainder of the field.

2.9 Plant biomass measurement and salt accumulation determination

Plant material was washed and dried in a 55ºC hotbox for 3 days to remove moisture. The dry weight was determined for samples to follow plant growth by weighing on a top loading

balance. An external analytical lab, ALS Environmental Inc. (Waterloo, ON), was used for analysis of sodium, chloride, magnesium, calcium and potassium content in plant biomass. The content of sodium, calcium, potassium and magnesium was determined by inductively coupled plasma atomic emission spectroscopy according to EPA method 200.7, and chloride was determined by ion-chromatography according to AHPA 4110B.

2.10 PAM fluorometry to determine photosynthetic capability

Photosynthetic health in plants exposed to saline field soils was tested using pulse amplitude modulated (PAM) fluorometery. Samples of tall wheatgrass from the Kindersley field site were taken from plots A, B, C, D, 1 , 2 and 3 in August 2009 during mid season sampling. Samples were dug up to include roots, and kept in a cooler with ice packs to prevent decomposition until measurements were taken. Samples were measured with a PAM-2100 (Heinz Walz GmbH, Eichenring, Germany). Plant samples were dark adapted by storage in a closed cooler for at least 30 minutes prior to analysis to ensure all PSII reaction centers were open for reaction with photons. The minimal fluorescence (F_o) in dark adapted tissue was adjusted to 0.300 ± 0.050 by adjusting the aperture size on the leaf clip. A single saturating pulse (0.6 s, 2000 μmol/m^2s) was used to measure the maximum fluorescence (F_m) of dark adapted tissue. The fluorescence in steady state (F_s) was measured using 640-700 nm actinic radiation (70 μmol/m^2s) after 30 seconds for 14 minutes after steady state was reached. A saturating light pulse (0.6 s, 2000 μmol/m^2s) was produced to measure maximal fluorescence during steady state photosynthesis (F_m') with actinic light. Parameters calculated using PamWin software (PamWin v 2.00, Heinz GmbH, Germany) were maximum PSII activity (F_v/F_m), photochemical quenching (qP, energy storage) non-photochemical quenching (qN, energy loss), and yield (steady state PSII activity).

2.11 Statistical Analysis

All statistical analyse were performed using the software GraphPad Prism 5 (GraphPad Software, Inc). All analysis was by one-way ANOVA followed by Boneferonni post test at $P < 0.05$.

3 Results

3.1 Hydrogen peroxide (H_2O_2) treatment of tall wheatgrass and response to saline stress

H_2O_2 has been shown to improve seed germination under salt stress (Wahid et al., 2007; Wang et al., 2010). The potential application of this treatment to improve plant growth under field conditions, both as a stand-alone treatment and in combination with PGPR, was examined.

3.1.1 Effect of H_2O_2 and PGPR treatment on root and shoot emergence in the laboratory

The effect of imbibing seeds with H_2O_2 on germination of tall wheatgrass both with and without PGPR treatment was investigated. It is not known what effect the treatment will have under salt stress in comparison to, and in combination with, PGPR. Tall wheatgrass seeds were used in a petri plate germination assay. The 75 mM NaCl solution used to produce saline conditions has an EC of approximately 7.5 dS/m, equating to a moderate level of salinity which is commonly found in salt impacted soils at field sites. On a daily basis the emergence of root and shoot radicals were each monitored, and the results are shown in Figure 3.1 and Figure 3.2 respectively. These graphs show the results of three separate replicates of the germination assay with results normalized to the average germination of all RO watered samples in a replicate.

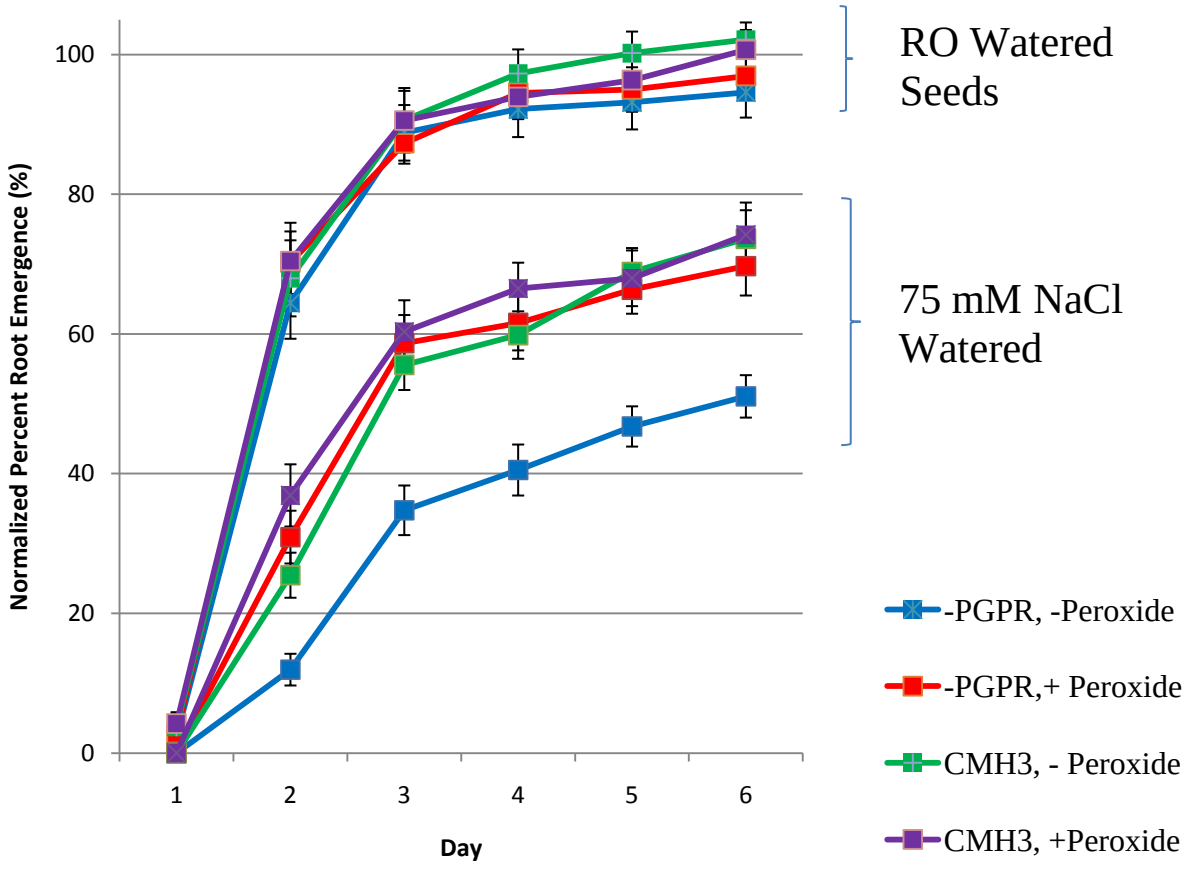


Figure 3.1: Emergence of root radicals from seeds with and without PGPR treatment and with and without H_2O_2 treatment. RO watered controls were averaged to obtain a 100% normalized benchmark. Error bars show standard error. All treatments (PGPR alone, H_2O_2 alone, and H_2O_2 and PGPR combined) showed statistically significant improvement in root emergence compared to the no-treatment control when watered with 75 mM NaCl at 90% confidence interval. Treatments watered with 75 mM NaCl were not significantly different from each other.

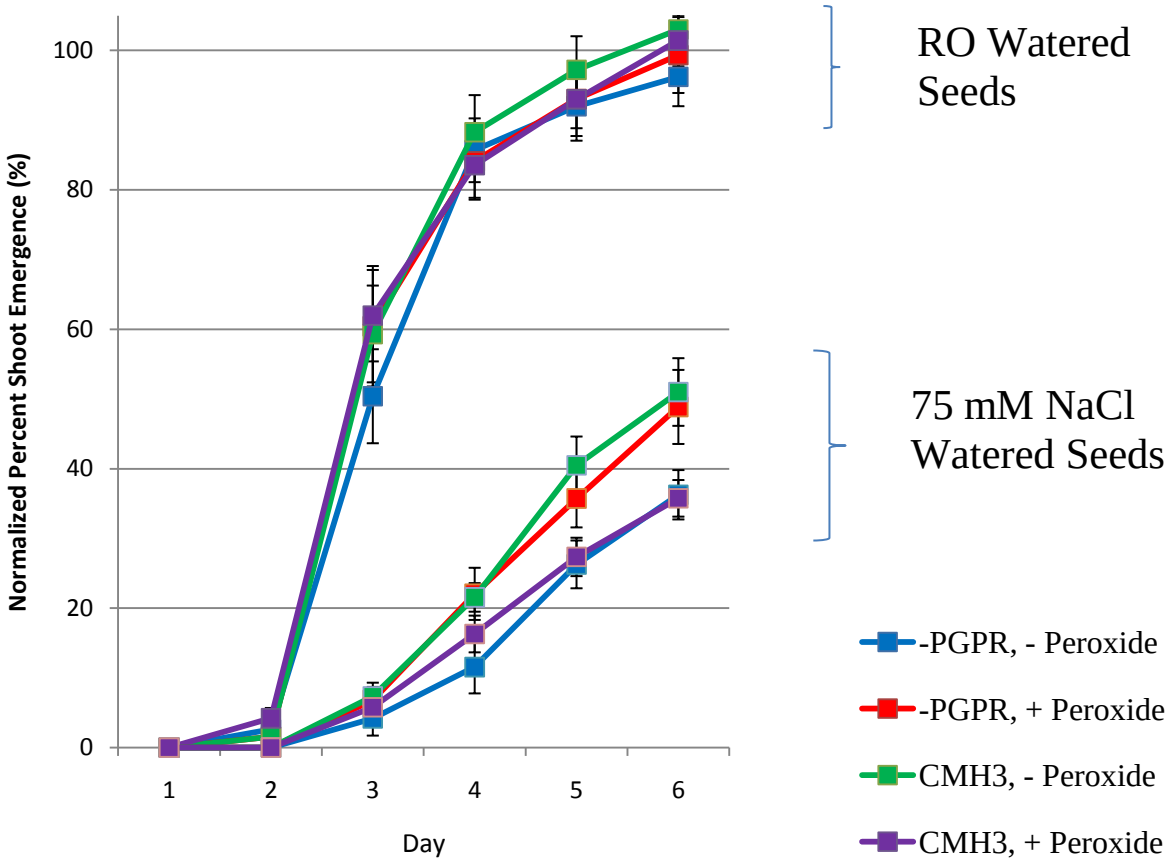


Figure 3.2: Emergence of shoot radicals from seeds with and without PGPR treatment, and with and without H_2O_2 treatment. Germination of RO watered controls were averaged to obtain a 100% normalized benchmark. Only the PGPR with no H_2O_2 treatment on days 5 and 6 showed statistically significant improvements in germination compared to the no-treatment control when watered with 75 mM NaCl, at 90% confidence interval.

This assay was done to assess the potential of hydrogen peroxide treatment in combination with PGPR treatment to provide improved germination and growth when compared to PGPR treatment alone. In both the root and shoot emergence data, seeds imbibed with H_2O_2 showed an increased rate of germination when compared to the untreated control seeds, and all treatments showed a statistically significant increase in germination compared to the untreated seeds. Root emergence of the three seed treatments all showed a statistically equivalent level of germination, and all treatments showed significantly higher rates of germination than the untreated control. Shoot emergence showed an improved level of germination for both H_2O_2 alone and PGPR alone treatments. This trend was not however seen with the combination of H_2O_2 + PGPR, in which the level of shoot emergence was equal to the untreated control seeds. This suggests that the mechanism by which PGPR and H_2O_2 function to improve shoot emergence by themselves result in no improvement in germination when the two treatments are combined under saline conditions.

3.1.2 Germination of H_2O_2 treated tall wheatgrass in salt impacted soil

The germination of peroxide imbibed seeds was tested in Kindersley field site soils, using control unimpacted soil and impacted saline soil (EC= 4.5 dS/m). The average number of seeds that emerged at the end of a 4 week greenhouse germination assay was determined (Figure 3.3A). By the end of the four week trial there was no significant difference between the H_2O_2 treatments. There was a small decrease in germination between control and saline soil, but no significant difference between the various peroxide treatments. Figure 3.3B shows the average time taken for the various treatments to reach their maximum germination level. There is a decrease in the time required for seeds to reach maximum germination with H_2O_2 treatment. The greatest improvement in rate of germination was seen in 60 mM H_2O_2 imbibed seed, reaching maximum germination approximately 6 days sooner on average than 0 mM imbibed seed under salt stress. This may be of importance for establishment of seeds in field conditions when competition of weeds species and unpredictable weather conditions are of concern.

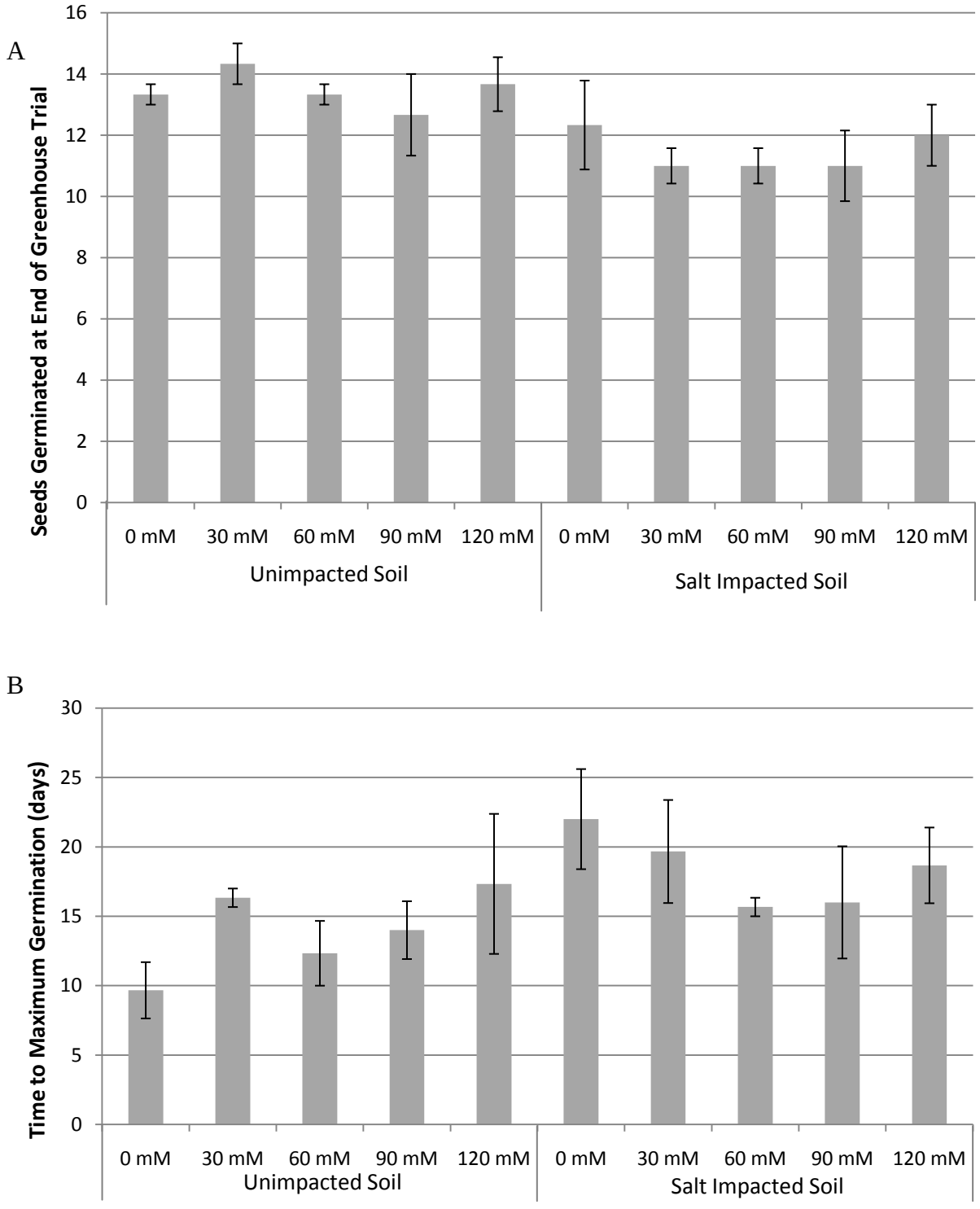


Figure 3.3: A) Germination of peroxide imbibed seeds at 0, 30, 60, 90 and 120 mM H_2O_2 in a greenhouse germination assay with clean and salt impacted (EC= 4.5 dS/m) field site soils. B) Time in days for each trial to reach its maximum germination. Error bars show standard error. N=3.

3.1.3 Antioxidant levels in H_2O_2 imbibed seed

The level of radical quenching antioxidant was determined by colour change of DPPH solutions when mixed with an extract of treated seed. Lower absorbance at 520 nm in Figure 3.4 shows a greater presence of antioxidant by quenching of the DPPH radical, as shown by the absorbance of the ascorbic acid positive control samples. None of the treatments of H_2O_2 showed a significant change in absorbance compared to the RO H_2O imbibed control, suggesting that there was no discernable change in the level of antioxidants that target radicals with H_2O_2 treatment. This suggests that the mechanism by which H_2O_2 improves germination may not be due to consuming radical scavenging antioxidants, or radical scavenging analogues.

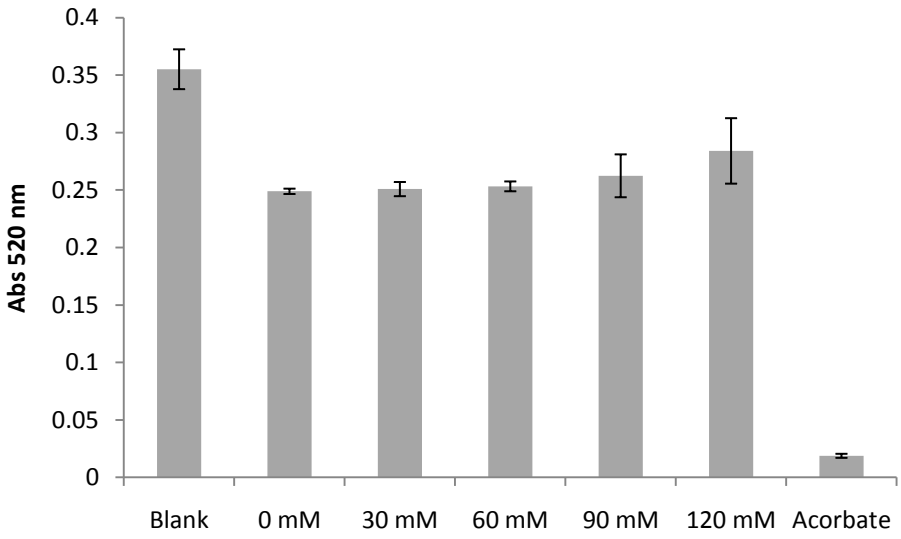


Figure 3.4: Antioxidant levels in H_2O_2 treated seeds. All treatments are not significantly different, but do show radical scavenging activity compared to the blank sample. Error bars show standard error. N=3.

3.2 Comparison of germination of switchgrass cultivars and western wheatgrass to tall wheatgrass and Inferno tall fescue

Salt tolerance, and the ability to produce high levels of biomass under saline conditions, is important for phytoremediation. A variety of plant species were sown under salt stress to compare their ability to germinate under saline conditions. These were western wheatgrass, common switchgrass from two suppliers (Agrecol and Ontario Seed Company (OSC)), Cave-In-Rock switchgrass, Southlow switchgrass, Forestburg switchgrass, Inferno tall fescue and tall wheatgrass. Seeds were sown in a 4x4 pattern in 2”x2” square pots for a greenhouse germination assay, with either control Kindersley soil and 4 dS/m Kindersley salt impacted soil. Each species was planted in 4 separate cells, and the emergence of shoots above the soil surface was monitored daily. The maximum emergence of shoots, defined at the number of shoots emerged after a 21 day time period, is shown in Figure 3.5A. It can be seen that Inferno tall fescue and tall wheatgrass have the highest level of emergence at the end of the 21 day time period, for both control and salt impacted soils. The time to reach maximum germination was also measured (Figure 3.5B). It can be seen that the time for all seed types to reach their maximum germination level is statistically equivalent under saline conditions when compared to the non-saline control.

A petri plate germination assay with tall wheatgrass, common switchgrass (Ontario Seed Company), western wheatgrass, and Cave-in-Rock switchgrass was performed (Figure 3.6). As in the greenhouse germination assay, western wheatgrass did not germinate during the entire assay time period, and tall wheatgrass showed the highest rate of germination by the end of the assay time period whether under salt stress or under no stress. The germination of tall wheatgrass and common switch grass at increasing salinities is shown in Figure 3.7 and Figure 3.8. This assay was carried out to determine if there is a point at which switchgrass is able to maintain

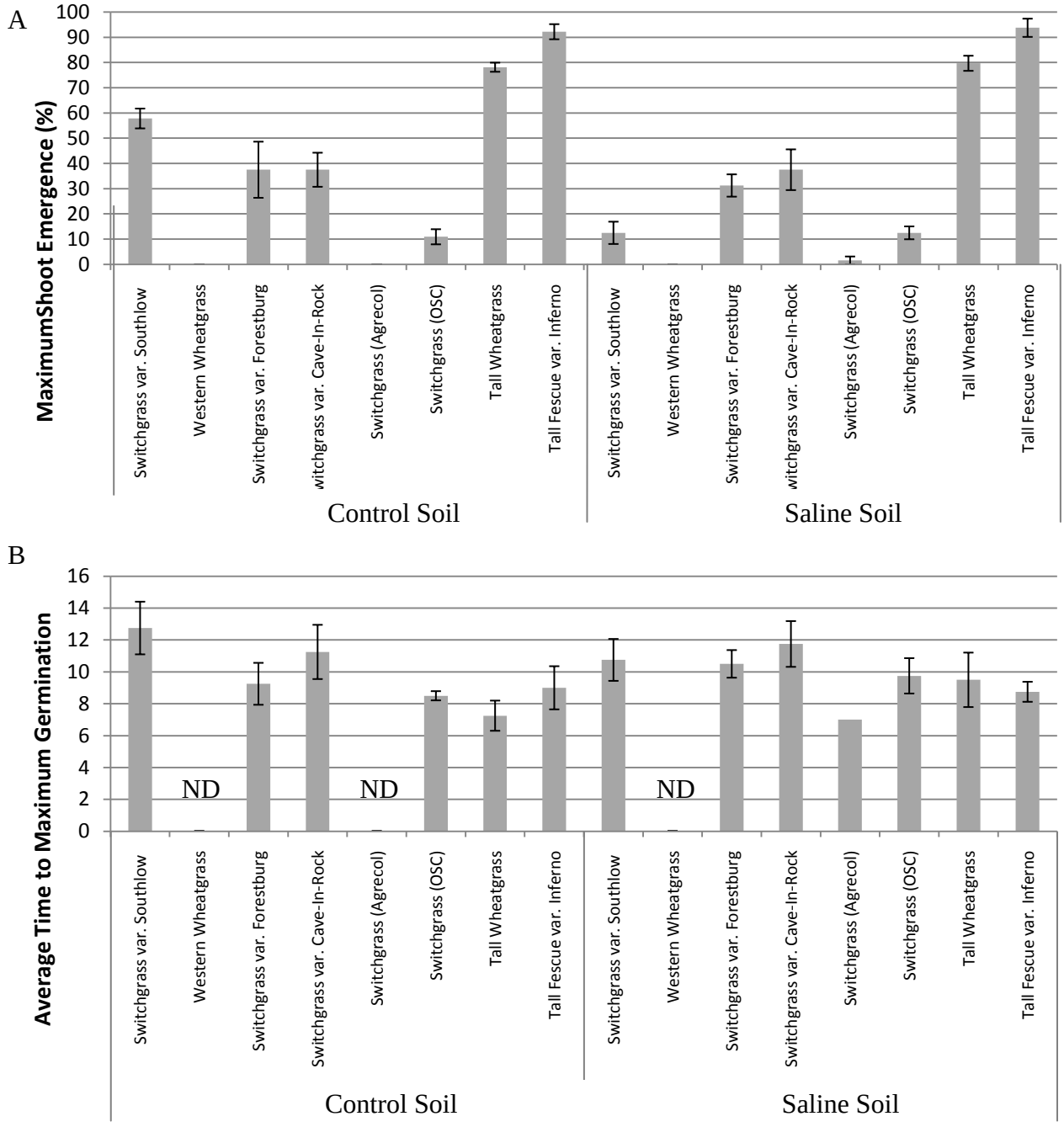
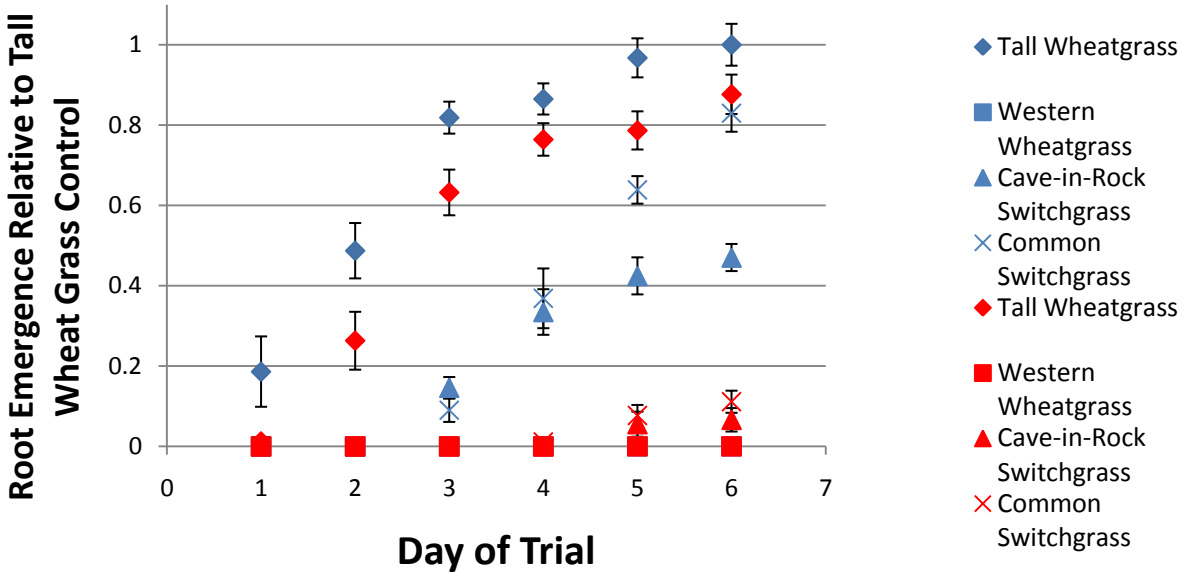


Figure 3.5: A) Maximum percent germination of various species under control and saline conditions at the end of a 4 week greenhouse trial. B) The average time for pots of seed to reach maximum germination. Error bars for both A and B are standard error. Common switchgrass (Agrecol) had only one pot germinate in saline soil and and so has no error bar for graph B. No germination was measured for Switchgrass (Agrecol) in control soil so the time to maximum germination was not determined. Western wheatgrass did not germinate so the time to maximum germination was not determined. Error bars are standard error. N = 3.

A



B

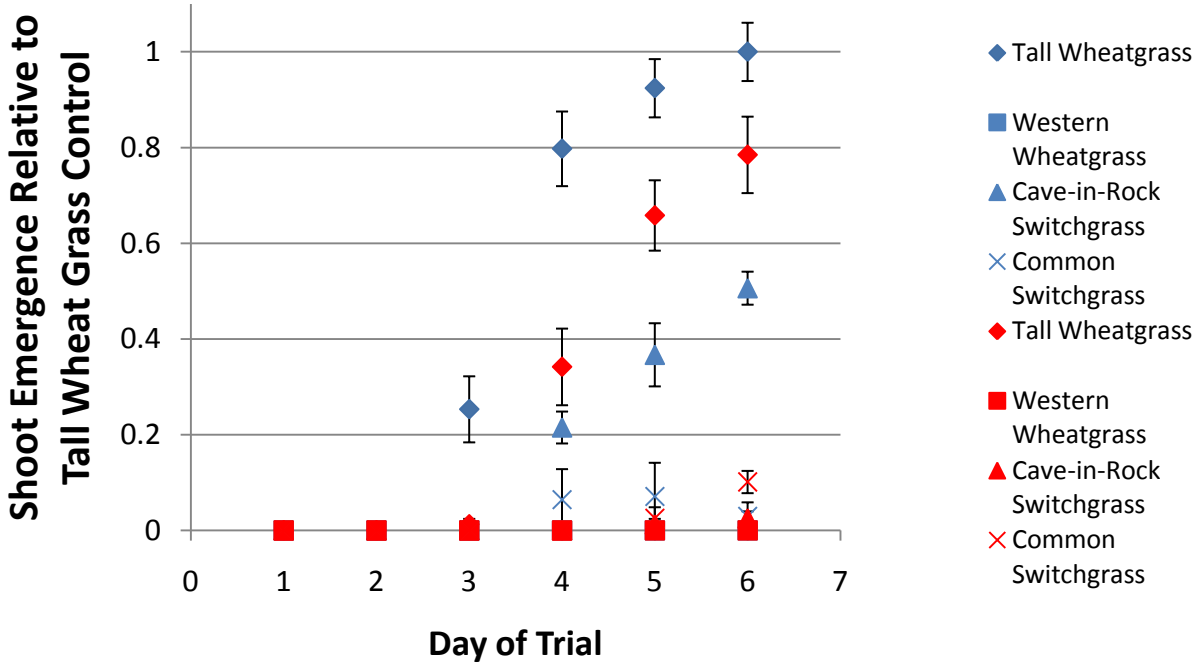


Figure 3.6: Root (A) and shoot (B) emergence of various species under control conditions and 75 mM NaCl salt stress during a petri plate germination assay. Blue points show control samples and red points show salt stressed samples. Error bars are standard error. N=3.

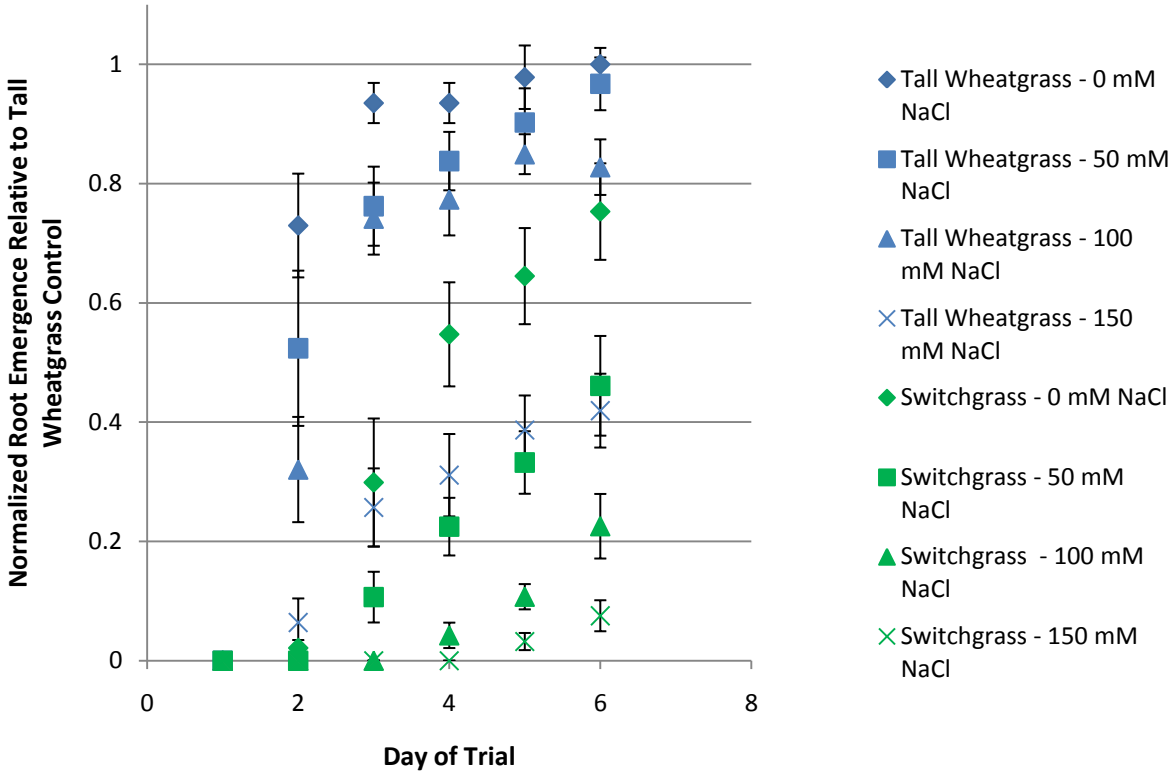


Figure 3.7: Root emergence of tall wheat grass and switchgrass at various salt concentrations in petri plate germination assay. Error bars are standard error. N=3.

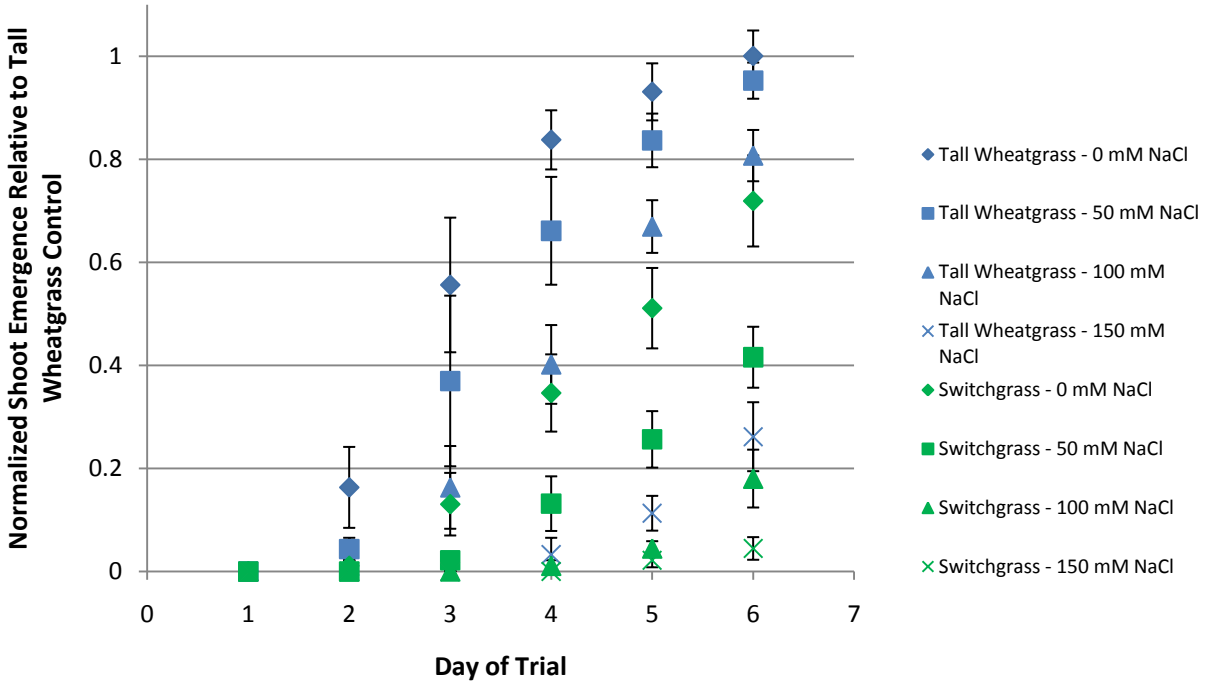


Figure 3.8: Shoot emergence of tall wheat grass and switchgrass at various salt concentrations in petri plate germination assay. Error bars are standard error. N=3.

germination to match tall wheatgrass. It can be seen, however, that Inferno tall fescue and tall wheatgrass perform better in all of the experiments, under all conditions, and so they may be better options for phytoremediation applications.

3.3 Field trials of phytoremediation with PGPR on salt impacted soils

Field trials were undertaken to test the applicability of PGPR enhanced phytoremediation. Field soils were sampled for salt analysis at planting, midway through the growing season and at the end of the field season. In addition to assessment of plant growth and changes in salinity, the vertical movement of salt during a field season and uptake of salt into plant tissue was examined.

3.3.1 Kindersley field site

3.3.1.1 Initial site salinity and k value determination

Soil samples for determination of electrical conductivity were taken prior to planting and application of fertilizer on the field. $EC_{1:2}$ and EC_e values of 5 soil samples were measured and compared, and an average k value of 3.3 was determined for the field site (Table 3.1). This value was used to convert all $EC_{1:2}$ values to EC_e for the 2008 and 2009 field season. The initial salinity of the field site was determined to be an average of 5.6 dS/m, with a wide range from 0.33 dS/m to 16.09 dS/m, making the site moderately saline.

3.3.1.2 Biomass production in Kindersley

Plots were planted with either – PGPR, CMH3 or UW3/4 treated seeds in a repeating block pattern Figure 2.2. Samples of plant biomass were taken from 0.25 m^2 squares from randomly determined points and the dry mass was measured. Plant production on the Kindersley field site in the test plots showed increased biomass production in PGPR treated plots (CMH3 and UW3/UW4) compared to –PGPR plots in 2008 (Figure 3.9). There was also high

Table 3.1: Measured EC_e and $EC_{1:2}$ values for Kindersley field site used to determine an average k-value. Error shown is standard error.

Sample Point	EC_e	$EC_{1:2}$	k-value ($EC_e/EC_{1:2}$)
1-A	0.94 dS/m	0.28 dS/m	3.3
1-C	4.38 dS/m	1.36 dS/m	3.2
2-C	2.19 dS/m	0.69 dS/m	3.1
8-C	4.67 dS/m	1.21 dS/m	3.8
11-C	1.31 dS/m	0.38 dS/m	3.3
		Average	3.3 ± 0.12

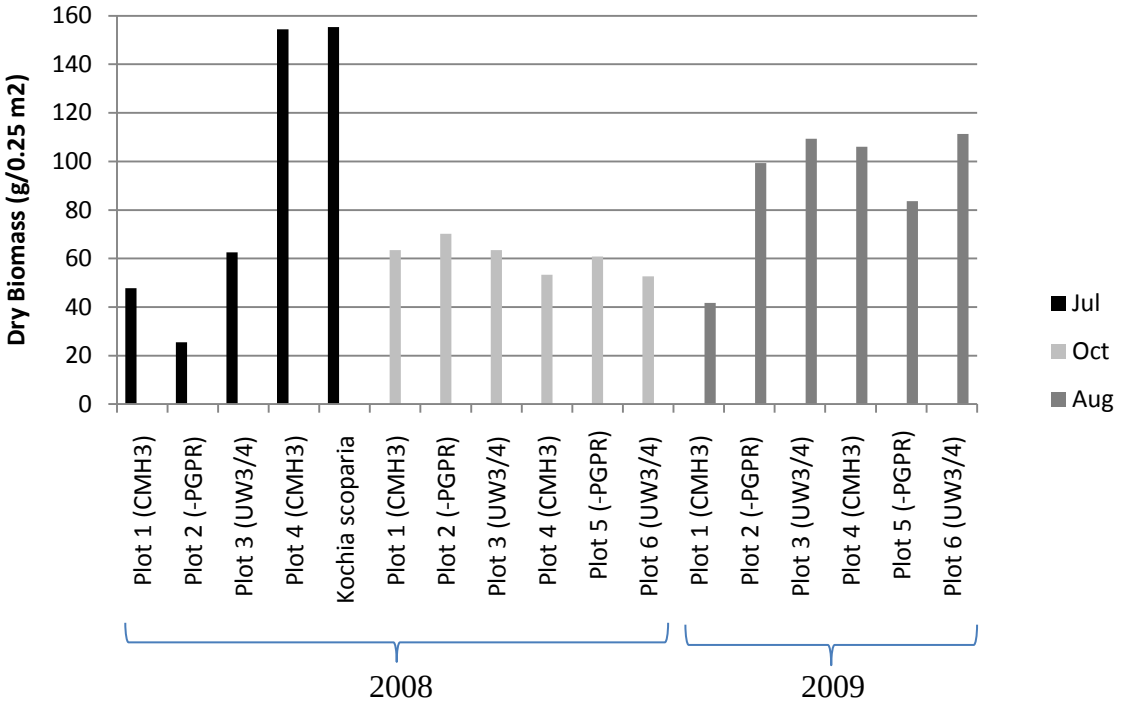


Figure 3.9: Biomass production in 2008 and 2009 at the Kindersley, SK field site. No plant material was sampled at the end of the planting season 2009. This was due to poor growth conditions and a lack of watering. All samples are a composite of three 0.25 m^2 samples. Plants sampled for the midseason time point in 2008 were combined by plot, and not by individual sub-point, so statistical analysis could not be done.

biomass production by a weed species (*Kochia scoparia*) at midseason (2 months after planting), present in unplanted areas surrounding the test plots. After this sampling time point the field was mowed to a height of 6-8 inches to remove plant material containing salt and prevent the establishment and spread *Kochia*. After 3 months, at the end of the growing season there was no discernable difference between CMH3, UW3/4 and –PGPR treated plants (Figure 3.9).

During the 2009 field season, plant growth on the plots did not follow the same trends seen in 2008, likely due to an invasion of weeds after planting. The field was overrun by *Kochia* weeds which had grown between the plots in 2008. Potential reasons for this will be discussed in the next paragraph. After midseason in 2009, as in 2008, the field was mowed to approximately 6-8 inches above the ground. At the end of season, no new plant growth was observed above this cut level, and so no plant sampling was done at the end of season. The reason for this lack of new plant growth after midseason is due to drought in the later half of the field season.

The difference in the establishment of plants on the field between 2008 and 2009 may be weather related. The weather data from Environment Canada for 2008 and 2009 are shown in Figure 3.10 and Figure 3.11 respectively. In the 2009 field season the site experienced a hard frost 4 days after planting. The timing of this frost may have been such that the young, germinating plants were killed by the frost, allowing *Kochia* to establish on the plots. A decrease in rainfall after midseason in 2009 was also observed compared to 2008, and the 2009 total rainfall during the field season (159 mm) was less than the average rainfall from 1985 to 2009 (199 mm) for the same time period and 198 mm in 2008. This decrease in rainfall in conjunction with a lack of watering due to reported rainfall levels from personnel in the region of the field site likely lead to the lack of plant growth between midseason and end of season.

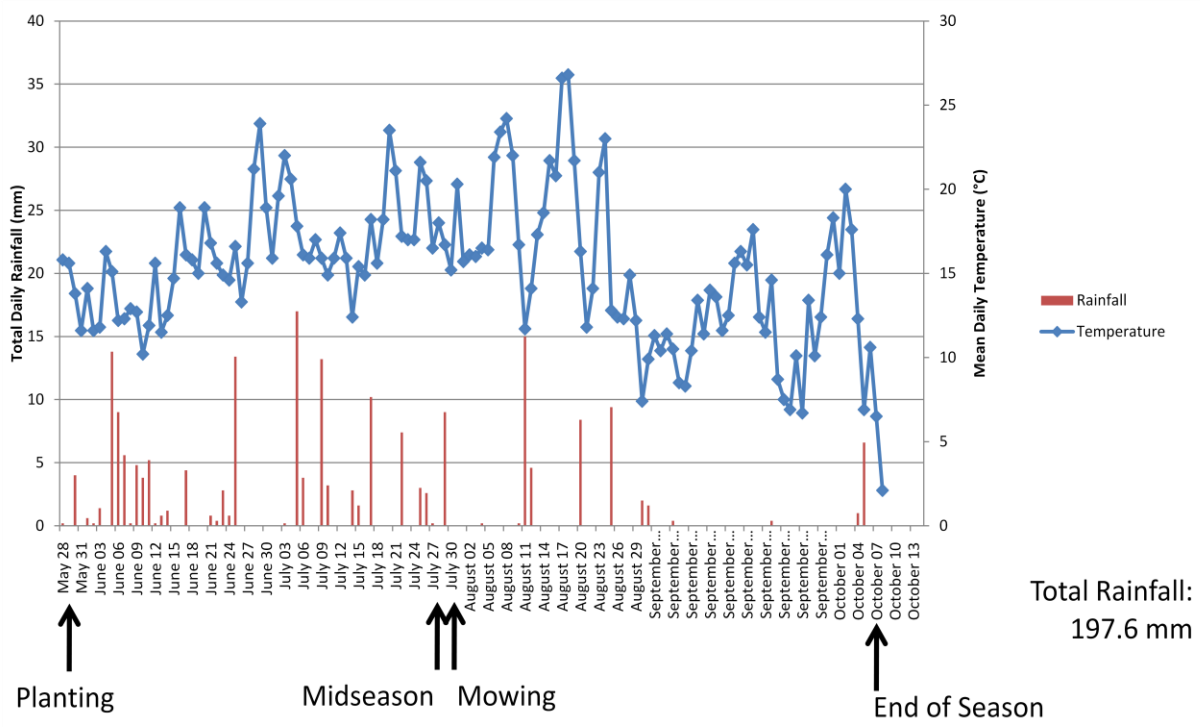


Figure 3.10: 2008 weather data for Kindersley, SK. In addition to the recorded rainfall, 1000 m^3 of water was applied to the field three times during the planting season. Planting, midseason sampling, end of season sampling and midseason mowing dates are shown.

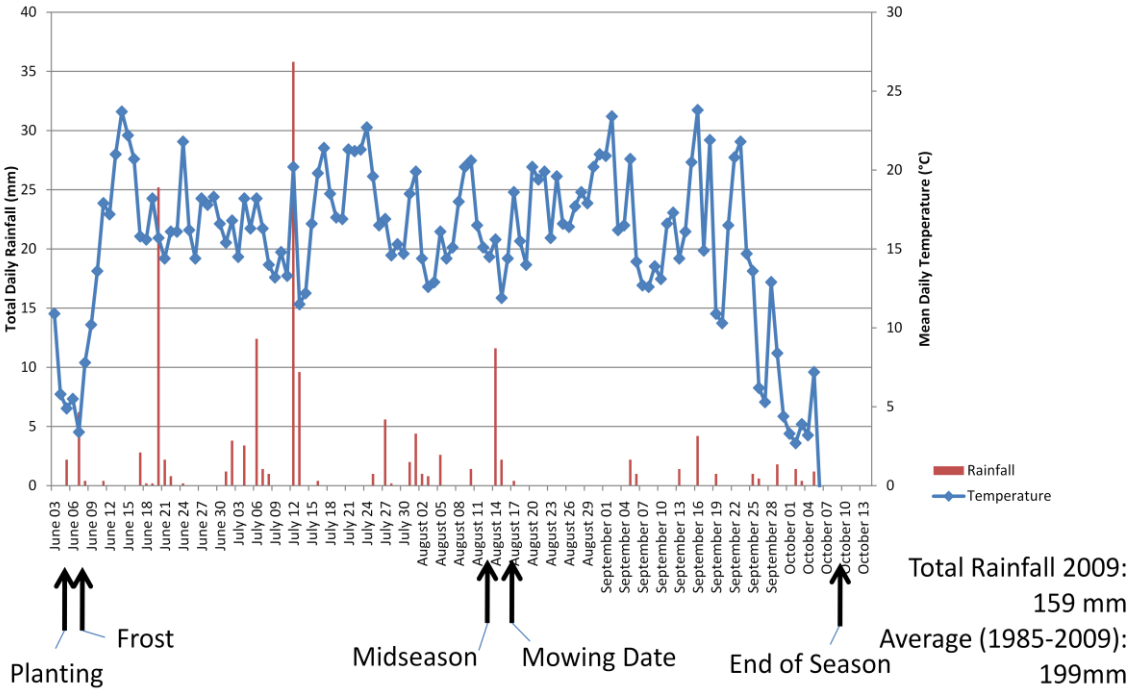


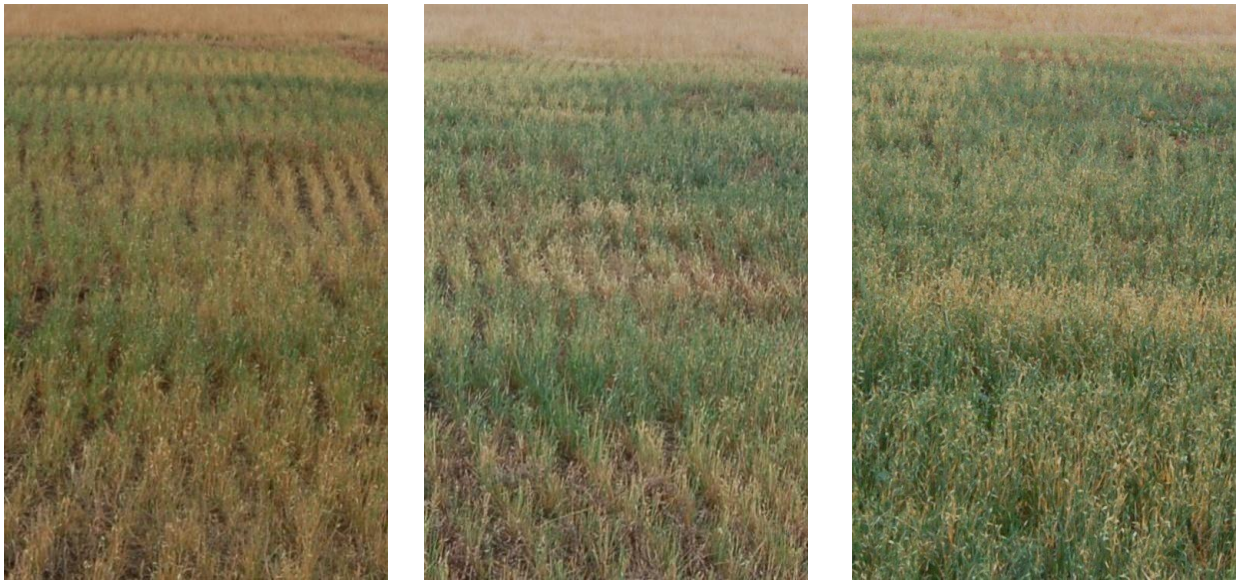
Figure 3.11: 2009 weather data for Kindersley, SK. A hard frost was observed shortly after planting, which may have had a large impact on plant growth during the remainder of the season. A lack of rainfall over the entire season is observed, in contrast to reports from the field that additional water was not needed for the plants, and so none was added. The lack of rainfall after midseason mowing may explain why no growth was observed between midseason and end of season sampling.

Photographs of plant growth in 2008 and 2009 are shown in Figure 3.12 and Figure 3.13 respectively. The difference in growth between 2008 and 2009 field seasons can be seen, with 2008 photos showing the distinct rows of planted species created by the seeder, and 2009 showing the unorganized growth of weeds. The change in growth between midseason and end of season in 2008 and 2009 can also be observed. In both years, the field was mowed after the midseason sampling to the same height, and the lack of growth after mowing in 2009 is visible in this comparison. This may be attributed to the lack of rainfall observed in the 2009 and lack of watering in 2009, compared to the additional watering of 1000 L three times during the 2008 growing season. It may also be due to the response of *Kochia* to mowing as it appeared to have started producing seed. In both years the planted area showed 100% plant coverage, so salt remediation is expected regardless of the growth of weeds versus the growth of planted oats and grasses.

A

CMH3 -PGPR UW3/UW4

B

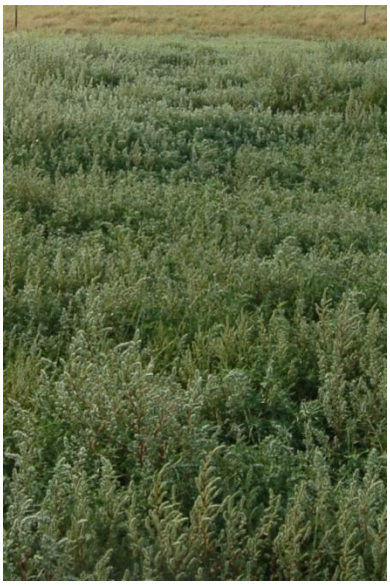


CMH3 -PGPR UW3/UW4

Figure 3.12: Kindersley 2008 plant growth photographs. A) Plant growth at the midseason sampling point. B) Plant growth at the end of season sampling point. The difference in green colour between the CMH3, –PGPR and UW3/UW4 treatments is an artefact of photography in changing light conditions. This was determined by comparison of colours in adjacent plots in uncropped photos.

A

-PGPR



CMH3

UW3/UW4

B

CMH3

-PGPR

UW3/UW4

Figure 3.13: Kindersley 2009 plant growth photographs. A) Plant growth at the midseason sampling point. B) Plant growth at the end of season sampling point. The difference in green colour between the CMH3, –PGPR and UW3/UW4 treatments is an artefact of photography in changing light conditions. This was determined by comparison of colours in adjacent plots in uncropped photos.

A

B

Figure 3.14: A) Example of plant growth in 2008 B) Example of plant growth in 2009

3.3.1.3 PAM fluorometery of field site plant tissue

Tolerance of salinity has been correlated to the ability to maintain photosynthetic activity (Belkhodja, Morales, Abadia, Gomez-Aparisi, & Abadia, 1994). In 2009, the photosynthetic capabilities of tall wheatgrass in plots A-D, and 1-3 and *Kochia* were tested using PAM fluorometery. Example PAM traces for Kindersley plots and Kochia are shown in Figure 3.15 and Figure 3.16. The average values for F_v/F_M, Yield, qP (photochemical quenching) and qN (non-photochemical quenching) were determined (Table 3.2). An unstressed plant will typically have a F_v/F_M value of 0.8 (Björkman & Demmig, 1987). Comparison of F_V/F_m values showed little variation between treatments, with all samples in the normal yield range of 0.8. Values for qP were slightly more variable, with an increased value for plots C (H_2O_2 +CMH3, qP=0.921), plot D (–PGPR, qP=0.910) and *Kochia* (0.950). Other plots were in the normal range of 0.8. The qN values were much more varied than the qP values, but most were in the normal range of less than 0.6 with the exception of plot 3 (UW3/4) which was higher than all other samples. The similarity of PAM values among the plots of varying treatments on the Kindersley field site is expected, as little visual difference was observed between plots. Previous work (Wu, 2009) with salt tolerant species used in our phytoremediation trials observed that stressed plants tend to show more difference in PAM fluorometery when a visual stress on the plant is observed. As shown in Figure 3.12 and Figure 3.13, there was no visible difference in plant health between plots.

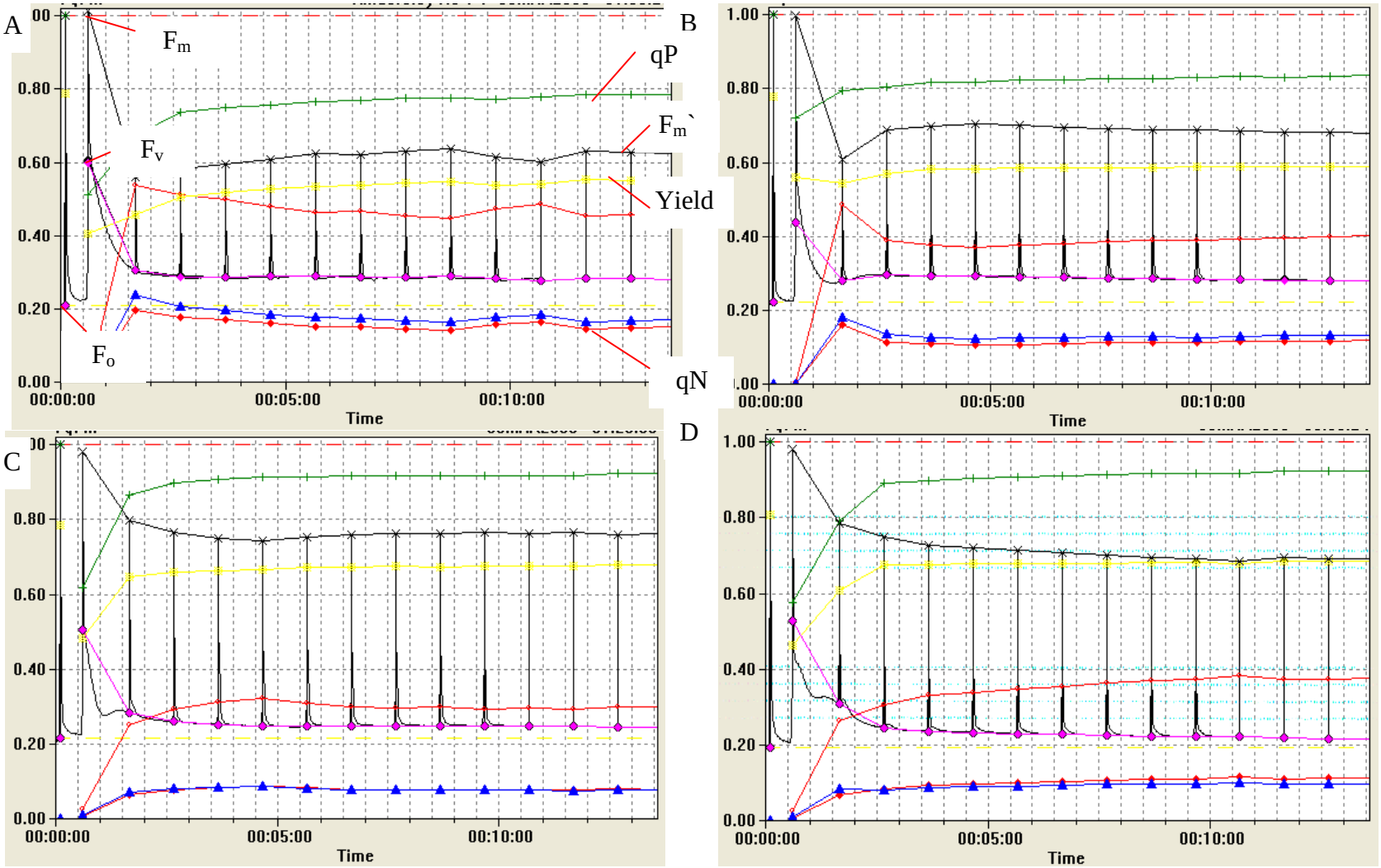


Figure 3.15: PAM traces of A) Plot A, H_2O_2 + CMH3 B) Plot B, H_2O_2 - PGPR C) Plot C, H_2O_2 + CMH3 D) Plot D, No treatment

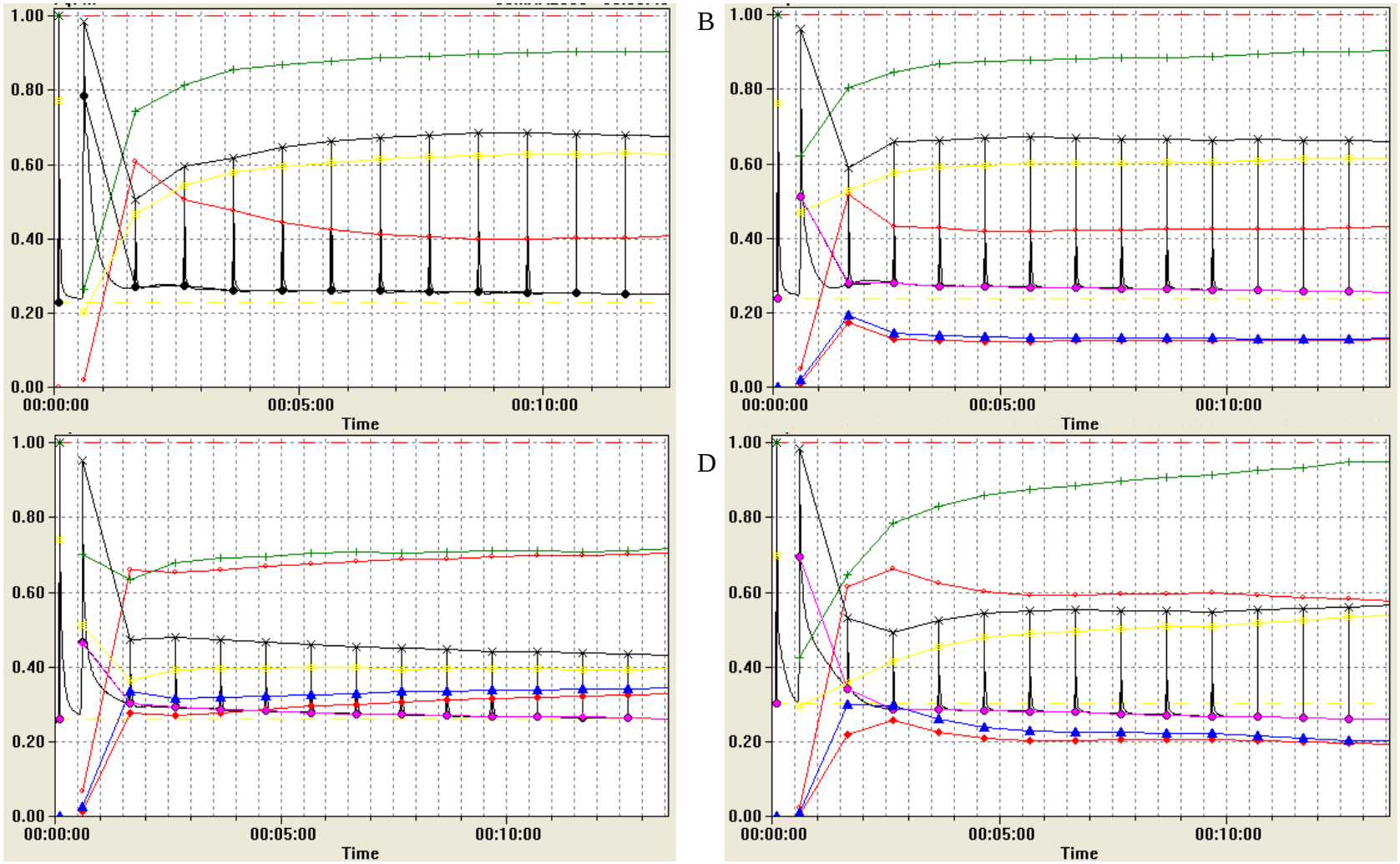


Figure 3.16: PAM traces of A) Plot 1, CMH3 B) Plot 2, -PGPR C) Plot 3, UW3/4 D) Kochia, weed species with no PGPR treatmen

Table 3.2: PAM fluorometery data from 2009 field samples from August 2009 and salinity data.

Plot (Treatment)	F_v/F_M	Yield	qP	qN	Salinity (dS/m)
Kin 1 (CMH3)	0.732	0.383	0.789	0.600	4.26
Kin 2 (No Treatment)	0.710	0.458	0.795	0.597	3.69
Kin 3 (UW3/4)	0.741	0.426	0.780	0.715	4.76
Kin A (H_2O_2 + CMH3)	0.794	0.551	0.778	0.452	2.58
Kin B (H_2O_2 – PGPR)	0.781	0.595	0.854	0.439	4.92
Kin C (H_2O_2 + CMH3)	0.787	0.681	0.921	0.290	2.80
Kin D (No Treatment)	0.800	0.670	0.910	0.370	3.14
Kochia (No Treatment)	0.697	0.537	0.950	0.576	4.76

3.3.1.4 Remediation of salt contamination in the rooting zone of the Kindersley field site

An overview of EC_e values for the entire 2008 field season is given in Figure 3.17, The samples taken at the end of May, the end of July, and the beginning of October, and shows a decrease at midseason followed by an increase between midseason and end of season. There was an overall decrease of 0.91 dS/m during the growth season. The salinity of surface soils during the 2009 field season decreased between planting and midseason followed by an increase between midseason to end of season (Figure 3.18). The overall decrease in soil salinity during the 2009 field season was only 0.45 dS/m, approximately half of the 2008 field season salinity change. This decrease in remediation during the 2009 field season compared to the 2008 field season may due to decreased biomass production. As discussed above, the factors that may have impacted plant growth during the 2009 field season include a frost soon after planting and drought during the growing season. The average decrease in salinity for plots with various PGPR treatments in the 2008 and 2009 field season is shown in Figure 3.19. In both 2008 and 2009, CMH3 planted plots show the greatest decrease in salinity, and UW3/UW4 plant plots show a consistent decrease in salinity over the two years of the field trial. The –PGPR planted plots showed an overall decrease in salinity in 2008, and a small average increase in salinity in 2009.

1	2	3	4	5	6	7	8	9	10	11	12
4.61 3.17 3.10	2.36 2.00 3.16	3.22 2.95 2.44	3.58 3.44 3.03	4.80 3.05 3.16	4.08 2.75 4.86	3.00 1.42 2.09	4.09 3.43 5.44	0.33 1.32 2.04	4.31 3.51 4.95	1.31 3.81 4.29	6.07 4.86 5.68
3.90 2.31 2.72	10.37 5.16 5.83	9.69 5.46 5.55	6.07 5.60 6.30	8.33 6.29 4.66	7.47 4.53 5.12	10.14 5.55 5.28	4.80 4.04 5.08	6.63 3.61 5.40	8.16 6.11 5.94	7.49 4.74 6.25	4.48 5.00 3.82
0.96 1.54 0.97	4.68 2.46 4.28	5.70 5.84 6.97	16.09 8.30 9.11	8.96 5.53 5.14	8.97 4.95 11.11	0.67 1.29 0.62	14.40 8.37 9.93	7.50 5.07 6.14	1.36 2.57 2.29	1.10 1.31 2.86	2.05 1.85 3.31
3.16 2.34 2.26	5.80 3.21 4.42	6.20 4.75 4.99	8.58 5.78 6.15	7.36 4.96 4.32	6.84 4.08 7.03	4.61 2.75 2.66	7.76 5.28 6.82	4.82 3.33 4.53	4.61 4.06 4.39	3.30 3.29 4.47	4.20 3.90 4.27

May 5.6 dS/m ± 0.41
July 3.98 dS/m ± 0.28
October 4.69 dS/m ± 0.38

Figure 3.17: EC_e of Kindersley field site during the 2008 field season. Salinities are reported in dS/m, and represent the salinity of a composite of 3 points, sampled to a depth of 25 cm. Samples were taken using a Dutch hand auger (3" diameter x 10" length). Values shown for the entire field are averages of all plot values with errors shown in standard error (N=12). Plot values are the average of the three composite points.

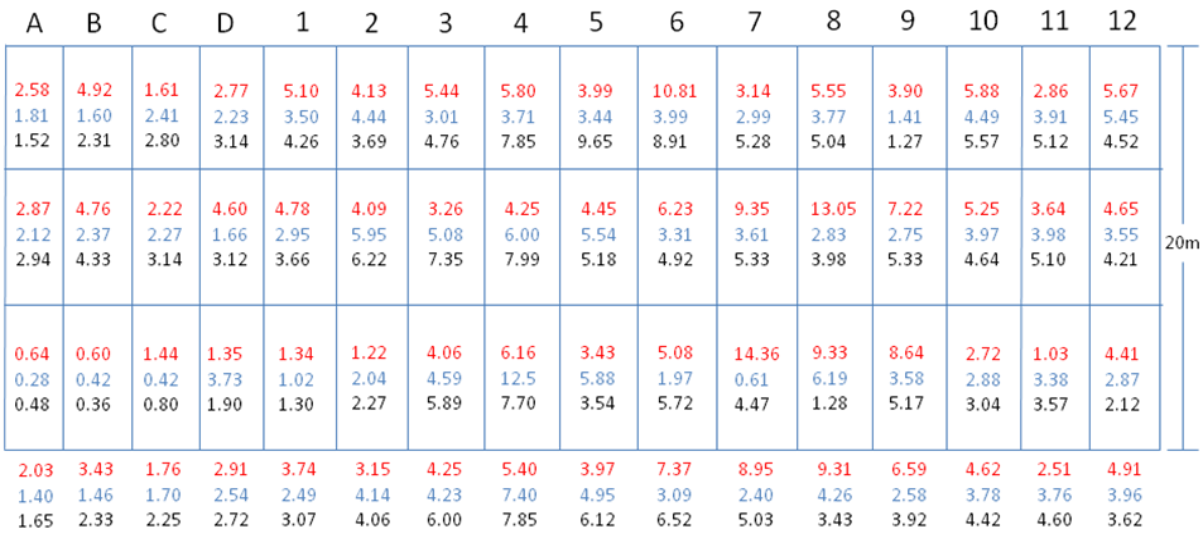


Figure 3.18: EC_e of Kindersley field site during the 2009 field season. Salinities are reported in dS/m, and represent the salinity of a composite of 3 points, sampled to a depth of 25 cm. Samples were taken using a Dutch hand auger (3" diameter x 10" length). Values shown for the entire field are averages of all plot values with errors shown in standard error (N=12). Plot values are the average of the three composite points.

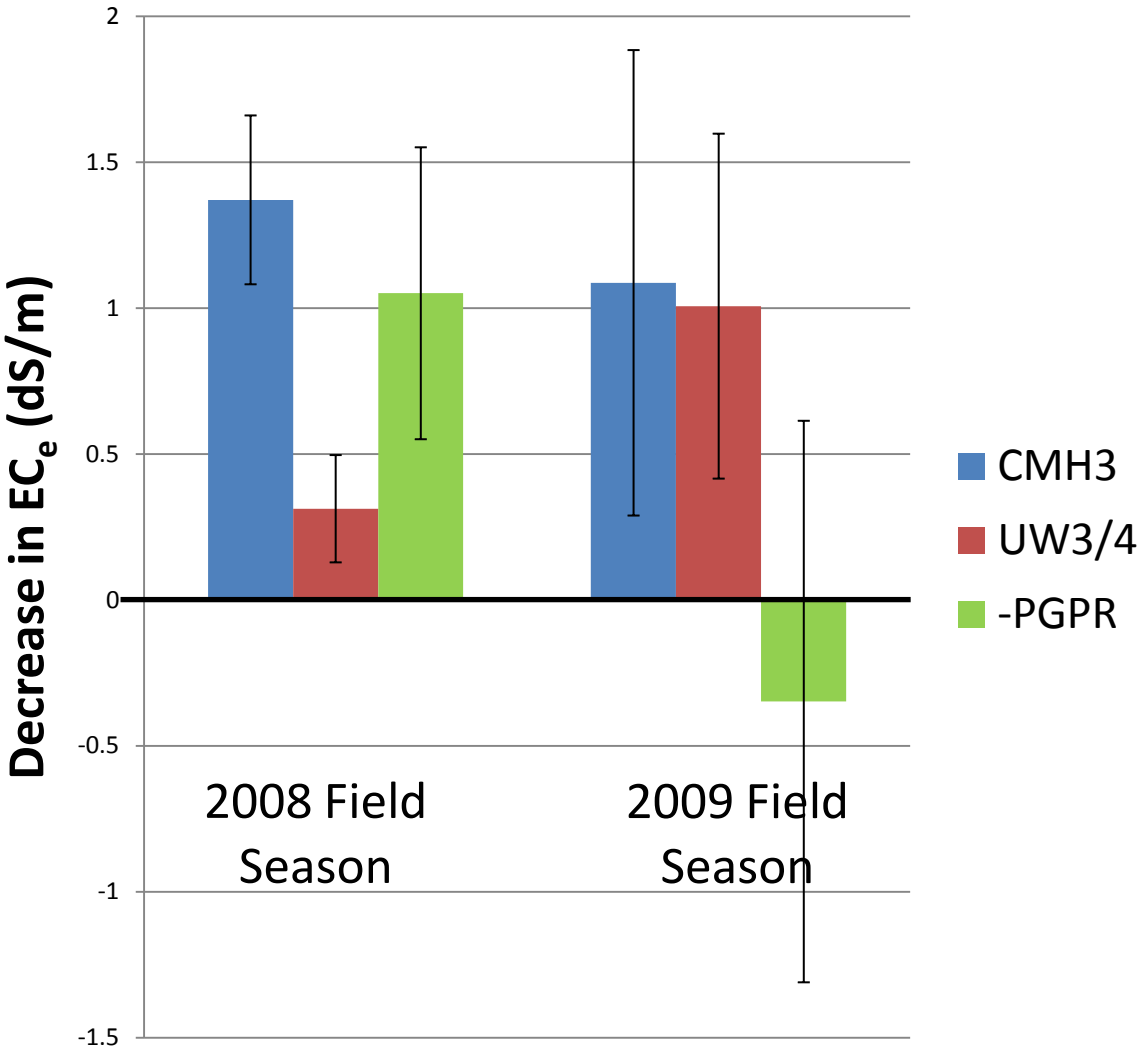
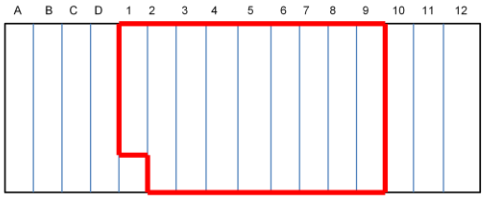


Figure 3.19: Change in EC_e of Kindersley field site soils. A decrease was seen in all averages except –PGPR plots in 2009, where an increase in salinity was seen between the beginning and end of the field season. N=4. Error bars are standard error.

3.3.1.5 Soil salinity below the rooting zone

During the 2009 field season, soil samples were taken in plots 1 through 9 to a depth of 1 m at planting and end of season sampling. Three depths of 0-30 cm, 30-60 cm, and 60-100 cm were sampled for salinity at beginning and end of season to assess if there is vertical movement of salt in the soil. The salinity data at these three depths at the beginning and end of season are provided in Figure 3.20. The average salinity for 0-30 cm and 30-60 cm decreased between the beginning and end of season from 6.03 dS/m to 5.25 dS/m and 6.41 dS/m to 5.76 dS/m, respectively. An increase in salinity was observed at the 60-100 cm depth from 6.15 dS/m to 6.59 dS/m. In the sampled area, 50% of the sample points increased in salinity over the course of the field season, 46% of the sample points decreased in salinity, while 4% remained the same (defined as a less than 10% change in salinity).

	1		2		3		4		5		6		7		8		9	
C	5.10	4.26	4.13	3.69	5.44	4.79	5.80	7.85	3.99	9.65	10.81	8.91	3.14	5.28	5.55	5.04	3.90	1.27
	4.00	4.17	3.16	6.41	4.75	3.32	4.55	4.62	3.03	5.44	12.09	10.49	1.84	6.71	14.04	6.07	13.56	1.51
	3.18	3.13	2.67	4.38	4.45	3.60	5.31	3.93	3.19	4.39	12.64	2.84	9.54	12.12	14.63	17.39	13.94	2.62
B	4.78	3.66	4.09	6.23	3.26	7.35	4.25	7.99	4.45	5.18	6.23	4.92	9.35	5.33	13.05	3.98	7.22	5.33
	4.81	4.23	3.75	5.06	3.55	5.89	4.38	8.55	4.22	5.35	5.74	4.02	9.67	13.27	12.34	5.72	9.68	7.22
	4.06	5.58	3.52	4.28	3.57	3.46	4.02	5.71	3.93	5.11	5.57	2.50	6.99	7.78	9.00	14.76	9.46	7.44
A			1.22	2.27	4.06	5.89	6.16	7.70	3.43	3.54	5.08	5.72	14.36	4.46	9.33	1.23	8.64	5.17
			2.05	1.57	3.15	5.91	4.58	6.21	3.38	2.99	3.11	5.77	9.72	4.39	11.19	1.86	10.18	12.90
			3.19	2.49	2.94	5.28	4.96	4.69	2.85	3.92	2.89	5.55	5.00	13.52	5.00	10.29	13.51	14.70
	4.94	3.96	3.15	4.06	4.25	6.01	5.40	7.85	3.96	6.12	7.37	6.52	8.95	5.02	9.31	3.42	6.59	3.92
	4.41	4.20	2.99	4.35	3.82	5.04	4.50	6.46	3.54	4.59	6.98	6.76	7.08	8.12	12.52	4.55	11.14	7.21
	3.62	4.36	3.13	3.72	3.65	4.11	4.76	4.11	3.32	4.47	7.03	3.63	7.18	11.14	9.54	14.15	12.30	8.25



2009 Field Data	t_0	t_{126}
0-30cm	6.03 dS/m	5.25 dS/m
30-60cm	6.41 dS/m	5.76 dS/m
60-100cm	6.15 dS/m	6.59 dS/m

Figure 3.20: Salinity at the beginning and end of 2009 field season, sampled at 0-30 cm, 30-60 cm, and 60-100 cm. Each sample point is a composite of two bore hole, taken with the use of a truck mounted drill. Salinities in red show results from the start of the field season, and black shows the end of season salinity. The listed salinities are ordered by depth, with 0-30 cm shown first, then 30-60 cm and 60-100 cm third in the list.

3.3.1.6 Salt mass balance by ion uptake into plant tissue from Kindersley field site

According to Alva *et al*, 1991, electrical conductivity can be related to ionic strength by the following equation

$$IS = 0.013 \times EC \qquad \text{Equation 4}$$

In this equation, the ionic strength (IS) expressed in terms of mol/L and is the sum of the contributions of all ions, as shown in the following equation

$$\text{IS} = \sum_{i=1}^{n} c_i z_i^2 \qquad \text{Equation 5}$$

In Equation 5, *c* is the concentration of the ion in mol/L and *z* is the total charge of the ion. Monovalent ions like chloride and sodium will have a *z* value of 1, while divalent ions such as magnesium and sulphate will have a *z* value of 2. Due to *z* being squared, ions with a higher charge will have a much higher influence on the ionic strength, and therefore have a larger effect on the electrical conductivity of a solution than with monovalent ions.

All extractable ions in the soil will contribute to the conductivity of a soil extract, and thus all ions must be considered when examining the effect of phytoremediation on salt uptake and change in soil salinity. The concentrations of Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+} in Kindersley soil from 2008 are given in Table 3.3. These average values show that Na^+ and Cl^- are in lower concentration in soil when compared to calcium and magnesium. The change in salinity of the Kindersley field site in 2008 of 0.91 dS/m equates to a change of the ionic strength of 0.012 mol/L. Plant material from the Kindersley field site was analyzed by ALS Laboratories Inc. in 2008 for the concentration of various ions. The results of these analyses are shown in Table 3.4,

Table 3.3: Average amount of ions in Kindersley field site soil in terms of mass and concentration. Errors shown are standard error, n=3.

Ion Species	Mass in Soil (mg ion / kg soil)	Concentration in Soil (mmol ion/kg soil)
Ca^{2+}	2477 ± 465	61.8 ± 11.5
Cl^{-}	396 ± 207	11.2 ±5.8
K^{+}	1503 ± 249	38.4 ± 6.4
Mg^{2+}	3020 ± 122	124.3 ± 5.0
Na^{+}	1123 ± 344	49.0 ±14.9

Table 3.4: Uptake of various ions into four plant tissue samples from Kindersley field site, and $\Delta EC_{e\ predicted.}$ The average $\Delta EC_{e\ predicited}$ for the field site is shown, with the standard error.

Ion Species	Concentration in Plant (mg/kg dry weight)	Concentration in Plant (mmol/kg)	Total mmol in Plant Tissue for the Entire Field	Equivalent mmol/L in Soil Extract	Contribution to Ionic Strength	Theoretical $\Delta EC_{e\ predicted}$
Plant 1						
Calcium	4 450	111.0	100 759	0.100	0.400	
Chloride	15 000	423.1	383 978	0.381	0.381	
Magnesium	2 960	121.8	110 517	0.110	0.439	
Potassium	25 100	642.1	582 693	0.578	0.578	
Sodium	3 810	165.7	150 390	0.149	0.149	
				Ionic Strength	1.947	0.49 dS/m
Plant 2						
Calcium	6 950	173.4	157 366	0.156	0.624	
Chloride	15 000	423.1	383 978	0.381	0.381	
Magnesium	3 960	162.9	147 853	0.147	0.587	
Potassium	32 900	841.6	763 769	0.758	0.758	
Sodium	2 470	107.4	97 497	0.097	0.097	
				Ionic Strength	2.447	0.62 dS/m
Plant 3						
Calcium	6 250	155.9	141 516	0.140	0.562	
Chloride	24 600	693.9	629 724	0.625	0.625	
Magnesium	5 390	221.8	201 245	0.200	0.799	
Potassium	43 200	1105.1	1 002 882	0.995	0.995	
Sodium	12 400	539.4	489 457	0.486	0.486	
				Ionic Strength	3.465	0.88 dS/m
Plant 4						
Calcium	2 480	61.9	56 154	0.056	0.223	
Chloride	18 500	521.9	473 573	0.470	0.470	
Magnesium	3 240	133.3	120 971	0.120	0.480	
Potassium	45 000	1151.2	1 044 669	1.036	1.036	
Sodium	8 880	386.3	350 514	0.348	0.348	
				Ionic Strength	2.557	0.65 dS/m
					0.66 dS/m ±0.08	
				Average $\Delta EC_{e\ predicted}$		

and the equivalent concentrations of ions in mmol/kg plant biomass (dry weight) were calculated from these data. In 2008, an average biomass of dry weight of foliage of 72.43 g/0.25 m^2 was produced between planting and midseason and an additional 62.61 g/0.25 m^2 between midseason and the end of season, giving a total annual biomass of 135.04 g/0.25 m^2. Using the extrapolated total biomass production for the field, mmol of each ion taken up by plants from the entire field were calculated by multiplying the total biomass per square meter times the field area times the concentration of ions within plant tissue. Using chloride data from plant 1 as an example (Table 3.4), the mmol Cl^- taken up by the plant is given in equations 6 to 8.

mmol Cl^- = Biomass per Area x Area of Field x mmol of ion in Biomass — Equation 6

mmol Cl^- = 0.54016 kg/ m^2 x 1680 m^2 x 423.1 mmol/kg — Equation 7

mmol Cl^- = 383950.0 mmol — Equation 8

It can be assumed that the ions taken up by the plants are in the rooting zone, defined as the top 30 cm of the entire field. The volume of this soil was calculated to be 504 m^3 (1680 m^2 area x 0.30 m^2 depth) which is equal to 5.04 x 10^8 mL. The soil has a high organic content giving a lower than average density of approximately 1 g/mL, which means the rooting zone has approximately 5.04 x 10^8 g of soil. To determine the effect of ions taken up into plant tissue, their effect on the salinity of the rooting zone must be determined. $EC_{1:2}$ measurements use a ratio of 30 mL H_2O to 15 g soil, and the salinity of the soil extract is measured. This ratio equates to 1.008 x 10^6 L of water for the salinity testing of the entire rooting zone. This value was used to calculate the concentration of ions in mmol/L in Table 3.4. Using the example calculation of chloride in plant 1 (Table 3.4), the calculations are shown in equations 9 to 11.

Equivalent mmol/L in Soil = mmol in plant tissue / Water volume for extraction Equation 9

Equivalent mmol/L in Soil = 383950 mmol / 1.008 x 10^6 L Equation 10

Equivalent mmol/L in Soil = 0.381 mmol/L Equation 11

These values are used in Equation 5 to calculate the ionic strength that a solution with these ions would give. For plant sample 1, this would give the following predicted change in ionic strength

$$IS = \sum_{i=1}^{n} c_i z_i^2 \quad \text{Equation 5}$$

$$\Delta IS_{predicted} = (0.100 \times 2^2) + (0.381 \times 1^2) + (0.110 \times 2^2) + (0.558 \times 1^2) + (0.149 \times 1^2)$$

$$\Delta IS_{predicted} = 1.947 \text{ mmol/L}$$

The ionic strength is used to calculate a ΔEC using a rearranged Equation 4, with the units for ionic strength converted from mmol/L to mol/L.

$$\Delta EC_{1:2\ predicted} = \frac{IS}{0.013} \quad \text{Equation 4 (Rearranged)}$$

$$\Delta EC_{1:2\ predicted} = \frac{1.947 \text{ x } 10^{-3} \text{mol/L}}{0.013}$$

$$\Delta EC_{1:2\ predicted} = 0.148 \text{ dS/m}$$

This value can be then multiplied by the previously determined k value for the Kindersley site of 3.3 to find the equivalent change in EC_e based on the ion uptake of 0.49 dS/m based on plant sample 1. The calculated change in EC_e by this method by averaging all plant sample changes is 0.66 dS/m ± 0.08. This average calculated change in EC_e accounts for 72.5 % of the reported change in salinity in 2008 of 0.91 dS/m, with a range of 63.4% to 81.4% when the standard error is taken into account for all four plant samples (Table 3.4). Through these

calculations, the majority of the change in conductivity in soil can be accounted for using the ion uptake into plant tissue.

In 2009, only Na^+ and Cl^- concentrations in plant tissue were determined. The averaged values of sodium and chloride concentrations for 2008 and 2009 are shown in Table 3.5. This table also shows the levels of these ions in plant tissue taken up over the entire field using the same calculations for Table 3.4. As sodium and chloride are focused on when dealing with brine water salinity, the theoretical change in salinity based only on these two ions was also calculated for 2008 uptake data (Table 3.5). The same conversion between the $EC_{1:2}$ calculated value and the EC_e calculated value is carried out here as before with the k value of 3.3.

$$\Delta IS_{predicted} = (0.464 \times 1^2) + (0.270 \times 1^2) \quad \text{Equation 5}$$

$$\Delta IS_{predicted} = 0.734 \text{ mmol/L}$$

$$\Delta EC_{1:2\ predicted} = \frac{0.734 \text{ mmol/L}}{0.013} \quad \text{Equation 4 (Rearranged)}$$

$$\Delta EC_{1:2\ predicted} = 0.056 \text{ dS/m}$$

$$\Delta EC_{e\ predicted} = 0.186 \text{ dS/m}$$

If only Na^+ and Cl^- are taken into consideration, only 0.186 dS/m of the 0.91 dS/m salinity change is accounted for in the 2008 field data. This shows the impact of other ions on salinity in a field trial.

The same calculations were carried out using the 2009 field data, when most of the growth was not from planted grasses and cereals but instead was *Kochia*. The values for these calculations are also taken from Table 3.5.

Table 3.5: Sodium and chloride uptake into plant tissue in 2008 and 2009 Kindersley field seasons

Field Season	Ion Species	Concentration in Plant Tissue (mg/kg)	Concentration in Plant (mmol/kg)	Total mmol in Plant over Entire Field	Equivalent mmol/L in Soil Extract
2008	Na^+	6890	299.7	271969	0.270
	Cl^-	18275	515.5	467782	0.464
2009	Na^+	2894	125.9	103646	0.103
	Cl^-	23414	660.4	543670	0.544

$$\Delta IS_{predicted} = \left(0.103 \times 1^2\right) + \left(0.544 \times 1^2\right) \quad \text{Equation 5}$$

$$\Delta IS_{predicted} = 0.647 \text{ mmol/L}$$

$$\Delta EC_{1:2 \text{ predicted}} = \frac{0.647 \text{ mmol/L}}{0.013} \quad \text{Equation 4 (Rearranged)}$$

$$\Delta EC_{1:2 \text{ predicted}} = 0.0498 \text{ dS/m}$$

$$\Delta EC_{e \text{ predicted}} = 0.164 \text{ dS/m}$$

This calculated change in salinity in 2009 accounts only for biomass produced between planting and midseason sampling, as there was no new growth after that point in the season. During this period of time, the salinity decreased by 1.29 dS/m, and the overall change between the beginning and end of season is 0.45 dS/m, 13% of the change at midseason, and 36% of the overall field season change.

In the previous paragraph, only Na^+ and Cl^- were used for 2009. However, by comparing the ratio of Na^+ and Cl^- to the other ions in 2008 (Table 3.4) an estimate for uptake of Ca^{2+}, Mg^{2+} and K^+ in 2009 can be made by assuming an equivalent proportion of ions is taken up from one year to the next. The total amount of Na^+ and Cl^- in 2008 is 0.734 mmol/L in soil extract. The ratio of the concentration of other ions (Ca^{2+}, Mg^{2+}, and K^+) to NaCl in 2008 is shown in Table 3.6 with the calculated concentrations of these ions in 2009. The adjusted numbers give the equivalent mmol/L in soil extract, which are used to calculate the ionic strength and conductivity, as was performed in Table 3.5.

$$\mathrm{IS} = \sum_{i=1}^{n} c_i z_i^2 \qquad \text{Equation 5}$$

$$\Delta \mathrm{IS}_{\mathrm{predicted}} = (0.103 \times 1^2) + (0.544 \times 1^2) + (0.742 \times 1^2) + (0.127 \times 2^2) + (0.100 \times 2^2)$$

$$\Delta \mathrm{IS}_{\mathrm{predicted}} = 2.295 \text{ mmol/L}$$

$$\Delta \mathrm{EC}_{1:2 \text{ predicted}} = \frac{2.296 \times 10^{-3} \text{ mol/L}}{0.013} \qquad \text{Equation 4 (Rearranged)}$$

$$\Delta \mathrm{EC}_{1:2 \text{ predicted}} = 0.177 \text{ dS/m}$$

$$\Delta \mathrm{EC}_{\mathrm{e\ predicted}} = 0.58 \text{ dS/m}$$

This calculation shows that by estimating the uptake of ions in 2009 based on 2008 analytical data accounts for 130% of the total season change, and 45% of the change in salinity from planting to midseason when all ions are included. In conclusion, by taking into account the 5 analysed ions the observed change in salinity can be largely accounted for. This shows that the uptake of ions into plant biomass on a salt impacted site contributes to the decrease in salinity during phytoremediation.

Table 3.6: Calculated ion content and change in salinity in 2009. Sodium and chloride levels in 2009 plant tissue and the relative proportion of these ions to Mg^{2+}, Ca^{2+} and K^{+} from 2008 data was used to estimate the magnesium, calcium and potassium in 2009 plant tissue. Ratios for Na^{+} and Cl^{-} were not calculated because their concentration in plant tissue were measured directly.

Ion Species	Ratio of ion concentration/NaCl concentration in 2008	Concentration in 2009 (mmol/L)	Theoretical ΔEC_e
Na^{+}	--	0.103	
Cl^{-}	--	0.544	
Mg^{2+}	0.196	0.127	
Ca^{2+}	0.153	0.099	
K^{+}	1.147	0.742	
	Ionic Strength	2.295	0.58 dS/m

3.3.2 Brazeau field site

The soil for this site was excavated from a contaminated sump at another location, and spread on an impermeable barrier to a depth of approximately 75 cm prior to site preparation and planting in 2008. Soil for salinity determination was sampled prior to planting and fertilizing. Comparison of the $EC_{1:2}$ and EC_e values of 10 soil samples provided an average k value of 2.7 for the field site, which was used for calculation of EC_e values for the 2008 and 2009 field seasons. The EC_e values that were calculated are shown in Figure 3.21 for 2008 and Figure 3.22 for 2009 data. These figures show very low salinity across the entire field, with an average salinity of approximately 1.1 dS/m in 2008. The salinity throughout the 2008 and 2009 field seasons stays constant, but is well below the regulatory guideline of 2 dS/m. However, poor plant growth during the 2008 field season was observed (Figure 3.23). This poor growth was attributed to low nutrient quality in the subsurface soil, and not salinity. Addition of fertilizer improved growth at the end of 2008, and a marked improvement in growth in 2009 was observed when fertilizer was applied from the beginning of the growth season. The improved growth between planting and midseason in 2008 and 2009 is clearly visible by comparison of Figure 3.23A and Figure 3.24A. There is less bleaching in 2009 and fuller visible growth beyond the rows planted by the drill seeder, which are clearly visible in the 2008 photos, shows improved plant growth with application of fertilizer.

In 2009, soil samples were taken beyond the 0-30 cm at midseason and end of season to determine if salt had been leached to a lower depth due to the heavy rainfall encountered on this site. As such, any present salt would be found within 75 cm from the surface. As shown in Figure 3.21, no salinities above 2 dS/m were found below the 0-30 cm soil depth in

1	2	3	4	5	6
1.12 1.06 1.42	1.24 1.07 1.40	1.02 1.03 1.39	1.63 1.67 1.40	1.09 0.95 1.18	0.95 1.02 1.59
1.14 0.85 1.25	1.15 1.16 1.19	1.02 0.86 1.36	0.98 1.06 1.28	1.14 0.95 1.14	1.03 1.45 1.17
1.09 1.40 1.17	0.94 1.27 1.08	1.16 1.33 1.33	1.27 0.95 1.18	1.06 1.23 1.96	1.07 1.07 1.19
1.11 1.11 1.28	1.11 1.17 1.22	1.07 1.07 1.36	1.29 1.23 1.28	1.09 1.04 1.43	1.02 1.18 1.32

June 1.12 dS/m ± 0.038
July 1.12 dS/m ± 0.030
October 1.32 dS/m ± 0.030

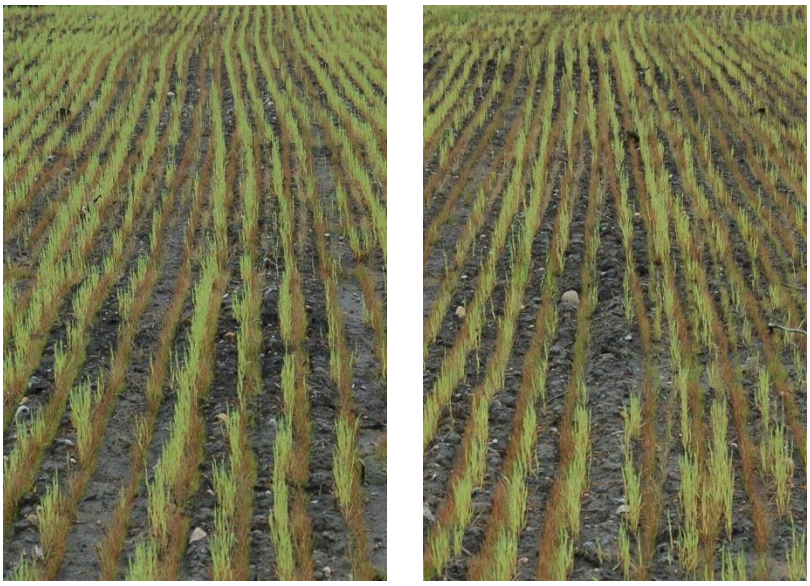
Figure 3.21: 2008 Brazeau EC_e data. It is seen that no sample point of surface soil is above the environmental criteria regulation of 2.0 dS/m. Values shown for the entire field are averages of all plot values with errors shown in standard error (N=12). Plot values are the average of the three composite points.

1	2	3	4	5	6
0.98/1.07 1.00 0.82	1.14/1.02 0.69 1.07	0.83 0.99 1.28	1.48 1.24 1.62	0.94/0.93 0.57 0.81	0.87 0.71 0.84
0.90/0.97 0.75/0.78 1.47/0.62	1.15/1.03 0.72/0.68 1.40/0.75	0.72 0.67/0.76 0.85/0.68	1.31 1.17/1.02 1.62	1.35/1.24 0.74/0.97 1.11	0.52 0.66/0.45 0.73
0.95/0.85 0.99 1.00	0.73/0.77 0.89 1.70	1.02 1.29 2.79	1.30 1.24 1.23	1.21/1.38 0.80 1.45	0.74 0.70 0.88
0.94/0.96 0.91/0.78 1.10/0.62	1.01/0.94 0.77/0.60 1.39/0.75	0.86 0.98/0.76 1.64/0.68	1.36 1.22/1.02 1.49	1.17/1.18 0.70/0.97 1.12	0.71 0.69/0.45 0.82

	Surface	Sub Surface
June	1.01 dS/m ± 0.094	1.03 dS/m ± 0.077
August	0.82 dS/m ± 0.083	0.78 dS/m ± 0.088
October	1.18 dS/m ± 0.123	0.68 dS/m ± 0.037

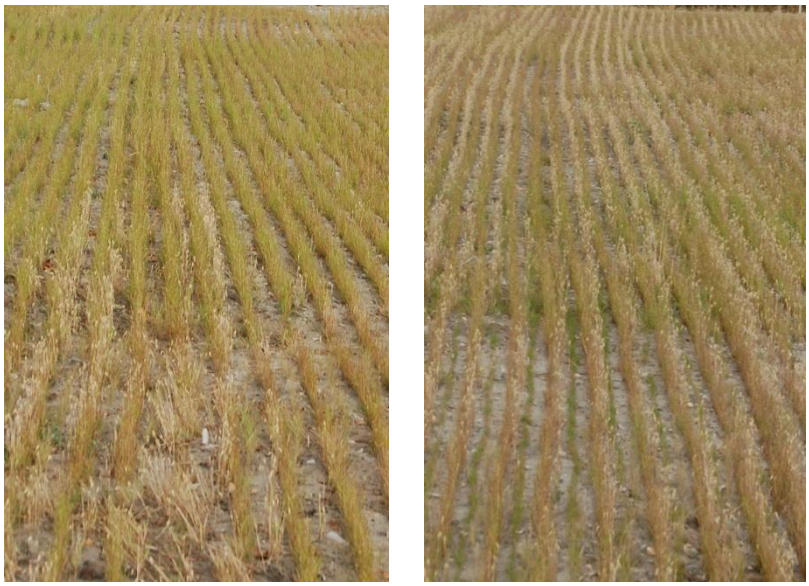
Figure 3.22: Brazeau field EC_e data from 2009 field season). Values shown for the entire field are averages of all plot values with errors shown in standard error (N=6). Plot values are the average of the three composite points.

A



UW3/UW4 - PGPR

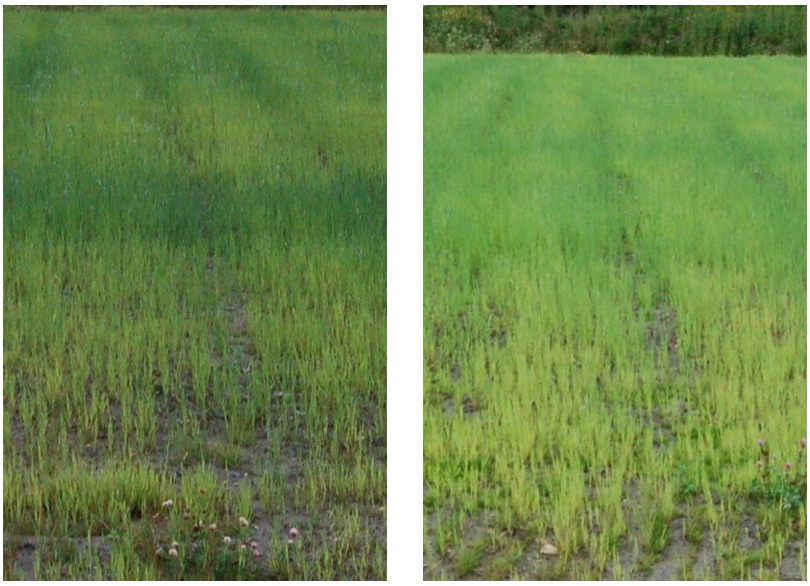
B



UW3/UW4 - PGPR

Figure 3.23: Comparison of plant growth during 2008 Brazeau field season A) Plot 1 (UW3/UW4) and Plot 2 (-PGPR) July 2008 B) Plot 1 (UW3/UW4) and Plot 2 (-PGPR) October 2008

A



UW3/UW4 - PGPR

B

UW3/UW4 - PGPR

Figure 3.24: Comparison of plant growth during Brazeau 2009 field season A) Plot 1 (UW3/UW4) and Plot 2 (-PGPR) August 2009 B) Plot 1 (UW3/UW4) and Plot 2 (-PGPR) October 2009.

2008. One sample point in 2009 was found to have a higher-than-criteria salinity of above 2 dS/m (point 3A). The soil that was excavated from the sump was not retested for salinity exceedances after spreading on the prepared site pad. Additional soil around the sump was excavated along with the contaminated sump soil, and was mixed during excavation, transport and spreading. This may have resulted in a dilution in soil salinity, explaining the lack of salt contaminated samples found on the site, and the lack of salt deeper in the site.

Production of biomass during the 2008 and 2009 is shown in Figure 3.25. As suggested by the photographs of biomass in Figure 3.23 and Figure 3.24, there was not a significant difference in biomass production when comparing the PGPR treatments. No biomass sampling was done at midseason in 2008 due to very low plant growth, and sampling was only done at the end of the field season. Growth in 2009 was greatly improved with the addition of fertilizer at planting, as shown in the graph of biomass production. The lack of difference between PGPR treated and control plots in terms of biomass is likely due to the low salinity. The salinity of 1 dS/m is not enough to cause a stress response, and as such, PGPR should have no significant effect on the production of biomass.

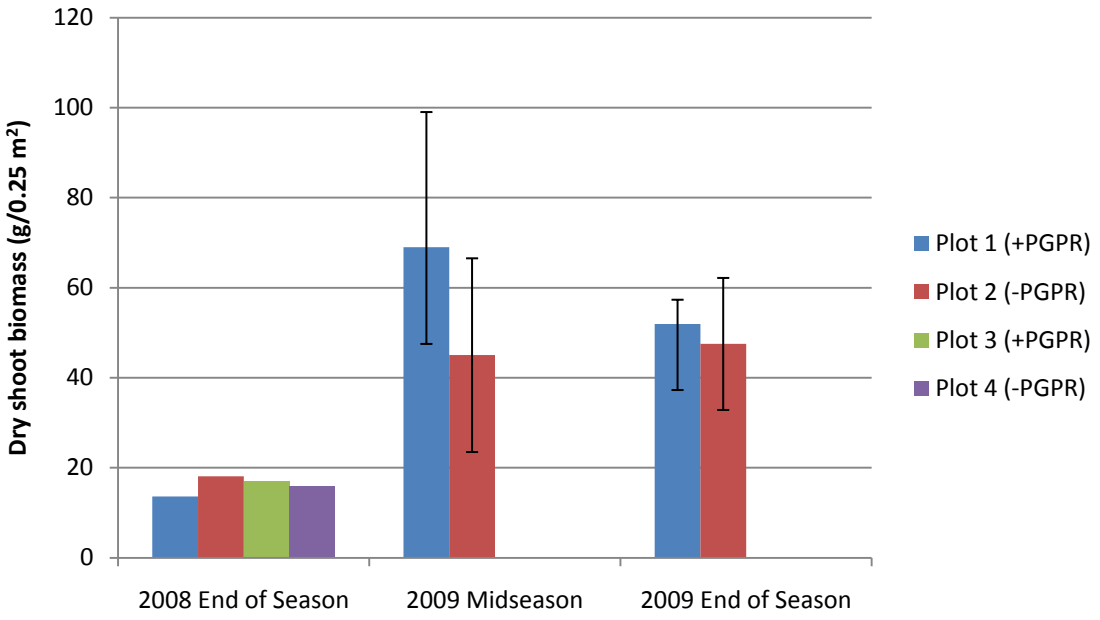


Figure 3.25: Biomass production on the Brazeau field site during the 2008 and 2009 field seasons. In 2008, all subpoints for each plot were collected into a single bag, so statistical analysis could not be carried out. There is an obvious improvement between the 2008 field season, during which fertilizer was not applied until after midseason, and 2009 when fertilizer was applied to the field at planting. There is not, however, a statistical difference between the +PGPR and –PGPR treatments in any time point. N=3. Error bars show standard error.

4 Discussion

Several studies were carried out to improve phytoremediation of salt impacted soils. The effects of hydrogen peroxide treatment both alone and in conjunction with plant growth promoting rhizobacteria (PGPR) on seed germination under salt stress were examined. In petri plate assays, peroxide was able to improve root and shoot emergence of tall wheatgrass. In combination with PGPR, hydrogen peroxide treatment improved root emergence when compared to control treatments, but did not show any improvement in shoot emergence. Five switchgrass cultivars and western wheatgrass were compared to tall wheatgrass and Inferno tall fescue, which are currently used in PGPR-enhanced phytoremediation. Under salt stress tall wheatgrass and tall fescue out-performed all other species in germination rate and maximum germination. In field trials on the Kindersley salt impacted site, a decrease in salinity was observed over a two year period, with improved biomass production compared to prior work conducted on the site. There was an increase in biomass production for PGPR treated plants compared to the non-treated plants in the first half of 2008. This was not observed after mowing in 2008, or in 2009 when *Kochia* comprised the majority of the field biomass. Calculations comparing ion content of plant tissue and the change in soil salinity over the course of the field season accounts for approximately 70% of the observed change in salinity on the site. This provides direct evidence that the removal of ions from the soil by plants is responsible for the change in soil salinity during a phytoremediation project.

4.1 Germination of seeds under salt stress

Determining methods to improve germination of PGPR-treated seeds in salt impacted soils is important during phytoremediation. The methods studied here were a chemical treatment to

improve germination of tall wheatgrass by imbibition of seeds in H_2O_2 and a comparison of plant species under salt stress in an attempt to choose more salt tolerant plant species.

Imbibing tall wheatgrass seed with H_2O_2 was shown to improve emergence of both roots and shoots when the seeds were germinated under salt stress. This is in agreement with a previous study involving H_2O_2 imbibed wheat seeds under salt stress (Wahid et al., 2007). The improvement in emergence of roots for H_2O_2 imbibed seeds was 150% of the untreated control under salt stress. This matched the germination observed for CMH3 treated tall wheatgrass. There was also an increased level of shoot emergence for H_2O_2 and for CMH3 treated seeds when compared to the control. The emergence of shoots with H_2O_2 and CMH3 in combination, however, showed the same level of germination as the untreated control. This suggests that under salt stress, the mechanism by which H_2O_2 and PGPR improve germination may not be compatible, potentially cancelling the effect of the other treatment. The improved germination of H_2O_2 imbibed seed was suggested to be due to decomposition of anti-oxidant analogue germination inhibitors in seeds (Ogawa & Iwabuchi, 2001). An assay of extracts from seeds imbibed with increasing concentrations of H_2O_2 did not show a difference in quenching of the DPPH radical. This may however, only indicate that a more specific anti-oxidant analogue that affects germination is decomposed by H_2O_2.

The germination of various species was compared with tall wheatgrass and Inferno tall fescue, both salt tolerant perennial grass species which have been used in PGPR-enhanced phytoremediation field trials. Switchgrass, listed by the United States Department of Agriculture as moderately salt tolerant (United States Department of Agriculture, National Plant Data Center, 2011), develops an extensive root system and is capable of producing high amounts of above ground biomass and so it was investigated for its potential use in phytoremediation trials. The

deep root system and high biomass production associated with switchgrass can improve remediation by expanding the depth of rooting zone by the plants and providing more shoot biomass for ion sequestration. Three cultivars of switchgrass, common switchgrass from two suppliers, and western wheatgrass were used in germination assays. During trials, all western wheatgrass seeds and all common switchgrass seeds, except one switchgrass seed, from Agrecol failed to germinate. This is in contrast to common switchgrass from Ontario Seed Company (OSC), which showed high germination rates in all trials. Both species that failed to germinate were from the same supplier. One of these species (Common switchgrass) was also obtained from another supplier and was able to germinate. Since the failure of germination may be linked to the supplier, more studies should be done with western wheatgrass from other suppliers to assess its suitability.

As shown in Figure 3.5A, Cave-in-Rock switchgrass and Forestburg switchgrass were able to maintain a higher level of germination than other switchgrass cultivars under salt stress in greenhouse trials. However, they were not able to compete with the germination of tall wheatgrass or Inferno tall fescue. Southlow switchgrass, which showed a higher level of germination than other switchgrass cultivars, was the only cultivar that showed a significant decrease in germination with the addition of salt stress. Other cultivars may be useful for lower salinity levels, depending on the rate of ion uptake.

Common switchgrass, Cave-in-Rock switchgrass and western wheatgrass were used in petri plate germination assays under 7.5 dS/m (75 mM NaCl) salt stress to compare with germination of tall wheatgrass. As seen in the greenhouse experiment tall wheatgrass was able to germinate at a higher rate than other tested species regardless of salinity and under all conditions western wheatgrass did not germinate. In a germination assay conducted with switchgrass and

tall wheatgrass at increasing salinities (Figure 3.8 and Figure 3.9), it was observed that tall wheatgrass was able to germinate at a higher rate under 100 mM NaCl (10 dS/m) than the common switchgrass no-stress control. This was observed in both root and shoot emergence. A very large decrease in germination was observed in tall wheatgrass between 100 mM and 150 mM NaCl treatments, although the germination of tall wheatgrass at 150 mM NaCl was still greater than the germination of common switchgrass at 100 mM NaCl. These assays show that the salt tolerance of tall wheatgrass is much greater than the tolerance of the other switchgrass cultivars tested and as such should continue to be used for remediation. Further work should be carried out, however, to determine the efficacy of establishing switchgrass stands to remediate deeper into soils at lower contamination levels. Many salt tolerant species maintain the ability to grow under saline conditions by actively excluding salt ions or selectively taking up some ions instead of others. Lower Na^+ concentrations relative to other ions may result in slowed uptake of Na^+ ions. As such the uptake of salts at lower concentrations should be compared between the salt tolerant species currently used in the PGPR enhanced phytoremediation system and less salt tolerant alternatives, such as switchgrass, which may not be able to selectively take up salt ions as effectively.

4.2 Plant growth in field trials

Plant growth on the Kindersley and Brazeau field sites both showed 100% field coverage in 2008 and 2009. The growth observed on the Brazeau site when comparing PGPR treated seeds to non-treated seeds was approximately equal. However, plant growth on the Kindersley site was higher for the CMH3 and UW3/4 treated seeds when compared to the –PGPR seeds. This may be attributed to the difference in salinity between the sites. The Brazeau site has an average salinity of 1 dS/m, which is well below established criteria for field soil salinity. The increase in growth

in 2008 after application of fertilizer at midseason sampling, and the improved growth in 2009 which had fertilizer application at the start of the season, supports the idea that the only major issue with the Brazeau field site is the low nutrient content of subsurface soil and not a stress due to salinity. The higher nutrient content of the Kindersley field site, which is top soil, allowed for better plant growth than at Brazeau despite higher salt levels.

In 2008, there was good growth on the field and 100% plant coverage. The variation in soil salinity across the site, even within individual plots, made a statistical comparison of plant growth with the various PGPR treatments unfeasible. However, between planting to midseason there was a trend of more growth observed in the CMH3 and UW3/4 plots when compared to the –PGPR plots. The amount of growth after mowing at midseason until the end of season was approximately equal between the different PGPR treatments. The biomass produced between midseason and end of season was, however, approximately equal to the amount produced between planting and midseason, showing that these species are able to grow well after a midseason harvest. The health of the plants by visual inspection was very good with no signs of stress, such as yellowed tips, and the planted species were able to maintain a green colour well beyond the time that surrounding plant species native to the site, but outside of the impacted zone, had died back and become dormant for the season.

The production of plant biomass in 2009 on the Kindersley field site is attributed mainly to *Kochia scoparia,* not the planted mixture of oats and grass species. The hard frost at the beginning of the 2009 field season, shortly after planting, was likely a major factor in the lack of planted species on the field which allowed *Kochia* to invade the test plots. Although the intended species did not grow, it was expected that there would be a change in salinity due to salt uptake by *Kochia*. This was observed between planting and midseason, when *Kochia* was able to

produce high levels of biomass. At midseason, the biomass was mowed to a height of approximately eight inches in order to remove salt that had been taken up into plant tissue and to prevent the *Kochia* from producing seed. Removing biomass at midseason from phytoremediation field sites planted with oats and grasses has been done with success at other sites (Chang, 2008; Wu, 2009), and in 2008 on the Kindersley site. The removal of biomass in 2009 was carried out in a similar fashion, but resulted in no additional growth between midseason and end of season. There are two potential factors that could have resulted in poor growth.

The first is the response of *Kochia* to mowing. At the time the field was mowed, the *Kochia* had begun to flower. As *Kochia* is an annual plant, it may not be able to recover from being cut after flowering had begun. The removal of plant biomass to prevent its spread to nearby fields may have resulted in the observed change in growth. The second potential factor is a lack of rainfall and a lack of additional water. As shown in Figure 3.11 there was very little rainfall after the midseason sampling point. Further, no additional water was applied to the field unlike the 2008 field season in which 1000 L of water was applied to the field three times during the year. This change was due to reports of adequate water from workers near the field site, and so no additional water was given to aid in plant growth. The lack of water is likely a major factor because, in addition to the lack of new growth of *Kochia*, there was also no observed additional growth of the grasses that had germinated within the *Kochia* stands. Although *Kochia* is unable to grow after mowing, the planted grass species should have been able to continue growing throughout the entire field season. Plant growth in grasses was not observed, making a lack of water a likely reason for the lack of additional growth between midseason and end of season. In

the future, this should be taken into account and the observed weather data should be used to make decisions as to whether or not additional water is needed for plant growth.

4.3 PAM fluorescence of field trial biomass

PAM fluorescence of field tissue in 2009 showed minor variation in photosynthetic health. The average yield from plots A through D and *Kochia* were all better than the tall wheatgrass from plots 1 through 3. This may be due to the trend of lower salinity found in plots A through D, or better health due to a lack of competition from *Kochia* which was seen growing in plots 1 through 3, but not A, B C or D. There was not an observed difference between +PGPR plots and –PGPR plots, as was observed in PAM fluorescence on salt impacted soil in other phytoremediation sites (Wu, 2009), but this may be due to the low stress from the salt on the soil due to high quality nutrients, and the only major stress observed during the remediation trial being a lack of water, which would affect all plants. The competition for these nutrients, namely K^+ and Ca^{2+} which may lower stress due to Na^+ exposure, may result in the observed decrease in photosynthetic yield in plots where *Kochia* was growing aggressively. Due to the variability of soil in the field and the limited data available, an analysis of qN and qP differences between plots could not be carried out. It was also observed previously that a visible physical effect on plant health was observed between treatments when a difference in PAM fluorescence showed decreased function (Wu, 2009), and this was not seen in the various PGPR treatments on the Kindersley site. The effect of PGPR on plants in field trials should therefore be further tested, if possible under more saline conditions and without weed infestation.

4.4 Soil salinity changes in field site soils

During the 2008 and 2009 field seasons, the salinity of surface (0-30 cm) field site soils was monitored. The salinity of the Brazeau field site during the two year field study stayed

relatively constant throughout the trial, with a starting and ending salinity within 0.06 dS/m of each other. As the salinity was already well below criteria and remained below criteria for the field trial, the lack of change in salinity is not a concern. After soil samples from the first sampling time point in 2008 were analyzed the soil was found to have a low salinity. It was suspected that the salt that was initially detected prior to excavation and spreading of the sump soil was driven deeper into the soil by rainfall, as the intended start of field work on this site was postponed by a year due to excessive rain and water logging of the soil. As such, in 2009 samples were taken to a depth of 75 cm, the reported depth of the impermeable pad on which the soil was spread. There was no observed increase in salinity with increased depth. Thus, it does not appear that the salt had migrated deeper into the soil. Additional soil from the same sump was spread on another field and remediation carried out was tested by consultants from NorthWind Land Resources. They also observed low salinity in the spread soil, despite the reported exceedances prior to excavation. It is suspected, therefore, that when the initial sump was excavated, additional soil was excavated from around the impacted soil. During transportation and site preparation the salt impacted soil was mixed with unimpacted soil and the salt concentration was diluted to the point that the salinity was below criteria.

The Kindersley site soil EC decreased by 1.37 dS/m during the 2 year field trial, with most of the change in salinity occurring in the first year. As covered in section 4.2, the plant growth in 2008 and 2009 were markedly different. It was expected that the decrease in biomass production in 2009 compared to 2008 would result in a smaller decrease in salinity. The biomass production between planting and midseason was comparable in 2008 and 2009 but the decrease in salinity was greater in 2008, with a decrease of 1.62 dS/m versus 1.29 dS/m in 2009 during the same time period. The increase in salinity between midseason and end of season in 2008 was

0.71 dS/m, compared to 0.84 dS/m in 2009. This gives a net decrease in salinity of 0.91 dS/m during the 2008 field season and a net decrease of 0.45 dS/m in 2009. The observed change in 2009 was half of the change in 2008. This may be attributed to the lack of plant growth between midseason and end of season in 2009, or may be attributed to the difference in ion uptake by *Kochia* compared to the planted species. The comparable level of plant growth between planting and midseason sampling during both years and the lessened decrease in salinity in 2009 compared to 2008 suggests that there is less ion uptake into *Kochia*. This is supported by the tissue analysis of *Kochia* from 2009 compared to grasses and oats which was shown in Table 3.5**.** The uptake of sodium into grass and oats was approximately twice the uptake into *Kochia*, suggesting that *Kochia* is able to better exclude sodium ions compared to oats and the planted grasses when grown on salt impacted soils. As such, the use of *Kochia* may be not as beneficial if sodium is of concern in the quality of the soil, instead of salinity caused by other salt ions. This, however, would require more research to determine the actual rate of total ion uptake into *Kochia* biomass.

During the 2009 field season, samples were taken at planting and at end of season sampling by drill truck to a depth of 1 m from plots 1 through 9. The soil was divided into three sections: 0-30 cm, 30-60 cm and 60-100 cm. The salinity of these samples is given in Figure 3.19. The EC_e measurements of the 0-30 cm, which is the rooting zone, and 30-60 cm soil horizons both decreased over the course of the field season by 0.8 dS/m and 0.65 dS/m, respectively. The deepest sampled horizon, from 60-100 cm, had an increase in salinity of 0.44 dS/m over the course of the field season. The vertical movement of salt through the soil is important to understand for phytoremediation, as the purpose of this method of remediation is to remove ions from the soil into plant biomass where it can be harvested and the salts removed the

site. Remediation was observed both in the rooting zone and the samples below the rooting zone, but not at the deepest level. This can be caused by movement upwards of salt ions with an excess of ions below the 100 cm to replenish the displaced ions in the 60-100 cm depth, or the downward movement of ions from higher soil horizons into the 60-100 cm depth. The movement of ions in the soil is caused by the movement of water, in which the ions dissolve, as it either percolates through the soil to deeper horizons or as the water is brought to the surface through evaporation and transpiration (i.e. uptake by plants). Previous field studies of the applicability of phytoremediation to salt impacted soils has generally occurred in regions of high rainfall (Qadir et al., 2003)(Batra, Kumar, Manna, & Chhabra, 1997), which would result in increased percolation and leaching of salt through the soil. As there were drought conditions during much of the field season in 2009 there would be less percolation of rainfall through the soil, and an increase in evaporation and transpiration. This would result in an upward net movement of water toward the surface. The observed decrease in the top 60 cm, despite the low amount of rainfall, suggests that phytoremediation was able to occur without as high rainfall. This salt to percolation through the soil to a deeper horizon would have been limited. While this does provide some information as to the direction of salt movement, the presence of ions in plant tissue should be measureable in levels to account for the change in salinity if the net movement of salts is upwards into the plant tissue and not deeper into the soil.

4.5 Salt uptake in field trials

Due to the low salt content of the Brazeau field site, ion uptake analysis was not carried out on collected plant material. The salt uptake of the biomass from the Kindersley field site, however, was used to calculate the ion uptake and compared to the change in soil salinity of the site over the course of growing seasons. The ion uptake data in Table 3.3 shows high levels of

potassium uptake compared to all other ions, and fairly high levels of calcium and magnesium. All of these ions contribute highly to salinity in soil. The ratio of uptake between ions was also not consistent between different plant samples. The uptake ratio of chloride ions to sodium ions into plant tissue in 2008 was between 2.0 to 6.1, with an average 4.1. In 2009, the average uptake ratio in *Kochia* was 8.1. This same variability in ion uptake was seen in previous phytoremediation studies involving salt impacted soil (Wu, 2009). The variability in ion uptake may be due to variations in the ion content in soil rhizosphere. As was previously mentioned, one of the mechanisms by which plants tolerate high levels of toxic ions such, as sodium, is to selectively take up ions that they compete with, such as potassium and calcium(Li et al., 2010; Parida & Das, 2005). As such, one would expect to see a preferential uptake of these ions if the soil has a sufficient level of these ions. Chloride and magnesium are required for various cellular functions in plants, including chloroplast biosynthesis and photosynthesis (Li et al., 2010), and so we would expect that these ions would be taken up in high amounts, which was also observed.

The uptake of ions into plant biomass was used to estimate the total ion content remediated from the field over the planting season. By using these values from four plant samples, the change in salinity accounted for in the soil by removal of these ions into plant biomass was calculated for the 2008 field season. The $\Delta EC_{estimated}$ was found to be between 0.45 dS/m and 0.88 dS/m with an average of 0.66 dS/m ± 0.08, which was 72.5% ± 8.7 of the observed ΔEC in the soil. Thus, the uptake of salt in 2008 was able to account for the majority of the decrease in soil salinity. Calculations showing this type of connection between phytoremediation of saline soils and ion uptake have not previously been reported in literature.

In 2009, only the Na^+ and Cl^- content of plant tissue was measured. The uptake of these ions was calculated to result in a theoretical change in salinity of 0.164 dS/m, or 36% of the

overall change in salinity during the 2009 field season. By estimation of the concentration of other ions in the 2009 plant tissue based on the uptake ratios from 2008 data, the estimated change in salinity was 0.58 dS/m, 130% of the observed 0.45 dS/m change during the 2009 field season. This discrepancy implies that either the ions removed from the soil into the plant biomass were replaced by additional ions moving upwards through the soil during the field season, or the ratio of ions in *Kochia* is not represented by the uptake data of planted grasses and oats. The uptake data for Na^+ and Cl^- in Table 3.5.

Table 3.5 shows a large average difference in uptake of Na^+, with a lower uptake in *Kochia* biomass, and a slightly higher uptake of Cl^- into *Kochia*. This suggests that the extent of uptake of the other ions may also be varied when compared to the uptake into the planted grasses and oats. There should be a distinct upward movement of salt ions if more salt was removed from the soil into plant biomass than is expected based on the observed decrease in soil salinity. It appears more likely that the estimation of uptake of Ca^{2+}, Mg^{2+} and K^+ into *Kochia* is not reliable enough for an accurate estimate. As such, when calculations of estimated change are carried out based on ion uptake into biomass, it is important that all ions of concern are measured directly, rather than estimated from previous observations.

The ion uptake shown in Table 3.4 demonstrates the potential variability in ion uptake from soil, even when the same species are analysed. This is likely due to variations in the salinity and salt composition within the soil, as the competitive uptake of ions will vary with concentration, and the varied production of biomass of the plant species that were planted during the phytoremediation trials. It is important to note that, despite these potential sources of error, by directly measuring the uptake of ions approximately 70% of the change in salinity in 2008 was accounted for by five ions. By addition of the amount of Na^+ and Cl^- removed in 2008 and 2009 (Table 1.5) and their respective molar masses, it can be found that and estimated 22.81 kg of NaCl was removed in 2008, and additional 21.65 kg was removed in 2009. These calculations provide evidence that the uptake of ions from the soil into plant biomass plays an important role in the decreased salinity observed in phytoremediation field trials.

4.6 Conclusions

During the course of this work, the efficacy of phytoremediation was tested and similar results were obtained when compared to previous work conducted using PGPR enhanced

phytoremediation (Chang, 2008; Huang et al., 2005; Wu, 2009). During the 2008 field season at the Kindersley site a decrease in salinity of 0.91 dS/m was observed, which matches with the approximate change of 1 dS/m observed in other field tests (Chang, 2008; Wu, 2009). In 2009, the change was approximately half the observed change in 2008, but a decrease in salinity was still observed. By sampling to a depth of 1 m it was seen that the top 60 cm had a net removal of salt, while the 60-100 cm horizon showed an increase in salinity. It could not be determined from the two sampled time points the net movement of salt throughout the field season. It was possible, however, to account for 72.5% of the change in salinity by measuring the amount of Na^+, K^+, Cl^-, Ca^{2+} and Mg^{2+} that had been taken up into plant tissue during the 2008 field season. In 2009, the Na^+ and Cl^- uptake data was used to account for 36% of the overall change while excluding K^+, which tends to be taken up in greater proportions than Na^+, and does not account for Ca^{2+}, which can have a large influence on salinity due to being divalent. These calculations show that the uptake of ions during phytoremediation does have a substantial impact on the change in salinity.

Switchgrass species and western wheatgrass were compared with tall wheatgrass and Inferno tall fescue under saline conditions. Although switchgrass is moderately salt tolerant according to the USDA, it had very poor germination in comparison to the tall wheatgrass and Inferno tall fescue. As such, it is not likely a good candidate for phytoremediation at moderate to high soil salinities if tall wheatgrass and tall fescue can be used.

Tall wheatgrass seeds imbibed with 60 mM H_2O_2 was able to germinate at a higher rate than tall wheatgrass imbibed with RO water when under 7.5 dS/m salt stress. The improvement in germination with H_2O_2 was statistically equivalent to treatment with the PGPR strain CMH3, and both had a greater germination level than the control seeds. The combination of H_2O_2 and

CMH3 improved root emergence levels to the same statistical level as H_2O_2 or CMH3 alone, but the combination of these two treatments resulted in no improvement of shoot emergence when compared to the control treatment. A comparison of general antioxidant levels in seed extracts from various levels of H_2O_2 imbibition showed no statistical difference between 0 mM (pure RO water) and 120 mM H_2O_2.

4.7 Recommended future work

Further studies into the vertical movement of salts with and without vegetation with rainfall should be studied, to verify the extent to which salt ions are taken up into plant tissue or percolate through the soil with varied levels of water. The uptake of ions is selective, and plants avoid stress by exclusion of toxic Na^+ in favour of preferable ions. As such, when remediation of a site proceeds a point may be reached where toxic ions are not taken up into plant biomass if highly tolerant species are used. The uptake of specific ions in different plant species at varying soil-salt concentrations should be examined. This would help determine which species are most effective to use in a phytoremediation trial.

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