Effects of bacterial inoculation on osmolyte production, salinity tolerance, and transcription in recurrently-selected saline-tolerant alfalfa breeding lines

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ABSTRACT

Previous studies have highlighted the effectiveness of both plant breeding and soil inoculation in the mediation of salinity stress in alfalfa (*Medicago sativa* L.), however few have examined the effects of utilizing both of these techniques in tandem. The objectives of this study were to: 1) assess the effectiveness of *Ensifer meliloti* and *Halomonas maura* as inoculants to improve salinity tolerance in alfalfa, 2) assess the effectiveness of recurrent selection in improving fitness of alfalfa in saline conditions, and 3) assess whether inocula would confer additional benefits to salt-adapted alfalfa populations as indicated by both phenotypic and RNA sequencing data. To achieve these aims this study grew three alfalfa generations sequentially selected for salinity tolerance in association with inocula *E. meliloti* and *H. maura* alone and in tandem and compared against plants given a 60 kg/ha nitrogen amendment under 0, 8, or 16 dS/m of salinity. Results showed that while biomass was only increased in saline conditions through the nitrogen amendment, *E. meliloti* did confer increased salinity tolerance through increases of the osmoprotectant proline. Recurrent selection for salt tolerance in alfalfa increased chlorophyll content, stem count, and plant height, however generation G2 displayed equal or superior performance compared to generation G3, suggesting the onset of an inbreeding depression. The RNA-seq study found that generation G2 plants displayed an upregulated component of the TOR signalling pathway which modulates growth under stressful conditions, and that *E. meliloti* modulated genes responsible for metabolism of the osmoprotectants proline and glycine betaine as well as sulfur uptake in salt stressed plants. In summary, despite strong inhibition of mutualism by 8 dS/m salinity rhizobium confer beneficial effects on alfalfa include significantly higher proline production and improved sulfur uptake; recurrent selection increased chlorophyll content, stem count, and height, however a potential inbreeding depression caused by high selection pressure may have prevented gains in biomass from becoming significant in later generations.

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LIST OF ABBREVIATIONS

C—Control

CN—60kg/ha Nitrogen

dS/m—Decisiemens per meter

EM—*Ensifer meliloti*

EH—*E.meliloti/H.maura*

GO—Gene Ontology

HM—*Halomonas maura*

KEGG—Kyoto Encyclopedia of Genes and Genomes

NR—NCBI Non-Redundant database

RNA-seq—RNA sequencing

PE—Paired end

SE—Single end

1. INTRODUCTION

Salinization of arable land is one of the drivers causing reduction in overall global crop yields and revenue. In 2015, the Food and Agriculture Organization (FAO) reported 412 million hectares and 618 million hectares of land were affected by salinity and sodicity respectively (Montanarella Luca et al., 2015). The salinization rate of arable land is set to increase due to the high temperature and altered precipitation patterns caused by climate change (Mukhopadhyay et al., 2021). This will be especially true in arid and semi-arid regions of the world (Allison et al., 1954; Butcher et al., 2016). Canada is not immune, with up to 1 million ha of land experiencing moderate to severe levels of soil salinity in the western regions alone (Wiebe et al., 2007).

While salinization detracts from the acreage of global arable land the nutritional needs of a growing global population will continue to increase. With this rise in population will come a concomittant increase in affluence, which in turn will lead to an increased demand for animal based protein (Henchion et al., 2021). Finding ways to mediate salinity stress while also supporting the protein demands of a population of over 9 billion people by 2050 necessitates the production of high quality and resilient forage crops for animal feed. As western Canadian land suffers from the highest rates of salinity in the country while supporting the greatest beef production in the country, there will be few regions where this issue will be of greater concern.

Alfalfa is the most important forage crop in Canada with 3.8 million ha cultivated in 2016 (Statistics Canada, 2021), and it supports both the beef and dairy industry. While alfalfa is called the 'Queen of Forages' for its high yield, high quality, and beneficial effects on soil health, it is only moderately salt-tolerant. Steppuhn et al., (2012) report alfalfa being susceptible to saline soil above 8 dS/m. In addition, many of the saline-tolerant cultivars commonly available do not have the winter hardiness necessary to provide consistent survival in harsh Canadian winters (Bhattarai et al., 2020).

Despite it's limited tolerance, alfalfa nevertheless compares favourably with many cash crops in terms of salinity tolerance, yet breeding efforts remain modest. Canola (*Brassica napus*) and wheat (*Triticum spp.*) experience yield losses above 4 and 6-8 dS/m respecively (Alberta Agriculture and Rural Development, 2001), while alfalfa becomes increasingly susceptible to salinity only above 8 dS/m (Steppuhn et al., 2012). Although there has been work done to

improve the salinity tolerance of alfalfa (i.e Steppuhn et al., 2012), superior tolerant varities with good winter hardiness are needed in Canada.

Alfalfa possesses many of the salt-stress related genes reported in other crops that offer potential for improvement by selection including; reactive oxygen species scavengers, protein transporters, and of greatest interest to this paper, osmolytes (Bhattarai et al., 2020). The quantity of beneficial alleles responsible for these traits can be increased through conventional plant breeding (i.e phenotypic recurrent selection in the case of alfalfa). As osmolytes can also increase the resilience of bacteria in salt stress (Wood, 1988), some of these genes may serve to make legume a more hospitable habitat for the mutualistic soil microbes (including rhizobium spp.) which naturally associate with it. This means that these genes might serve to both increase overall plant fitness in saline environment while also facilitating the soil microbes which provide it with limiting nutrients. This interaction, if existent, could cause a positive-feedback loop which would increase alfalfa's stress tolerance.

While rhizobium are beneficial to legumes in nutrient limiting conditions, alfalfa will abort nodulation in times of abiotic stress (Ferguson et al., 2019). There have been attempts to solve this problem by supplying mutualistic bacteria and/or rhizobium with intrinsically higher tolerances to salinity stress (Noori et al., 2018; Bertrand et al., 2020a). While success has been reported, the species of rhizobium which associate with alfalfa already have much higher tolerances to salinity than alfalfa (Talebi et al., 2008), meaning that a stress-related breakdown in the relationship may be more a function of insufficient tolerance on the part of the host plant. By this logic, any improvement in the salinity tolerance of the host crop should improve the odds of successful mutualism, thereby further increasing the salinity tolerance of the legume.

Several aspects of the aforementioned issue have previously been researched; for example salt-tolerant rhizobium strains were grown with both unimproved and improved alfalfa (Bertrand et al., 2015, 2020a), and the authors found increases in yield resultant from improved genetics; however these studies were only 4 weeks in duration, did not subject alfalfa plants to more than one cut as would be normal in field conditions, or use any plant growth-promoting rhizobacteria (PGPR) inoculants. Conversely, Liu et al., (2019b) and Martínez et al., (2015) experimented with the use of plant growth promoting bacteria instead of (or in addition to) rhizobium and found an increase in dry matter yield, however these studies did not look at genetic improvement of alfalfa.

To fill the knowledge gaps left be the aforementioned works, this study conducted a 120 day long trial subjecting three previously existing breeding lines of alfalfa developed for salinity tolerance to 0, 8, and 16 dS/m salinity stress in association with both rhizobium and the halotolerant plant-growth promoting rhizobacteria *Halomonas maura.* We predicted that soil bacteria would induce increased resilience to salinity stress in the form of heightened yield and production of osmoprotectants. We also predicted that associations between bacteria and more salt-adapted populations would be more effective than less-adapted populations in combatting stress. Finally, we predicted that soil bacteria would regulate the transcription of salt-stress related genes in alfalfa to improve salinity tolerance. To test these hypotheses this paper:

1) Assessed whether recurrent selection for salt tolerance of alfalfa populations increased biomass, chlorophyll, height, stem count, or elevated levels of proline, trehalose, and glycine betaine under salt stress after 120 days of growth.
2) Assessed whether salt-tolerant PGPR and/or rhizobium conferred salinity tolerance to alfalfa compared to single bacterial treatments and/or non-inoculated controls through biomass, chlorophyll, height, stem counts, and quantification of proline, glycine betaine, and trehalose levels.
3) Assessed through mRNA sequencing whether improved alfalfa populations and bacterial inoculation increased levels of differently expressed genes associated salinity tolerance relative to non-stressed plants.

2.1 Soil Salinization

2.1.1 Mechanisms of Soil Salinization

Soil salinity is primarily induced through natural processes, although human activities such as irrigation also can cause or exacerbate issues. Salinity most commonly manifests in arid and semi-arid regions, where the annual rainfall is less than annual evaporation (Allison et al., 1954). Because precipitation is not guaranteed to flush salts from the soil, small salinity issues can compound to become serious issues over the course of time (Kwiatkowski et al., 1996). Groundwater is almost always the vector by which salts reach the soil surface, although in coastal areas seawater can also sometimes be the cause (Mukhopadhyay et al., 2021). While western Canada is currently landlocked, it was once a inundated by seawater, and the bedrock beneath much of the northern Great Plains is now a marine shale that is saturated with salt (Florinsky et al., 2000). Groundwater can dissolve the salts embedded in this shale, most commonly magnesium sulphate and sodium sulphate (Dodd et al., 1964). If this groundwater is allowed to rise to the top of the soil profile these salts can be deposited when the solvent is evaporated (Wiebe et al., 2007). This rise in groundwater can either occur naturally via precipitation events, or artificially through anthropogenic irrigaton (Butcher et al., 2016).

While groundwater is most often the culprit in regard to soil salinity, the exact mechanism by which it brings salts to the soil surface varies. The broadest delineation between types of salinization is between primary and secondary salinization. Primary salinization is naturally occurring, while secondary salinization is human caused (i.e., irrigation) (Butcher et al., 2016). Primary salinization can be further subdivided into different mechanisms; Groundwater can rise through artesian wells, hillside seeps, irrigation, depressions, outcrops, the edges of sloughs, and off the sides of lotic water bodies (Kwiatkowski et al., 1996; Anderson et al., 2015). Hillside seeps and outcrop seeps happen at the edge of hillsides where water is drawn out either because an increase in pressure brought on by the elevation change, or because of a permeable layer close to the surface. Artesian wells occur when a break in otherwise an impermeable layer above the water table creates a natural pressure-relief valve. Seasonal depressions can become saline when rainwater saturates the soil, connecting non-saline upper soil horizons to the saline water table below; thereby allowing salt ions to travel to the surface. Even after soil dries, water can be drawn to the surface through capillary action. Similarly, groundwater can be drawn out of

the edges of sloughs/lotic water bodies and to the surface through capillary action. While the many vectors by which salinization occurs can be disorienting, management is simple as it must always focus on water management.

2.1.2 Effects of Salinization on Soil

Once salts are established in the soil profile, they alter many soil characteristics. Sodium salts specifically can destroy soil structure through deflocculation (Chibowski, 2011). Sodium ions have a dispersive effect on clay particles, whereby they weakly bond with negatively charged clay particles because of their Na^{1+} ionic charge while the negative charge of their hydration shell (the water molecules with polar bonds to the cation) breaks any bridges bonding that clay particle with the next clay particle. This phenomenon is called deflocculation and results in an amorphous slurry which cements when dry. Conversely, calcium ions (which are present in some salts) have a flocculating effect whereby they use their stronger Ca^{2+} positive charge and smaller hydration shell to act as a bridge; bonding with 2 separate clay particles, thereby binding them together into a "floc" and creating improved soil structure.

In the Canadian System of Soil Classification (CSSC), saline soils with a B horizon characterized by an exchangeable Calcium: Sodium ratio of 10:1 or less and columnar structure are classified as "Solonetzic" soils (Soil Classification Working Group, 1998). While other soil types can have saline features, Solonetzic soils are the quintessential result of soil salinization. The hardpan B horizon is created through the mechanism already outlined, and results in limitations on crop growth. The B horizon is a wet slurry when wet, and when dried solidifies into an impenetrable layer through which roots cannot grow.

2.1.3 Prevention of Salinization

Prevention of soil salinity should always be the preferred management option as it is the simplest and least costly. The simple practice of constant cropping is attributed with much of the improvement in saline soils in western Canada since the 1980's (Government of Canada, 2023) when it was an overriding concern (Kwiatkowski et al., 1986). The converse practice, summer fallowing, allows groundwater (and it's associated salt ions) to rise unimpeded as no vegetative material is present to inhibit it through evapotranspiration. If prevention is no longer an option, or in more severe cases where conventional crops cannot be grown due to pre-existing salinity, the focus must shift to remediation. While there are several high-cost remediation options,

including deep ripping, gypsum amendments, and the installation of tile drainage (Hadrich Joleen C., 2011; Simundsson et al., 2016; Chhabra, 2021). A less costly option is the planting of highly saline tolerant perennial forage species such as Altai wild rye (*Leymus angustus.* Trin), slender wheatgrass (*Elymus trachycaulus.* Link), or green wheatgrass (*Elymus hoffmannii* Jenson & Asay) (Renault et al., 2004; Steppuhn and Grieve, 2005). By planting perennial forage species with high salt tolerance a water table can be mediated, preventing salt deposition reoccurring through a recharge cycle. Over time, precipitation will leach salts from upper horizons, breaking down the hardpan Bn or Bnt horizon (Soil Classification Working Group, 1998) and facilitating improved crop production. In this manner, the producer can plant successively less saline-tolerant crops until the soil produces healthy yields. A key component of this strategy is the deep rooting system of the listed forage grasses. While not a grass, the perennial legume alfalfa (*Medicago sativa*) also has deep tap root system which has the potential to lower the groundwater table over multiple growing seasons (Steppuhn, 2012). While not as tolerant to salinity as the listed grasses, alfalfa's nitrogen-fixing ability also makes it an attractive crop for use in remediating more moderately salt-affected soils. Development of more salt-adapted varieties should therefore be of great interest to producers.

2.2 Alfalfa

2.2.1 Effects of Salt Stress on Plant Metabolic Function

Salinity stress retards plant metabolism. Plant metabolism can be generally divided into photosynthesis, the process whereby the energy of the sun is stored as potential energy in the form of sugars, and respiration where these sugars are used as energy for growth and maintenance. The stored energy created by photosynthesis is used for three purposes: growth, maintenance, and stress tolerance (Munns and Gilliham, 2015). As the plant's energy resources are finite, an increase in demand in any of these metabolic areas must mean a concomitant decrease in supply to others. It is clear from this logic that salinity stress will reduce the plant's ability either to grow or maintain regular metabolic function.

Deleterious effects of soil salinity on plants manifests in two ways. First, soil salinity imposes osmotic stress on the plant through changing soil water potential, and secondly it can cause toxicity by interfering with normal metabolism (Butcher et al., 2016; Bhattarai et al., 2020). Osmotic stress occurs when salts accumulate in the root zone to create a lower water

potential in the soil than the plant root, resulting in water being osmotically drawn from the plant (Butcher et al., 2016). Alternatively, as was addressed in earlier sections, sodium ions can cause changes in soil structure. The ions can create sodic soils with "hardpans" through which many plant roots cannot penetrate. These hardpans can drastically limit the plants access to water or nutrients (Soil Classification Working Group, 1998). Both of these mechanisms can create effective drought conditions even if water is present in the soil. The effective drought conditions caused by salt stress cause the plant to close stomata to reduce water loss, and the resultant lack of CO_2 means that energy in the electron transport chain is not used to split CO_2, but instead can excite surrounding O_2 molecules to create superoxide radicals. Superoxide radicals, a reactive oxygen species (ROS), in turn can go on to create other species of radical (Das and Roychoudhury, 2014). Reactive Oxygen Species produced by superoxide include singlet oxygen, hydrogen peroxide, and hydroxyl radicals. These radicals can damage cell membranes, lipids, and proteins eventually leading to cell death in the plant (Das and Roychoudhury, 2014).

Salt can directly affect plant metabolism through nutrient deprivation and the creation of toxic compounds within the plant. The uptake of the essential nutrient K^+ can be reduced if the concentrations of Na^+ ions in the soil or plant are excessive (Cornacchione and Suarez, 2015). Over time, this dearth of potassium can starve the plant of an essential macronutrient. A buildup of Na^+ and Cl^- ions in the plant can also cause membrane damage (Butcher et al., 2016), which can be a further cause of stomatal closure. Furthermore, the normal K^+ and Cl^- concentration within chloroplasts are unbalanced in times of salt stress because of decreased K^+ uptake/ increased Cl^- uptake which brings important Cl^-/K^+ into disequilibrium, inhibits photosynthesis, and can be yet another cause of stomatal closure described earlier (Bose et al., 2017).

2.2.2 Salt Response of Alfalfa

Plants, including alfalfa, must respond to ionic stress through attempting to restore ionic homeostasis (Cornacchione and Suarez, 2015; Sandhu et al., 2017; Gao et al., 2019). Mechanisms to achieve ion homeostasis can either take the form of transport or compartmentalization. In alfalfa, the SOS (Salt overly Sensitive) signalling pathway is one of the primary pathways responsible for achieving ion homeostasis through transport (Zhu, 2003). In this system, high concentrations of Na^+ ions trigger a release of Ca^{2+} ions in the cytosol which triggers a signalling cascade leading to transport of Na^+ ions out of the cell. Conversely, NHX

genes are related to the alternate mechanism: ion compartmentalization of Na^+ ions within a vacuole within the cell which also prevents damage from occurring (Zhu, 2002).

The response of plants to the osmotic component of salinity stress relates to ROS scavenging and osmotic adjustment. ROS can be produced as a result of stomatal closure, already discussed. To manage this stress, the plant must create ROS scavengers. ROS scavengers can either by enzymatic (i.e. Superoxide Dismutase & Catalase) or non-enzymatic (i.e. glutathione & carotenoids) (Ashraf, 2009), and can act through several mechanisms including oxidation or dismutation; in the former the scavenger brings the ROS to a lower oxidation state by itself being the target of oxidation, and in the latter the scavenger (through dismutation) reacts to make the species into two species: one highly oxidized and the other less oxidized (Das and Roychoudhury, 2014). While the product of these reactions can sometimes be more ROS, the resulting species are less potent and can then be the subject of reactions with further scavengers to make them inert.

Osmotic adjustment becomes necessary when normal water potential between membranes become disturbed and is threatened by turgor loss. Alfalfa responds to this stress through the production of osmolytes (or osmoprotectants). These osmoprotectants are non-toxic organic compounds that can be safely synthesize at high concentrations without inhibiting metabolism. These compounds act by equalizing water potential across membranes (Holmström et al., 1994; Singh et al., 2015). By increasing the solute level on one side of the membrane, the osmoprotectant decreases the water potential and thereby prevents water loss-especially if there is a high-concentration saline solution on the other side of the membrane. Alfalfa produces several osmoprotectants and they can be divided into three classes. These are betaines, polyols/sugars, and amino acids. Osmoprotectants are of particular interest to this paper as they are important for both bacterial and plant salt-stress responses (Wood, 1988; Fichman et al., 2015), and because bacteria can stimulate osmolyte production in plants (Noori et al., 2018). In addition to osmotic adjustment, the production of osmoprotectants may therefore also offer protection to the alfalfa-rhizobium relationship.

2.2.3 Effects of Salinity on Alfalfa-Rhizobium Relationship

Alfalfa naturally associates with (and often depends upon) nitrogen-fixing bacteria rhizobium sp., specifically of the genus *Ensifer* (formerly *Sinorhizobium*) for mineral nitrogen in

N limited system. The typical Rhizobial symbionts of alfalfa are the gram-negative bacteria *Ensifer meliloti* and *Ensifer medicae*, however *E. meliloti* is most prolific partner (Talebi et al., 2008). *E. meliloti* is reported to be much more salt tolerant than its symbiont alfalfa (Zahran et al., 1999), and there are many strains which exhibit even higher tolerances (Zahran, 1999; Talebi et al., 2008). This ability is not even limited to *E. meliloti* strains found in saline soils but can be found in non-saline soils as well (Talebi et al., 2008).

In the mutualistic relationship between legume and rhizobium, the bacterial symbiont fixes nitrogen (N_2) from the atmosphere, converting it into ammonia (NH_3) with the enzyme nitrogenase (Yang et al., 2022). In exchange for this essential macronutrient, the plant provides the bacteria with carbohydrates and an environment suitable for nitrogen fixation (Ott et al., 2005; Ferguson et al., 2019). The plant facilitates an oxygen free environment (as oxygen can inhibit nitrogenase activity) in the nodules by producing leghaemoglobin, an oxygen binding protein (Ott et al., 2005). The mutualistic relationship between bacteria and alfalfa is generally mutually beneficial but is also costly to the host plant as it must divert a large quantity of its photosynthates to the bacteria to support their growth and metabolism (Ferguson et al., 2019). Thus, in times of stress the cost: benefit ratio of this mutualistic interaction may not be favourable for the alfalfa, meaning that the plant may limit or end the relationship through limiting essential nutrients to the bacteria (Prell et al., 2009; Ferguson et al., 2019; Chakraborty and Harris, 2022).

Several aspects of the symbiotic relationship between rhizobium and alfalfa are especially susceptible to environmental stress, including successful nodulation and nitrogen fixing efficiency. Salinity can sabotage inoculation by causing root hairs not to respond to rhizobium (i.e., no curling) or to shrink (Tu, 1980). Salt stress can cause nodules to shrink in size for soybean, chickpea, and alfalfa (Velagaleti and Marsh, 1989; Zurayk et al., 1998; Djilianov et al., 2003). The number of nodules can also be decreased in legumes when exposed to salt stress (Tu et al., 1980). Drought stress can cause a reduction in nitrogen fixation in legume species. as accumulated ROS damage leghemoglobin and nitrogenase (Mhadhbi et al., 2009; Ferguson et al., 2019; Chakraborty and Harris, 2022). While the symbiotic relationship between legume and bacteria is sensitive to stress, it may be possible to strengthen it through improved salinity tolerance of the host plant.

2.2.4 Mitigation of Stress in the Alfalfa-Rhizobium Mutualism

The alfalfa-rhizobium symbiosis may possibly be made more resistant to salinity stress through increased tolerance of both the host and symbiont to salinity. Because nitrogen fixation is the role of rhizobium in the relationship, the continued production of NH_3 in the presence of salinity stress is an indicator of health for the association (Bertrand et al., 2015). Alfalfa is always more salt sensitive than its normal symbionts, *E. meliloti* or *medicae* (Talebi et al., 2008; Bertrand et al., 2015), and when it reaches its salinity threshold it ceases to support its root associated rhizobium through a supply of carbohydrates. Instead, the plant uses these reserves to manage the abiotic stress (Ferguson et al., 2019). Increasing the salinity tolerance of the host plant may therefore increase the salinity tolerance of the symbiosis (Mhadhbi et al., 2009), although this may not be a consistent trend (Bertrand et al., 2015). While this trend may not be universal, it has been shown that soil bacteria (both Rhizobial and non-Rhizobial) can increase salinity tolerance in plants through various mechanisms (Martínez et al., 2015; Noori et al., 2018; Liu et al., 2019b), suggesting that a positive-feedback loop may be possible.

Means by which soil bacteria can increase the salinity tolerance of alfalfa include: increasing the accumulation of osmoprotectants, increasing levels of antioxidants, and increasing nutrient availability (Noori et al., 2018). Mutualistic bacteria can also exert a degree of transcriptional regulation over genes coding for the enzymes that create and degrade osmolytes such as trehalose and proline (López et al., 2008; Noori et al., 2018). Certain soil bacteria can induce decreased expression of the stress hormone ethylene (Liu et al., 2019b). Some Plant Growth Promoting Rhizobacteria (PGPR) can also induce a low shoot: root ratio, possibly increasing the plant's ability to access water (Bertrand et al., 2015). By increasing the salinity tolerance of the host plant, these bacteria may increase the ability and salinity threshold at which alfalfa cease supporting their bacterial symbionts.

High osmolyte concentrations induced by bacteria which protect alfalfa from salinity stress may, by extension, also protect the symbiotic relationship with bacteria. Both bacteria and host plant benefit from these compounds in times of water stress (Sauvage et al., 1983; Elsheikh and Wood, 1995; Zahran, 1999; Ashraf and Foolad, 2007; Fichman et al., 2015; Santantonio et al., 2019). Since certain bacteria can induce increased concentrations of these compounds in alfalfa (Bertrand et al., 2020), and since salt-tolerant alfalfa cultivars produce higher concentrations of osmoprotectants (Bhattarai et al., 2020) it is possible that stacking salt-tolerant,

osmolyte-overproducing bacteria and alfalfa cultivars could yield positively interactive effects on growth and salinity tolerance.

A possible example of such a bacteria may be *Halomonas maura,* a halophilic, gram-negative bacteria which has previously been shown to mediate salinity stress in alfalfa (Martinez et al., 2015*). H. maura* is capable of growth in mediums of 0.5-2.5 M NaCl (Argandoña et al., 2005). It creates biofilms through the production of an exopolysaccharide; *mauran* (Llamas et al., 2006). This biofilm aids in soil water retention, soil aggregation, and protection of plants from pathogens (Llamas et al., 2006). In addition to all of these characteristics, *H. maura* also possesses a facultative anaerobic metabolism which reduces nitrate to nitrite. Llamas et al., (2005) found that no $N0_2$ or N_2 is released from the bacteria, leading them to believe the bacteria change NO_2 to NH_4, meaning the species would not contribute to greenhouse gas emissions or loss of nitrogen from the soil. Lastly, *H. maura* has the ability to fix nitrogen in microaerobic conditions.

2.2.5 Breeding

Alfalfa is a common forage crop around the globe (Li and Brummer, 2012) with approximately 30 million ha cultivated globally (Annicchiarico et al., 2015). It is popular because of its high-protein content, high forage yield, rapid regrowth, and wide adaptability to varied climates and soil types (Kulkarni et al., 2018). In Canada, it is grown on 3.7 million ha of land (Statistics Canada, 2021), making it a staple forage crop for the livestock industry.

The majority of cultivated alfalfa is auto-tetraploid (2n=4x=32), although diploid subspecies exist, with reproduction occurring through cross pollination. There are eight recognized sub-species of alfalfa, with *sativa* and *falcata spp.* being common. The evolutionary relationship between the diploid and polyploid subspecies is complex (Annicchiarico et al., 2015; Yu et al., 2017). The outcrossing nature of alfalfa means that conventional breeding through the creation of inbred lines results in high rates of inbreeding depressions (Annicchiarico et al., 2015). Instead, populations perform best with higher levels of genotypic heterogeneity (Li and Brummer, 2012). Thus, the aim of alfalfa breeding programs is to increase the frequency of favourable alleles in a population without expecting to achieve the almost complete homogeneity seen in inbred lines.

Recurrent selection (with or without progeny tests), mass selection, and the development of synthetic lines are common breeding methods for alfalfa. Recurrent phenotypic selection is a common method where a population of alfalfa is grown in a spaced nursery for trait evaluation and selected superior plants are poly-crossed in a controlled environment; 'Bullseye' is an example of a cultivar developed through phenotypic recurrent selection. Pollen control can be conducted to produce a half-sib progeny family with the known female parent (i.e. Rokebul Anower et al., 2013), or full-sib progeny family with both parents known (He et al., 2022). Mass selection is generally applied to improve highly heritable traits in early generations by selecting superior genotypes from a large population (i.e., 'Bridgeview', as described by Steppuhn et al., 2012). Synthetics lines are created by mixing parent lines; lines can be narrow- (<10 parent lines) or large-based (>50 parent lines). Crossing in this system is based on general combing ability of parents (Annicchiarico et al., 2015). 'Halo', one of the industry-standard saline-tolerant alfalfa varieties, is the result of a large-based synthetic breeding effort with 192 parent plants (Steppuhn et al., 2012).

The rate of gain in relation to alfalfa's traits of interest (predominantly forage biomass) is much slower compared to that of many other crops species. Many estimates put the rate of gain for forage yield below 0.3 %/year for alfalfa (Annicchiarico et al., 2015). Even this small rate of gain may be predominantly the result of increased disease or stress resistance (Lamb et al., 2006). Annicchiarico et al., (2015) postulate that this low rate of gain is caused by the confluence of several factors including alfalfa's outcrossing nature, reduced funding relative to other crops, and complexities arising from alfalfa's autotetraploidy/outcrossing characteristics. Poor rates of gain are partially the result of the long cycles associated with selecting traits in a long-lived perennial; being a long-lived perennial, alfalfa selections may sometimes need to run the span of years rather than a growing season if traits such as persistence or winter survival are being selected for. These factors are compounded by alfalfa programs often suffering from already lower rates of funding than conventional "cash" crops which are utilized in higher profiting industries (i.e., intensive human food production vs extensive livestock production. An additional obstacle is that, to avoid inbreeding depressions alfalfa breeders cannot fix favourable alleles in a population through inbreeding, or fully capitalize on heterosis through the development of F1 hybrids (Annicchiarico et al., 2015).

Fortunately, with the increasing availability of genomics technologies forage breeders are gaining access to powerful "-omics" techniques with which to supplement phenotypic selection. While phenotypic selection is a proven technique, it has limitations including speed and accuracy. While high-throughput phenotyping efforts are currently aiming to mitigate these limitations, alfalfa breeding programs still stand to benefit from the information that genotypic selection and genomics has to offer. Techniques such as Genome Wide Associate Studies, Marker Assisted Selection (MAS), Genotyping by Sequencing, Single Nucleotide Polymorphism arrays (SNP arrays) are all related to finding plant traits which may allow them to succeed in stressful environments. Matching phenotypic traits to validated differentially expressed genes (DEGs) and Quantitative Trait Loci (QTLs) can only increase the effectiveness of current breeding efforts.

2.2.6 Use of Genomics in Salt-Stress Tolerance Determination in *Medicago spp.*

Genome Wide Association Studies can be used to discover various genetic markers (such as SNPs) associated with desirable phenotypic traits. Locations of high SNP abundance which are correlated with a desirable trait, are termed QTLs. Once discovered these QTLs and SNPs can be used in Marker Assisted Selection (MAS) to quickly select genotypes with greater genetic fitness who are more likely to survive in a given environment. Data can be collected for inputting into GWAS through either GBS or technologies such as SNP arrays. However, due to the falling cost of GBS, which captures far more data, GBS is quickly becoming the preferred method by researchers (Medina et al., 2021). In alfalfa breeding, a good example of GWAS being used for salinity tolerance is Liu et al., (2019) who conducted a GWAS study on *Medicago sativa* to find SNPs related to salt-tolerance. The authors found 53 significant SNPs, with the most important markers being correlated with higher dry weight.

While GWAS has been used successfully for selections in tetraploid alfalfa, its use is complicated by allelic dosages. In tetraploid crops, rather than genes having a set number of allelic combinations as is the case in diploid crops (AA, Aa, or aa), more combinations are possible (i.e. Aaaa, AAaa, AAAa, AAAA, aaaa) (Li and Brummer, 2012;Medina et al., 2021). Therefore, some researchers turn to using the aforementioned techniques on the model legume *Medicago truncatula* instead of *Medicago sativa* as this is a diploid version of alfalfa. For example, Kang et al., (2019) conducted a GWAS study to find salt-stress related traits. The

authors found traits such as vacuolar H^+-ATPase production, peroxidase production, and various transcription factors to be associated with salinity tolerance.

Contrary to GWAS studies, RNA-sequencing can be utilized to determine differentially expressed Genes (DEG), or genes whose transcription rate is regulated by the plant. RNA-seq can provide exact reads of mRNA which match to specific genes. These DEGs can then be validated through quantitative reverse transcription polymerase chain reaction (qRT-PCR). RNA-seq is a powerful tool as it not only provides the mRNA reads of putative genes, but also collects data as to the relative abundance of those reads. This relative abundance can be used to determine the causes of phenotypic differences observed in the field under (for example) saline vs non-saline conditions. Where GWAS can aid in selections by predicting patterns in potentially successful genotypes, RNA-sequencing can actually uncover transcriptional mechanisms behind successful phenotypes facilitating, aiding breeders in identifying important metabolic pathways associated with meeting a given breeding objective. The results of salt-stress related transcriptomic experiments are reviewed in the following sections.

2.3 Salt Stress Transcriptomic Analyses in Alfalfa

Recently, the utilization of RNA-sequencing (RNA-seq) technology to examine the DEGs intrinsic to different alfalfa genotypes has been increasing. Studies such as Bhattarai et al., 2021; Luo et al., 2019; Lei et al., 2018; Gruber et al., 2016; Postnikova et al., 2013) all aimed to examine the effects of salinity stress on various tissues in alfalfa in response to salinity stress. These studies found DEGs related to several key components of plant metabolism and function. These included: antioxidants, Ca^{2+} signalling, Na^+/K^+ transport, nitrogen uptake, plasma membrane stabilization, and various transcription factors. None of the listed studies discussed the importance of osmoprotectants, therefore these are discussed at the end of this section because of their relevancy to this paper.

2.3.1 Antioxidants

Oxidation stress is a damaging effect of salinity whereby elements such as oxygen are reduced, their electrons are excited to a higher energy state, and are thus given the ability to oxidize other compounds. This oxidation can damage cell structures and machinery, in extreme cases leading to plant death. It is therefore to be expected that antioxidants and reactive oxygen species

scavengers (ROS scavengers) would be upregulated in tolerant genotypes. While Gruber et al., (2016) report increased transcription for such ROS scavengers as superoxide dismutase (SOD) and peroxidase in tolerant genotypes and Postnikova et al., (2013) found increased transcription in roots for isoflavones and phenylpropanoids (both with antioxidative properties), Lei et al., (2018) found that many ROS scavengers including catalase and SOD were upregulated in a non-tolerant cultivar relative to a tolerant cultivar. The authors theorized that the tolerant cultivar did not experience increased levels of ROS, implying the plant had another mechanism for precluding the issue.

2.3.2 Ca^{2+} Signalling

Ca^{2+} plays an important role in stress signalling in many plants. Specifically, Ca^{2+} signalling is strongly involved in the plant's response to salinity stress. In response to salt shock and plant cell wall damage, Ca^{2+} is released, stimulating the salt overly sensitive 1 protein transporter (SOS1) to begin transporting Na+ ions out of the plant roots (Seifikalhor et al., 2019). Specifically, SOS2 and SOS3 complexes are activated through the release of Ca^{2+} ions, and they in turn stimulate the SOS1 antiporter to begin transporting Na^{+} outside of the cell (Zhu, 2003). Postnikova et al., (2013) found under expression of the inositol-145-trisphosphate gene in tolerant and non-tolerant alfalfa roots, which in turn causes the release of Ca^{2+} ions in the plant cell. Interestingly, Lei et al., (2018) report that a tolerant genotype showed increased transcription for SOS3 in non-saline conditions but showed roughly equal transcription in saline stress. Otherwise, the authors report upregulation of transcription for most other Ca^{2+} related genes in both cultivars.

2.3.3 Na^{+}/K^{+} Transport

Salinity stress can significantly reduce the uptake of K^{+} in plants, as the Na^{+} ions have the same valence arrangement. Na^{+} can therefore outcompete K^{+} ions at uptake sites and cause broadscale potassium deficiencies throughout the plant. Gruber et al., (2016) report a reduction in transcripts associated with K^{+} channels in a salt sensitive alfalfa genotype while they were maintained in salt-tolerant cultivars. Lei et al., (2018) report higher transcription for most (but not all) genes related to K^{+} transport in a salt-tolerant cultivar relative to the intolerant cultivar experiencing salt stress. In comparison, the salt-intolerant cultivar utilized in the experiment experienced a

decrease in transcription for Na^+ transport related genes in salt stress while these genes were almost unchanged in the salt-tolerant cultivar.

2.3.4 Nitrogen Uptake

Nitrogen uptake, in addition to potassium uptake, may be reduced in response to salinity stress. Hu and Schmidhalter (2005) review several studies which show the effects of drought and salinity on plant nutrition; their conclusion regarding nitrogen is that Na^+ and Cl^- ions may be able to effectively compete with NO_3^- and NH_4^+ ions for uptake by plants. Gruber et al., (2016) report decreased transcription of nitrate channels in a salt-sensitive cultivar in salt stress while Postnikova et al., (2013) report increased transcription of nitrogen uptake related genes in a salt-tolerant cultivar in salt stress.

2.3.5 Plasma Membrane

Plasma membranes are complex interfaces which control the juncture between the exterior world and the cell, or between a vacuole and the rest of the cell. These lipid-based membranes are key in relation to salinity tolerance in plants as the embedded proteins and transporters within the membrane control the flow of salts in and out of whichever compartment they house. This can mean excluding (with greater or lesser effectiveness) salt ions from the plant cell, or conversely storing salt ions in special vacuoles to prevent damage to the rest of the cell. The cell membrane is also integral to signalling within the plant, and by extension controls the plant's ability to respond to salinity stress. For example, the SOS1 protein complex, already discussed, is embedded within the plasma membrane and can be stimulated Ca^{2+} ions which in turn are released in response to ROS formed by the RBoH enzyme which is also embedded in the plasma membrane (Banik and Dutta, 2023). Postnikova et al., (2013) found that the gene encoding remorin (a protein embedded in the plasma membrane) was upregulated in a salt-stressed salt-tolerant cultivar. The authors report that this protein, in turn, may help to repair damaged plasma membranes. Lei et al., (2018) found higher expression of vacuolar membrane transporters, suggesting a larger role of compartmentalization than exclusion in both cultivars.

2.3.6 Lipid Peroxidation

Lipids, such as the those which constitute plasma membranes (phospholipid fatty acids/PLFAs) are prone to a special kind of oxidation by ROS. If salinity causes the production of ROS, individual fatty acid molecules can be oxidized which creates an intermediary lipid radical and then a lipid-peroxyl molecule (itself a radical). This lipid peroxyl oxidizes another lipid molecule and then chain reaction continues until there are no more PLFAs to oxidize (Das and Roychoudhury., 2014). Bhattarai et al., (2021) found that the salt-tolerant cultivar utilized in their study showed increased transcription of a gene responsible for producing an enzyme homologous to aldehyde-dehydrogenase which could serve to detoxify toxic aldehydes produced from lipid peroxidation.

2.3.7 Osmolytes

Osmoprotectants, such as trehalose, glycine betaine, and proline (representative of the three classes; sugars/polyols, betaines, and amino acids) are invaluable compounds made by stressed plants to equalize water potential across membranes (Singh et al., 2015). While there are a number of studies examining the effects of salinity on transcription in alfalfa, this paper found only one RNA-seq study which directly examined an osmoprotectant in alfalfa, although the only RNA-seq data reported was that transcription of proline metabolism related DEGs decreased over the course of 2-26hrs of salt stress (Li et al., 2022). In addition, there were several studies which used techniques such as RT-qPCR and/or Northern-Blot analyses. (Guo et al., 2022) Found through RT-qPCR that metabolic transcripts responsible for creating the osmoprotectant proline were upregulated in the presence of salinity stress in alfalfa, whereas catabolic enzymes were decreased. Through RT-qPCR (Jin et al., 2010) Also found that salt stressed alfalfa seedlings were induced to increase expression of enzymes responsible for osmoprotectant enzymes, including myo-inositol-1-phosphate synthase. While there appear to be few RNA-seq studies examining osmoprotectants, there is a plethora of research on their production and function, as well as knowledge pertaining to the genes responsible for their production. The following sections will examine the genes related to the metabolism of three osmoprotectants representative of the three classes of osmoprotectant already mentioned.

2.3.7.1 Trehalose

Trehalose is carbohydrate, and thus part of the sugar/polyol class of osmoprotectants. As an osmoprotectant, Trehalose is capable of stabilizing the water content of cells (Lopez et al., 2008). Trehalose is formed in a two-step reaction by the joining of two glucose molecules by Trehalose-6-Phosphate synthase and Trehalose-6-Phosphate Phosphatase (TPS and TPP) (Ramon et al., 2009). The genes coding for these two enzymes that are sensitive to salinity in *Medicago truncatula* are: (TPS2), (TPS7), (TPS8), (TPS9), and (TPS11) (Song et al., 2021). For T6PP, genes include (T6PPA) and its homologues (Santantonio et al., 2019). The only enzyme responsible for Trehalose degradation is Trehalase, and the production of this enzyme is controlled by the MTTRE1 gene in *Medicago truncatula* (López et al., 2008; Macovei et al., 2019).

2.3.7.2 Proline

Proline is an amino acid which naturally accumulates in alfalfa in response to salt stress (Armengaud et al., 2004). Proline acts as an osmoprotectant similar to trehalose, however in addition it also acts as an antioxidant thereby protecting plant and bacteria from oxidative damage caused by salt stress (Liang et al., 2013).

Enzymes responsible for proline's formation include Ornithine Amino Transferase (OAT), Pyroline-5-Carboxylate synthase 1 (P5CS1), and Pyroline-5-Carboxylate synthase 2 (P5CS2)(Liang et al., 2013). Its degradation is governed by proline Dehydrogenase 1 (PRODH1), PRODH2, as well as Pyroline-5-Carboxylate Dehydrogenase (PC5DH) (Liang et al., 2013). P5CS and PRODH govern the rate-limiting steps (Liang et al., 2013).

Expression of proline synthesis genes is different depending on the kind of osmotic stress the plant is experiencing as well as the individual plant. In most plants, P5CS1 is activated by salt stress, while P5CS2 is a housekeeping gene. In alfalfa, the converse is true with P5CS1 being the housekeeping gene while P5CS2 is salt induced (Armengaud et al., 2004). Expression of P5CS2 is higher in roots than in shoots, however synthate is accumulated in the roots possibly because of increased expression of the OAT enzyme and increased transport of proline from the shoots to roots (Armengaud et al., 2004).

2.3.7.3 Glycine Betaine

Glycine Betaine is a betaine which acts as an osmolyte, and may be able to stabilize nitrogen fixing activity in alfalfa during salinity stress (Pocard et al., 1991). Glycine betaine is produced in both bacteria and in plants (Pocard et al., 1991; Ashraf and Foolad, 2007), and protects cells from losing water during salinity stress. It may also be able to stabilize proteins against stress (Sakamoto and Murata, 2002). Glycine betaine synthesis is governed by Betaine Aldehyde Dehydrogenase (BADH) and the MSBD1 gene (Amjad et al., 2015) as well as Choline monooxygenase (CMO)(rate limiting) (Sakamoto and Murata, 2002; Annunziata et al., 2019). Glycine Betaine is not degraded, and so concentration is dependent upon biosynthesis (Annunziata et al., 2019).

2.4 Induction of Stress Related Genes by Soil Bacteria

Soil bacteria may offer a means by which to augment genetic improvement through regulating the transcription of stress related genes. While there are many mechanisms by which soil bacteria can increase plants' resistance to salinity stress, the mechanism of interest to this paper is the induction of stress related genes. Gene induction occurs naturally in plants in response to stimuli from the environment. Plants possess many DEGs which can be thus stimulated. Transcriptional control of these genes is exerted by the modulation of signalling chemicals, such as abscisic acid, which in turn are stimulated by environmental conditions; environmental stresses such as herbivory, infection, drought, or salinity stress can all stimulate chemical signalling cascades which can serve to alter the transcription of stress related genes (i.e., Fichman et al., 2015).

For example, proline is an important osmoprotectant and ROS scavenger in plants; when a plant receives a signal from a particular organ via alteration in abscisic acid levels that the plant is experiencing salinity stress, the transcription of the MsPC5S2 (Medicago Sativa Pyroline-Carboxylate-5-Synthase) gene could be stimulated (Fichman et al., 2014). This gene codes for the production of an enzyme which creates proline (pyroline-carboxylate-5-synthase) (Liang et al., 2012). When transcription is complete, the mRNA is transported to a ribosome where it is used as a template for the construction of the PC5S2 enzyme. When finished, this enzyme carries out it's function by creating the osmolyte proline. When the nucleus receives a signal that salinity stress has subsided, this MsPC5S2 gene is downregulated and transcription ceases, halting the creation of the PC5S2 enzyme and by extension the osmolyte proline.

Soil bacteria have been shown to be capable of causing plants to increase or decrease the transcription of some DEGs related to stress tolerance in plants. Examples include the modulation of expression in many stress related genes by a strain of *Trichoderma* in rice in response to drought (Pandey et al., 2016) The induced expression of salt-stress related genes in Tomato by a *Streptomyces* strain (Gong et al., 2020), and the up-regulation of genes which inhibit ethylene production in alfalfa by rhizobium in the face of drought (Defez et al., 2017). A host of similar studies are reviewed by Miller and Nielsen (2021).

This induction can occur because bacterial, fungal, and archaeal symbionts live in such close association with their host plants. The intimate relationship often involves the exchange of photosynthate (provided by the plant) for a host of available services from the microbe (N supply, P solubilization, water, defense, etc.). This relationship means that some microbes may have access to different plant signalling pathways, such as the SOS1 pathway used to induce salinity tolerance in Arabidopsis by *Bacillius oryzicola* (Baek et al., 2020). Because of this access these microbes are capable of altering plant gene expression with signals of their own. In times of environmental stress, these symbionts can act to increase the plant's resistance to the stress by manipulating expression of stress-related DEGs.

2.4.1 Effect of Bacterial Inoculation on Osmolyte Production

While soil bacteria are generally capable of mediating the transcription of stress related genes, they may specifically be able to alter the transcription of the osmolytes trehalose, proline, and glycine betaine which are of specific interest to this paper. In their review, (López et al., 2008) examine studies which show that *Ensifer meliloti* may retard the expression of the Trehalase gene, an enzyme which catabolizes trehalose in alfalfa. The authors report that this retardation was uncorrelated with trehalose content, suggesting a more nuanced mechanism of transcriptional regulation than the induction caused by substrate availability. Interestingly, most of the trehalose produced in the studies examined appear to be produced by the bacteroids themselves. As both the bacterial and plant cells benefit from the effects of this osmoprotectant, the decrease in trehalase transcription increases concentration of trehalose and may, by extension, increase the salinity tolerance of the symbiotic relationship between bacteria and alfalfa.

Similar to trehalose, studies have shown that various strains of soil bacteria can induce the production of proline in alfalfa. (Noori et al., 2018) found that proline content was induced in alfalfa in response to inoculation by *Ensifer meliloti, Klebsiella sp. A36, and Kosakonia cowanii* and salinity levels of 0-250mM. Proline can buffer the effects of salinity stress on both bacteria and host plant, and thereby can protect the symbiosis from damage.

Contrary to the mechanisms listed for trehalose and proline, (Mandon et al., 2003) found that transcription of the bacterial *bet* genes in *Ensifer meliloti* responsible for glycine betaine synthesis is controlled by the choline derived from the host plant. The authors also report it likely that the transcription of the host plants glycine betaine synthesis pathway is regulated by the same *betI* repressor as *Ensifer meliloti,* suggesting a complex interplay of transcriptional regulation.

By protecting both plant and bacterial cells from damage, as well as by stabilizing the nitrogen fixing capacity of nodules, osmoprotectants act to protect the symbiotic relationship between plant and bacteria. Ostensibly this could increase the salinity tolerance of the mutualism as a whole. The greater resilience of nitrogen fixation could therefore increase the host plants fitness in times of salinity stress. If this is the case, then understanding the transcriptional relationship between host and bacteria could be of vital importance for the development of saline-tolerant leguminous crops.

3. Effects of Soil-Mutualistic Bacterial Inoculation on Osmolyte Production and Growth in Three Generations of alfalfa (*Medicago sativa*) populations selected for salt tolerance

3.1 Abstract

The halophile *Halomonas maura* and biological N-fixing rhizobium may increase salinity tolerance of alfalfa (*Medicago sativa*), especially for alfalfa populations with improved salt tolerance. The objectives of this study were to 1) evaluate whether salt-tolerant bacteria, or a 60 kg/ha nitrogen amendment could increase the salinity tolerance of alfalfa; 2) assess whether recurrent selection for salt tolerance of alfalfa populations would interact with soil bacteria. To achieve these objectives three alfalfa generations sequentially selected for improved salt tolerance were inoculated with either salt-tolerant (*H. maura*), non-tolerant (*Ensifer meliloti*) bacteria, or a 60 kg/ha nitrogen amendment in either non- (0 dS/m), moderate-(8 dS/m), or highly (16 dS/m) saline soil in the greenhouse. Our results showed that N remains a limiting nutrient in moderate to high salinity stress. Furthermore, this experiment showed that recurrent selection of alfalfa in salinity stress causes several adaptations; however, yield loss occurred under a moderate salinity stress during 120 days of growth. Improved alfalfa generations in combination with nitrogen amendments were able to produce the highest yields in moderate salinity stress. *E. meliloti* had a positive effect during regrowth, and it increased proline content, especially in root tissue. However, *H. maura* provided almost no benefits to plants under salt stress. These results suggest that N is an important nutrient in alfalfa salt tolerance. Recurrent plant selection combined with N-fixing rhizobium could increase alfalfa growth under a multi-harvest system. Improvement of alfalfa traits related to nitrogen utilization and biological N fixation may be vital to alfalfa salt tolerance.

3.2 Introduction

Salinization of arable land is one of the drivers causing a reduction in overall global crop yields and revenue. In 2015, the Food and Agriculture Organization (FAO) reported 412 million hectares and 618 million hectares of land affected by salinity and sodicity, respectively (FAO, 2015). Salinization rate of arable land is set to increase due to high temperature and altered precipitation patterns caused by climate change (Mukhopadhyay et al., 2021). This will be especially true in arid and semi-arid regions of the world (Butcher et al., 2016). In western Canada alone, there are up to 1 million ha of land experiencing moderate to severe levels of soil

salinity (Wiebe et al., 2007). While salinization detracts from the acreage of global arable land, the nutritional needs of a growing global population are also increasing. With this rise in population, demand for animal-based protein has concomittantly increased (Henchion et al., 2021). Finding ways to mediate salinity stress while also supporting the protein demands of a population of over 9 billion people by 2050 (Hunter et al., 2017) necessitates the production of high quality and resilient forage crops for animal production. Alfalfa (*Medicago sativa*) is the most important forage crop in Canada with 3.8 million ha cultivated in 2016 (Statistics Canada, 2021) supporting both the beef and dairy industry. While alfalfa is called the 'Queen of Forages' for its high yield, high feed quality, and beneficial effects on soil health, it is only moderately salt-tolerant (Bhattarai et al., 2020). Nonetheless, by lowering the ground water table with its deep root system alfalfa can preclude the possibility of groundwater depositing salts on the soil surface, thereby mitigating or preventing further salinization.

While alfalfa becomes increasingly susceptible to salinity above 8 dS/m (Steppuhn et al., 2012), genetic improvement of alfalfa may increase its salt tolerance. Alfalfa possesses many salt-stress related genes including genes coding for reactive oxygen species (ROS) scavengers, salt transporters, and of greatest interest to this paper, osmolytes (Bhattarai et al., 2020). Osmolytes, or osmoprotectants, are non-toxic compounds synthesized in the plant that serve to equalize water potential across membranes to prevent water loss (Sakamoto and Murata, 2002). The quantity of beneficial alleles controlling the traits such as osmolytes production in a plant can be increased through conventional breeding (i.e., phenotypic recurrent selection in the case of alfalfa). As osmolytes can also increase the resilience of bacteria in salt stress (Wood, 1988), increasing the quantity of these alleles may create a positive feedback loop between alfalfa and it's natural symbiont *Ensifer meliloti* where both host and mutualists' salinity tolerance increases.

While developing salt-tolerant alfalfa is critical for promoting growth of alfalfa in saline areas, there has also been growing interests in understanding the beneficial effects of soil microbes on salt-stressed alfalfa (Liu et al., 2019). The typical rhizobial symbiont of alfalfa, *E. meliloti*, is a nitrogen-fixing species of bacteria, which naturally infects alfalfa's root hairs forming nodules. Plant-bacteria mutualism is an integral part of alfalfa's life cycle, however, beneficial effects on alfalfa growth can also be realized in association with salt-tolerant bacteria other than rhizobium. Noori et al., (2018) found several non-Rhizobial bacteria endemic to salt stressed alfalfa, in tandem with *E. meliloti,* induced greater root biomass and shoot nitrogen

content compared to controls. Liu et al., (2019) similarly found several strains of halotolerant plant growth-promoting rhizobacteria (PGPR) to increase levels of antioxidant enzymes and certain growth parameters of salt-stressed alfalfa. Other researchers also showed beneficial effects of PGPR such as *Pseudomonas sp., Hartmannibacter diazotrophicus,* or (of interest to this study) the free-living obligate halophile *Halomonas maura* on the growth of salt-stressed alfalfa (Martinez et al., 2015; Ansari et al., 2019). For the interested reader, a review of genetic mechanisms of salt-stress mediation in plants by mutualistic bacteria was recently completed by Miller and Nielsen (2021). While there are many studies examining the beneficial effects of soil microbes on alfalfa, few examine their use in tandem with conventional plant breeding.

Alfalfa, as with all legumes, will abort nodulation by rhizobium under stress (Ferguson et al., 2019), precluding nitrogen fixation. In relation to salinity stress, nitrogen-fixing rhizobium bacteria such as *E. meliloti* always have a higher tolerance than alfalfa (Talebi et al., 2008), which means the relationship between legume and symbiont is likely limited by the salinity tolerance of the host crop. For example, salt-tolerant rhizobium strains were grown with both unimproved and improved alfalfa (Bertrand et al., 2015;Bertrand et al., 2020), and the researchers found increases in forage yield resultant from improved alfalfa genetics. However, these studies were only 4 weeks in duration, which did not consider multiple harvests as would be normal in alfalfa management. Liu et al., (2019) and Martinez et al., (2015) experimented with the use of salt-tolerant bacteria *Halomonas maura* in addition to rhizobium and found an increase in forage yield. However, these studies did not look at genetic improvement of alfalfa.
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This study builds off of the previous work of both Bertrand et al., (2020) and Martinez et al., (2015) by utilizing a similar selection protocol as the former in tandem with the same bacterial inoculants of the latter. In this study it was predicted that soil bacteria could induce increased osmolyte production in alfalfa, and that this induction would improve salinity tolerance and be magnified over the course of multiple cycles of plant selections for salt tolerance in alfalfa breeding populations. To test this hypothesis the objectives of this paper were to assess whether recurrent selection for salt tolerance of alfalfa populations increased levels of the osmoprotectants proline, trehalose, and glycine betaine under salt stress after 120 days of growth. Furthermore, it aimed to assess whether salt-tolerant PGPR (*H. maura*) and/or rhizobium conferred salinity tolerance to alfalfa as compared to single bacterial treatments and/or non-inoculated controls through measuring biomass, chlorophyll, height, and osmoprotectant concentrations.

3.3 Materials and Methods

3.3.1 Experimental Design

The experiment included three treatments: salinity level, alfalfa generation, and bacterial inoculations. The experiment followed a split-plot design with three replicates, with the entire experiment replicated twice resulting in 2 x 3 = 6 total replicates. The bacterial treatment served as the main grouping factor, while salinity level and alfalfa generation were fully randomized within the main factor. Within the salinity treatment there were non-(0 dS/m), moderate (8 dS/m) and high (16 dS/m) salinity treatments. The alfalfa generations consisted of the S906 breeding lines selected sequentially through 3 generations, resulting in populations 1-3 (see details in the Plant Material session), with the salt-tolerant alfalfa cultivar ‘Halo’ serving as the check cultivar. Overall, there were 270 genotypes representing each generation over the course of the experiment (3 plants/pot*6 replications*15 pots/generation=270 genotypes). Within the bacterial treatment, there were five levels: a control (C), a 60 kg/ha nitrogen fertilizer application to compare bacterial nitrogen fixation to mineral nitrogen (CN), a rhizobium (*Ensifer meliloti* strain rm1021) treatment (E), a salt-tolerant PGPR (*Halomonas maura* strain S-31, ATCC 700995) treatment (H), and a combination rhizobium/PGPR treatment (EH). Thus, in each replication unit there were five plots set according to the five bacterial treatments (Control, 60kg/ha Nitrogen, *Ensifer meliloti, Halomonas maura,* and *E. meliloti* + *H. maura*. Within each plot the 12 pots

consisting of four generation treatments (1, 2, 3, and 'Halo') in addition to the three salinity levels (0, 8, and 16 dS/m) were fully randomized.

3.3.2 Bacterial Material

Bacteria were stored in a fridge at 4 ºC until propagated. *E. meliloti* strain rm1021 was sourced from Dr. Trevor Charles' lab at the University of Waterloo and propagated on yeast-mannitol agar plates or in yeast-mannitol growth broth. *Halomonas maura* strain S-31 (ATCC 700995) was sourced from Cedarlane (Burlington, ON, Canada) and was propagated on ATCC 1097 agar and growth broth normalized to pH of 7. In testing the bacteria, *E. meliloti* grew optimally on growth medium with no additional salts added, however in testing was able to grow on 600 mM NaCl yeast-mannitol agar with ~50% growth. Conversely, the halophile *H. maura* displayed optimal growth on 1450 mM mixed-salt ATCC 1097 medium. , also normalized to pH of 7. All handling of bacterial material was done aseptically in a laminar flow hood (Nu475 400 Biosafety Cabinet, MN, USA). Before inoculation of plant material, both strains were grown in their respective growths (YM and 1097) broth to a density of at least $1x10^8$ colony forming units (CFUs) as determined through plate counts.

3.3.3 Plant Material

From 2015-2021, three cycles of plant selections for improved salt tolerance were made using a silica sand based hydroponic system in the College of Agriculture greenhouse at the University of Saskatchewan to develop the three alfalfa generations used in this study. Plant nutrients were supplied using a modified Hoagland's nutrient solution. In the first cycle, alfalfa germplasm with a diverse genetic background were assembled for initial screening at a salt level of 16 dS/m (NaCl solution) to develop alfalfa breeding line S906(1) [Generation 1 (G1)]. In the second cycle, ~1200 genotypes of half-sib progenies of S906 (1) were seeded in the greenhouse and screened at 20 dS/m. The superior performing genotypes were crossed using bumble bees in the greenhouse to develop S906 (2) (G2). The third cycle was carried out on 1500 seedlings representing S906 (2) progenies at a salt level of 20 dS m^{-1} to develop S906 (3) (G3). For the 3rd cycle, salt solution was changed from single sodium chloride (NaCl) of previous cycles to a mix of calcium chloride ($CaCl_2$), magnesium sulphate ($MgSO_4$), sodium sulphate ($NaSO_4$) and NaCl to mimic the mixtures of salts that are commonly found in saline soils in western Canada (Dodd

et al., 1964). All seedlings were watered with salt solution twice per day using an automatic pumping system for a 15min period. The growth stages for evaluation include seed germination, and seedling to mature growth stages. The vigorous, surviving genotypes at the end of the screening process were recovered from salt stress and crossed using bumble bees. Plant selection was based on plant injury score, survival rate of half-sib family, seedling vigor, plant height, and plant biomass. The seeds of S906(3) were harvested in 2021. Seeds were collected from all generations and stored at -20ºC.

3.3.4 Growth Conditions and Experimental Treatments

Along with the check cultivar Halo, 2.4 g of seeds were weighed out per generation. Before inoculation these seeds were surface sterilized by immersion in 70% ethanol for 30s, 5% bleach for 10 min, followed by 6 rinses with sterile distilled water as per Bertrand et al., (2015). Seeds were then placed on pure agar plates and allowed to germinate for 3 days at 25 ºC before transplanting 6 seedlings to a pot in the greenhouse. Seedlings were inoculated with *E. meliloti* and/or *H. maura* by pipetting 0.3 mL of growth broth with at least 1 x 10^8 CFUs/mL on to the base of each seedling the day after sowing. Seedlings were re-inoculated 20 days after the initial inoculation, at which point a 60kg/ha nitrogen amendment was also applied only to plants within the nitrogen-amended treatment. The soil for this experiment was of a silt-loam texture and was sourced from the Livestock and Forage Center of Excellence site (51.95, -106.38) near Clavet, SK. Soil pH averaged 6.1 and electrical conductivity averaged 1.14 dS/m.

Seedlings were grown at 22 ºC, with a 16:8 h light:dark cycle and watered daily as needed with a measuring cup by hand from pre-mixed salt solutions. Other than plants in the nitrogen treatment, no fertilizer was applied. Salinity treatments were applied on the 10th day after planting. To avoid salt shock to the seedlings the solutions were initially applied at a salinity level of 2 dS/m and gradually increased by 2 dS/m every 2 days until target salinity levels had been reached. Salt treatments consisted of mixed salt solutions representative of many saline areas in western Canada (Dodd et al., 1964), and salt ratios were adapted from Steppuhn et al. (2012). Salts included sodium chloride, sodium sulfate, calcium chloride, and magnesium sulfate. The 0 dS/m treatment averaged 0.46 dS/m, the 8 dS/m treatment averaged an actual salinity level of 7.6 dS/m, while the 16 dS/m measured 13.6 dS/m. After 35 days, pots were thinned down to 3 plants.

3.3.5 Phenotypic Data Collection

Plant-growth characteristics, including height, chlorophyll content, and plant count were measured every five days for the first 20 days, and every 20 days thereafter in addition to immediately before each harvest. Stem counts were conducted in the second trial only. Shoot biomass was determined at 60, 90, and 120 days after planting. At 60 & 90 days biomass was harvested to a 5 cm stubble height but was harvested at ground level at 120 days. Root biomass was collected at 120 days. Root biomass was determined after washing roots over a 2 mm sieve. Nodulation was measured on a 0-9 qualitative scale adapted from Howieson and Dilworth (2016) with 0 representing no nodulation and 9 representing ≥20 large nodules. Root and shoot biomass was dried in an oven at 60 ºC for 72 h before weighing. The chlorophyll content was measured using a Spectrum Technology 502 SPAD Meter (Aurora, IL, USA). Three readings per plant were averaged to determine chlorophyll content, with readings coming from the fully developed third, fourth, and fifth leaf from the ground of a randomly chosen plant per pot, respectively. The percentage of surviving seedlings was calculated at 120 days by dividing number of surviving plants by the total number of original plants.

3.3.6 Osmoprotectant Determination

Approximately 1 g of fresh leaf and root samples were clipped and flash-frozen in liquid nitrogen. Samples were stored at -80 ºC prior to analysis. Proline content was determined colorimetrically on all replicates as per Shabnam et al., (2016). Briefly, 0.1 g of sample was ground in 1.25 mL of 3% sulfosalicylic acid. The solution was centrifuged at 10000 rpm for 10 mins. Then, 0.5 mL of the supernatant was mixed with 1 mL of 1.25% ninhydrin/glacial acetic acid and heated to 100 ºC for 30 min. The solution was removed, and the absorbance of the solution was then taken in a cuvette at 508 nm in a Thermoscientific Genesys 10 Bio spectrophotometer (Waltham, MA, USA). Absorbance values were referenced with values from a standard curve to determine proline concentrations.

Trehalose content of the leaves and roots was determined on three replicates from the second experimental run with the Megazyme trehalose assay kit (Megazyme, Wicklow, Ireland) according to the manual. Briefly, 0.5g of frozen plant tissue (root or shoot) was ground and placed in 20 mL of 80 ºC water. The resulting solution was agitated on a Cole Parmer model

4817 magnetic stirrer (Vernon Hills, IL, USA) for 15 min. The solution was then cooled and volume corrected to 25 mL in a volumetric flask. This solution was then filtered through Whatman No. 1 filter paper, and the filtrate stored at 4 ^{o}C until further use. About 0.2 mL of the solution was treated to remove D-glucose as per instructions. Finally, the resulting solution was mixed with supplied solutions and absorbance read on a microplate reader (Thermoscientific Varioskan Lux, Waltham, MA, USA) at 340 nm. Absorbance values were referenced with a standard curve to determine concentrations of trehalose/sample.

Glycine betaine content was determined colorimetrically on the three replicates from the second experimental run as per Senthilkumar et al., (2021) with slight modifications. Because of inherently low quantities of glycine betaine in tissues, 5 mg rather than 1 mg of ground tissue was utilized. Samples were placed in 1.5 mL of 2N sulfuric acid and heated to 60 ^{o}C for 10 mins. The solution was then centrifuged at 14000 rpm for 10 min at room temperature. Exactly 125 uL of supernatant was withdrawn and mixed with 50 uL of 4 ^{o}C KI-I_3. The resulting solution was incubated for 16h in the dark at 4 ^{o}C. This solution was then centrifuged at 14000 rpm at 0 ^{o}C for 30 min. All supernatant was then taken out of the microfuge tube and the pellet was dissolved in 1.4 mL of 1,2 Dichloroethane. After incubating for 2 hrs the absorbance of the solution was measured at 345 nm on a Thermoscientific Genesys 10 Bio spectrophotometer (Waltham, MA, USA). Determination of glycine betaine content was done by referencing absorbance values with those of a standard curve.

3.3.7 Statistics

Data were analyzed in R Version 3.6.3 (R Core Team, 2023). Linear mixed effects models and repeated-measure mixed-effects models were used to analyze most data. Data were square-root or log transformed both when heteroscedasticity was encountered to improve model fit. Packages ‘nlme’, ‘lme4’ and ‘survival’ were used to make models. Models for chlorophyll, height, stem and stem count were structured with repeated measures to capture changes over time and to account for autocorrelation. Biomass, glycine betaine, and trehalose models were linear mixed-effects models because of single/comparatively few timepoints involved. Proline models used a survival-type model to account for right-censorship in the data. Respectively, model structure was:

Chlorophyl/height/stem count<-lme(Response~Bacteria (or Generation)*Salinity*Time, random=1/R/Rep/Bac, data=data, correlation=corARMA(form=~1|R/Rep/Bac, p=2, q=2), na.action=na.exclude)

Biomass/glycine betaine/trehalose<-lmer(Response~Bacteria*Generation*Salinity + (1|R/Rep/Bac, data=data)

Proline<-Survreg(response~Bacteria (or Generation)*Salinity, data=data).

Where R was the temporal replication (1^{st} or 2^{nd}), Rep was the replication (1-6)_, and Bac was the main grouping factor of the split plot design (see section 3.3.4). In both repeated-measures models and survival models only 2-way interactions, (either Bacteria*Salinity OR Generation*Salinity), excluding the 'Time' variable, were modeled before convergence failure was reached. Least Square Means were utilized to determine the means within treatments at P =0.05.

3.4 Results

3.4.1 Chlorophyll

Chlorophyll was only modeled for the 0-60 day range as leaf size in the saline treatment was insufficient after this time. Chlorophyll content was affected significantly by salinity, inoculant, generation, and time (refer to supplemental Table A1 for P-values). In the 0-60 day period, salinity caused a 12.5% increase in chlorophyll content (Table 1) in 8 dS/m relative to 0 dS/m. N fertilizer application increased chlorophyll content 15.2% as compared to the control treatment, and plants in the rhizobium treatment also had an 8.4% higher chlorophyll content than control plants (Table 1). Alfalfa Generation 2 had the highest chlorophyll among generations and the check cultivar where Generation 2 exhibited 6.05% higher chlorophyll content than the worst performer, Generation 1.

Table 1.1. Responses of chlorophyll content, plant height, and stem count to main effects of alfalfa generation, salinity, and inoculant.

	Chlorophyll	Height (cm)	Stem Count (# per plant)
Alfalfa generation (G)			
G1	47.9c	19.8c	4.2ab
G2	50.8a	20.8b	4.0b
G3	49.6ab	22.8a	4.4a
Halo alfalfa	48.5b	20.4b	4.2ab
Standard error of means	1.4	0.9	0.3
P value	0.02	<0.001	0.02
Salinity level (dS/m)			
0	45.5b	25.7a	4.9a
8	51.2a	16.2b	3.5b
16	51.0a	-	-
Standard error of means	1.4	0.93	0.22
P value	<0.001	<0.001	<0.001
Inoculant			
Control	46.2c	20.1	3.6c
60 kg/ha nitrogen	53.2a	21.4	4.3ab
Ensifer meliloti (E)	50.1b	22.9	4.8a
Halomonas maura (H)	47.4c	19.3	3.7c
E + H	49.1b	21.2	4.5ab
Standard error of means	1.5	1.03	0.23
P value	0.03	0.16	<0.001

3.4.2 Plant Height

Plant height responded significantly to salinity, generation, and time (Refer to supplementary Table A1). The significant effect of alfalfa generation consisted of G3 growing 22.8cm, 15.2% taller than the shortest, Generation 1 (Table 1). Plants exposed to 8 dS/m salinity had significantly reduced height (being 36.9% shorter) compared to the non-saline check. Inoculation did not change plant height, but there was a significant inoculant x salinity level, which was due to the taller height of plants receiving applied N or inoculation with *E. meliloti* under salinity (a 14.7% and 11.3% increase over the worst performer, *H .maura* treated plants in 8 dS/m) (Table 2). It is also worth noting that *E. meliloti* treated plants also grew taller than *H. maura* treated plants when exposed to 8 dS/m salinity.

Table 3.2. Responses of plant height and stem count to salinity x inoculant interactive effects during three months of growth in the greenhouse. Letters display significant differences at P<0.05.

	Inoculant	Salinity level	
		0 dS/m	8 dS/m
Height (cm)			
	Control	23.6 ± 1.2^{abc}	16.6 ± 1.1^{c}
	60 kg/ha nitrogen	25.5 ± 1.1^{ab}	17.2 ± 1^{b}
	E	29.1 ± 1^{a}	16.7 ± 0.9^{bc}
	H	23.6 ± 1.1^{abc}	15 ± 0.9^{d}
	E + H	26.6 ± 1^{ab}	15.7 ± 1^{d}
	P value	0.01	
Stem Count			
(# per plant)	Control	3.9 ± 0.2^{bcd}	3.2 ± 0.1^{ef}
	60 kg/ha nitrogen	4.7 ± 0.1^{ab}	3.9 ± 0.3^{cdef}
	E	6 ± 0.5^{a}	3.6 ± 0.2^{bcdef}
	H	4.1 ± 0.2^{bce}	3.2 ± 0.1^{df}
	E + H	5.7 ± 0.4^{a}	3.3 ± 0.2^{cdef}
	P value	<0.001	

3.4.3 Stem Number

Alfalfa stem count was affected by salinity, inoculant, generation, and time (Refer to supplementary Table A1 for P-values). Generation effects consisted of G3 having an average of 0.4 more stems/plant than G2 (Table 1). The salinity treatment of 8 dS/m significantly reduced stem count by 1.4 stems/plant relative to 0 dS/m (Table 1). Inoculant treatments interacted significantly with salinity even while there was no significant generation x salinity interaction. Alfalfa plants inoculated with *E. meliloti* or a combination of *E. meliloti* and *H. maura* displayed the highest stem counts between all levels of the inoculant treatment. Over 120 days under the 0 dS/m treatment. *E. meliloti* and *E. meliloti/H. maura* treated plants had higher stem counts (2.1 and 1.8 more stems/plant, respectively) than control plants, while plants treated with 60 kg/ha nitrogen had marginally higher stem counts (0.8 and 0.7 more stems/plant respectively) than control plants in both 0 and 8 dS/m (Table 2).

3.4.4 Shoot and Root Biomass

Shoot biomass responded significantly to salinity and inoculants at all harvests with a significant interaction between the two treatments except at 60 days (for all P-values refer to supplementary Table A2). Conversely, there was no difference in shoot or root biomass among the alfalfa generations and check cultivar Halo (Table A2). At 60 days, shoot biomass was reduced in 16 dS/m (0.46g +-0.002) and 8 dS/m (0.67g+-0.002) relative to 0 dS/m (1.28g+-0.004) salinity. The inoculant effect consisted of the N amended plants having higher biomass than either control, *H. maura*, or *E. meliloti*/*H. maura* (Fig 1A). A marginal generation effect appeared where G2 had

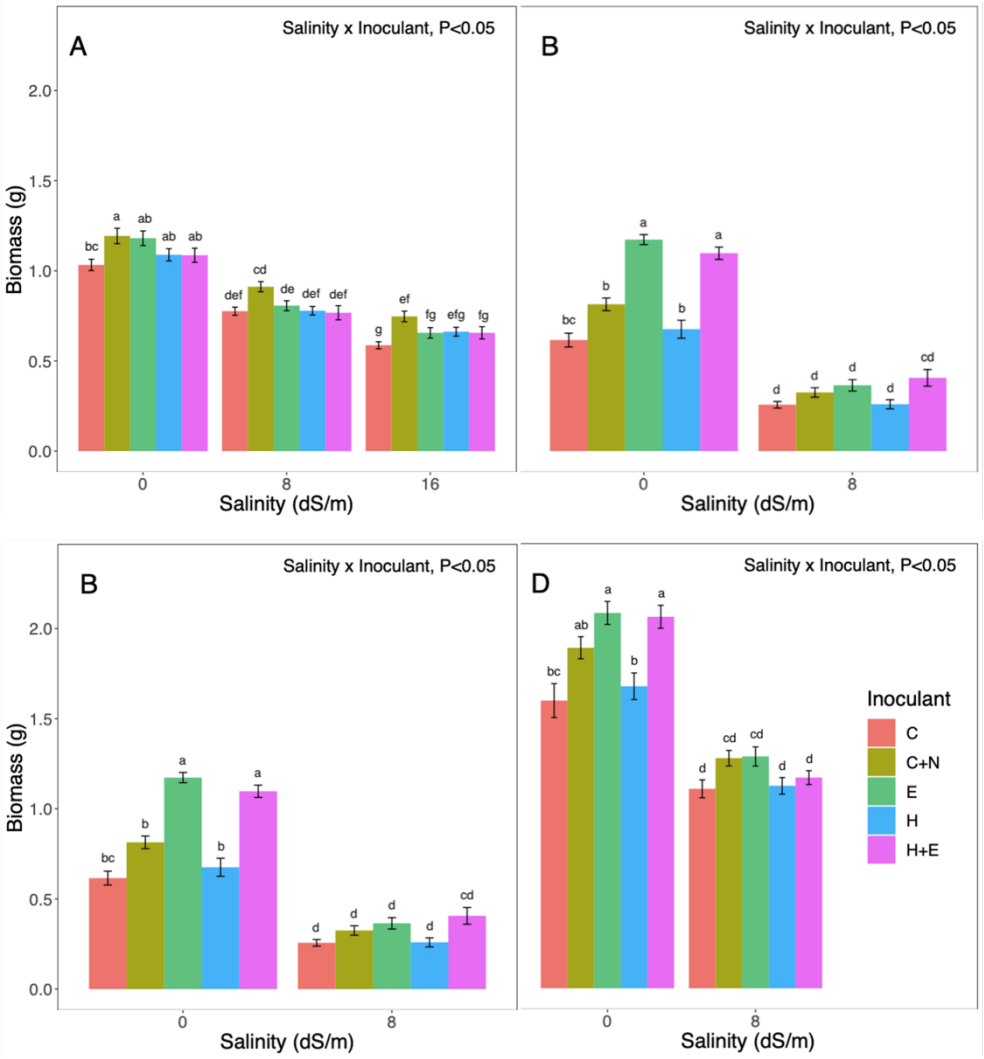

Fig. 3.1. The response of alfalfa shoot biomass A) 60 days, B) 90 days, C)120 days, and D) root biomass at 120 days to a salinity x inoculation treatment. C=Control, CN=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura,* EH=E+H. Different lower-case letters represent difference between treatment at P = 0.05.

higher biomass than G1 (0.86 +- 0.006 vs 0.76 +- 0.005g). We did not detect alfalfa generation x inoculant interaction effects on shoot biomass despite numerically higher biomass of G2 in the N amended treatments at the 1st cut in 8 dS/m (Fig. 3.2A). This effect was not as noticeable at 120 days (Fig. 3.2B). N amended plants grew numerically larger than rhizobium treated plants at 60 days (Fig. 3.1A) with the converse being true at 90 days (Fig. 3.1B), with both treatments appearing equal at 120 days (Fig. 3.1C). It is possible that given a larger population than the modest population used in this project that these results could be significant.

At 90 days, there were a bacteria x salinity interaction, which was because of plants in the *E. meliloti* and *E. meliloti /H. maura* treatments had higher shoot biomass than any other treatment, and N fertilized plants had higher biomass than the control within 0 dS/m, but no difference was found in salinity treatment (Fig. 3.1B). At 120 days, there were the same suite of effects as at 90 days, with the interaction consisting *of E. meliloti* and *E. meliloti* / *H. maura* produced higher biomass than any other treatment within 0 dS/m (Fig. 3.1C).

There was a salinity x inoculant effect on root biomass as E and EH had higher root biomass than *H. maura* or control treatments within 0 dS/m, but no difference was found within other salinity treatments (Fig. 3.1D). The root:shoot (R:S) ratio showed a salinity main and salinity:bacteria interaction effect (Table A2). The interactive effect consisted of nitrogen-amended plants having a higher R:S ratio (4.57 +-0.089) than *E. meliloti* (2.01 +-0.026) within the 0 dS/m treatment where otherwise 8 dS/m had uniformly higher R:S ratio than 0 dS/m (11.49 +-0.190 vs 3.83 +- 0.037)). At 120 days, Root + Shoot biomass, and total biomass (3 cuts + root) all displayed salinity and bacteria main effects and a salinity x bacteria interaction. In all of these categories, the interaction consisted of *E. meliloti* and *E. meliloti* + *H. maura* having higher biomass than all other treatments only within 0 dS/m (Data not shown).

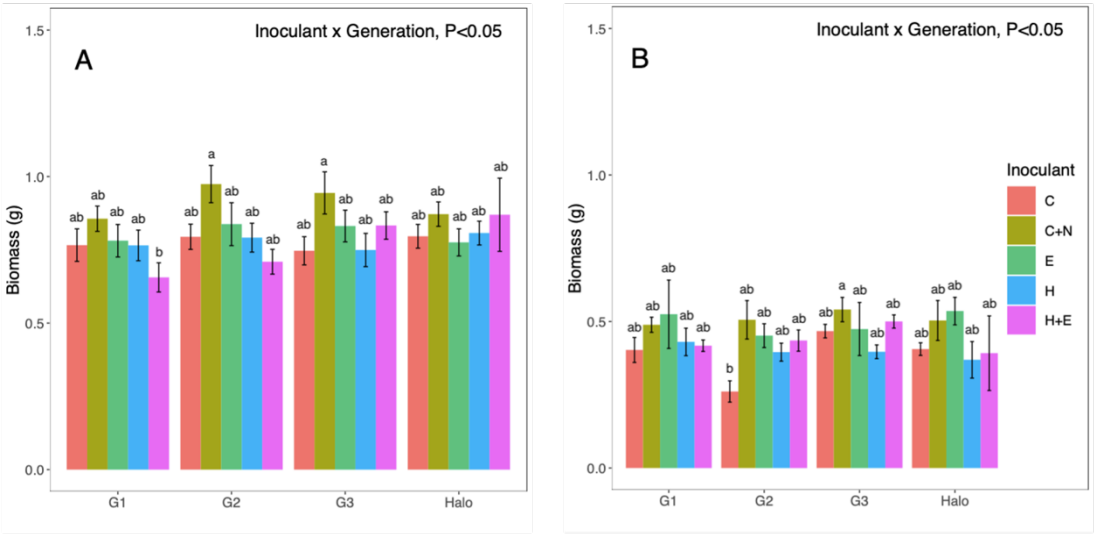

Fig. 3.2. The response of shoot biomass to a generation*inoculant interaction within 8 dS/m at 60 days (A) and 120 days (B). C=Control, C+N=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura,* H+E=H+EC=Control, CN=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura.* EH=E+H. Different lower-case letters represent

3.4.5 Alfalfa Survival

Significant differences in plant survival count were observed between salinity treatments, but no difference was found among alfalfa generation, inoculant, or their interactions (data not shown). There were no mortalities among 0 dS/m plants, however mortality was significantly higher in 8 dS/m than 0 dS/m (59% survival vs 100% survival). There was almost 100% mortality of plants in 16 dS/m plants after 3 months of growth.

3.4.6 Osmolytes

Salinity and inoculant significantly affected shoot and root proline content, but no alfalfa generation main effect was observed for proline content (Table A2). The alfalfa generation x bacteria interaction for root proline content was significant (Table A2). The application of *E. meliloti* and *E. meliloti +/H. maura* elevated proline levels (3.87+/-0.395 and 3.02 +/-0.436 mg/g, respectively) in the shoots, with a lesser increase in nitrogen amended plants (2.48 +/-0.415mg/g). As expected, shoot and root proline content was higher in the 8 dS/m treatment relative to 0 dS/m (Fig 3A-D). The significant bacteria x alfalfa generation interaction effect in

the roots was due to elevated proline levels in shoot of alfalfa G1, G2, and G3 relative to Halo after inoculating with *E. meliloti* (Fig. 3E). While there were no salinity x inoculant interactions on proline content in shoots, the elevated proline levels were most noticeable in 8 dS/m.

Root proline content was also affected by salinity and inoculant main effects (Table A2, Fig. 3F). The salinity effect consisted of 8 dS/m having higher root proline contents than 0 dS/m salinity. The bacteria effect consisted of *E. meliloti* and *E. meliloti* + *H. maura* having elevated levels of proline relative to the control, especially in the 8 dS/m. An inoculant x alfalfa generation interaction manifested in which elevated proline levels were observed in G2 root tissue after inoculating with *E. meliloti* and *E. meliloti* + *H. maura* (Fig. 3.3F).

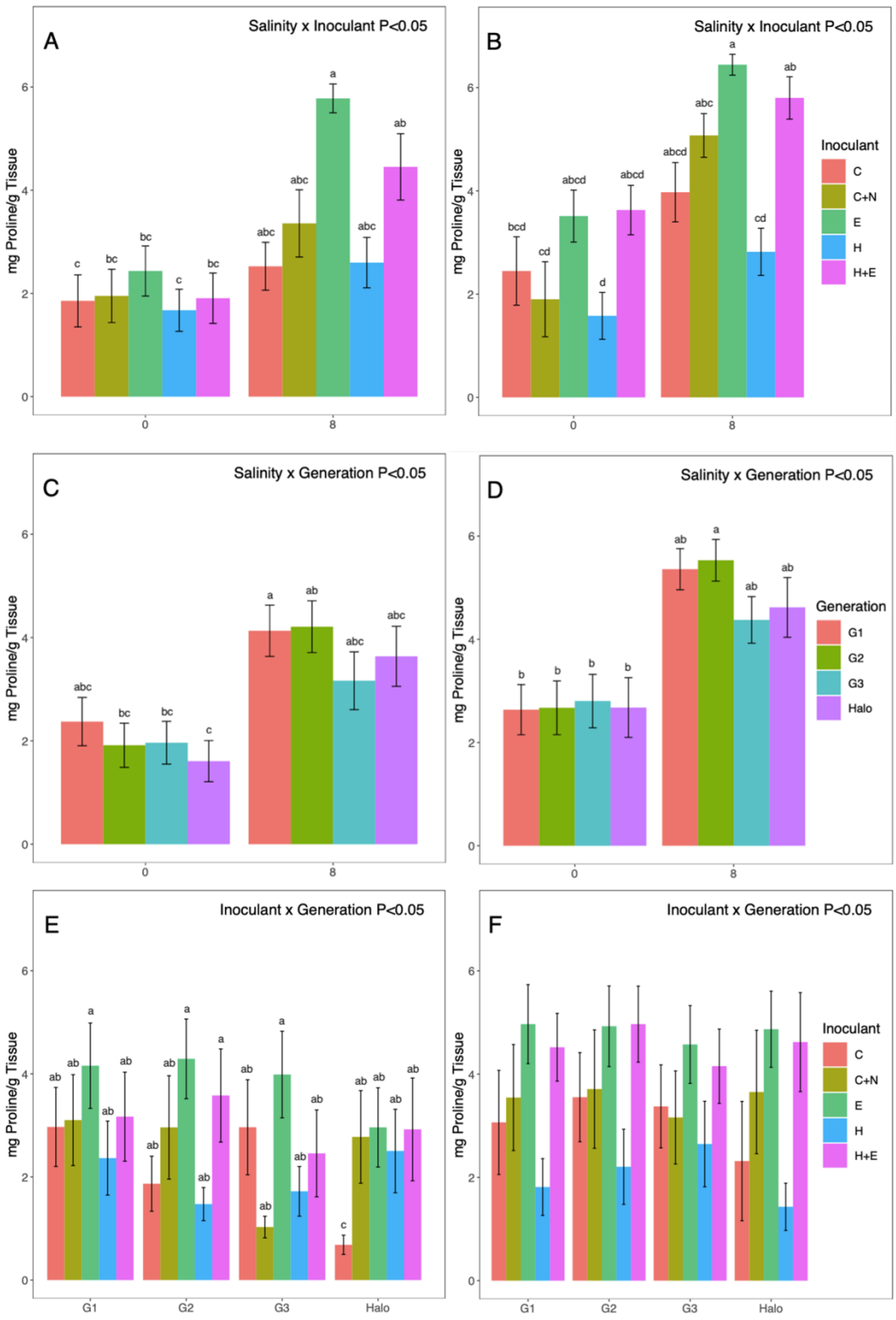

Fig. 3.3. The response of proline in alfalfa shoot (A, C, E) and root (B, D, F) to interactions among salinity, inoculant, and alfalfa generation. C=Control, C+N=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura,* H+E=H+E. Different lower-case letters represent difference between treatment at P = 0.05.

Neither shoot or root glycine betaine content responded to any treatment, but elevated levels of glycine betaine appeared in *E. meliloti* treatment (Fig. 3.4A, B), and was elevated in G2 with exposure to 8 dS/m salinity (Fig. 3.4D, C) although this effect was not significant. Trehalose content was not significantly different among treatments (Table A2, Fig. 3.5A-D), but trehalose did appear to follow the trend of proline and glycine betaine in that it was elevated in *E. meliloti* treatments as well as in G2 alfalfa population in shoot tissue (Fig. 3.5C). Shoot tissue contained noticeably higher trehalose content than did roots (Fig. 3.5A-D).

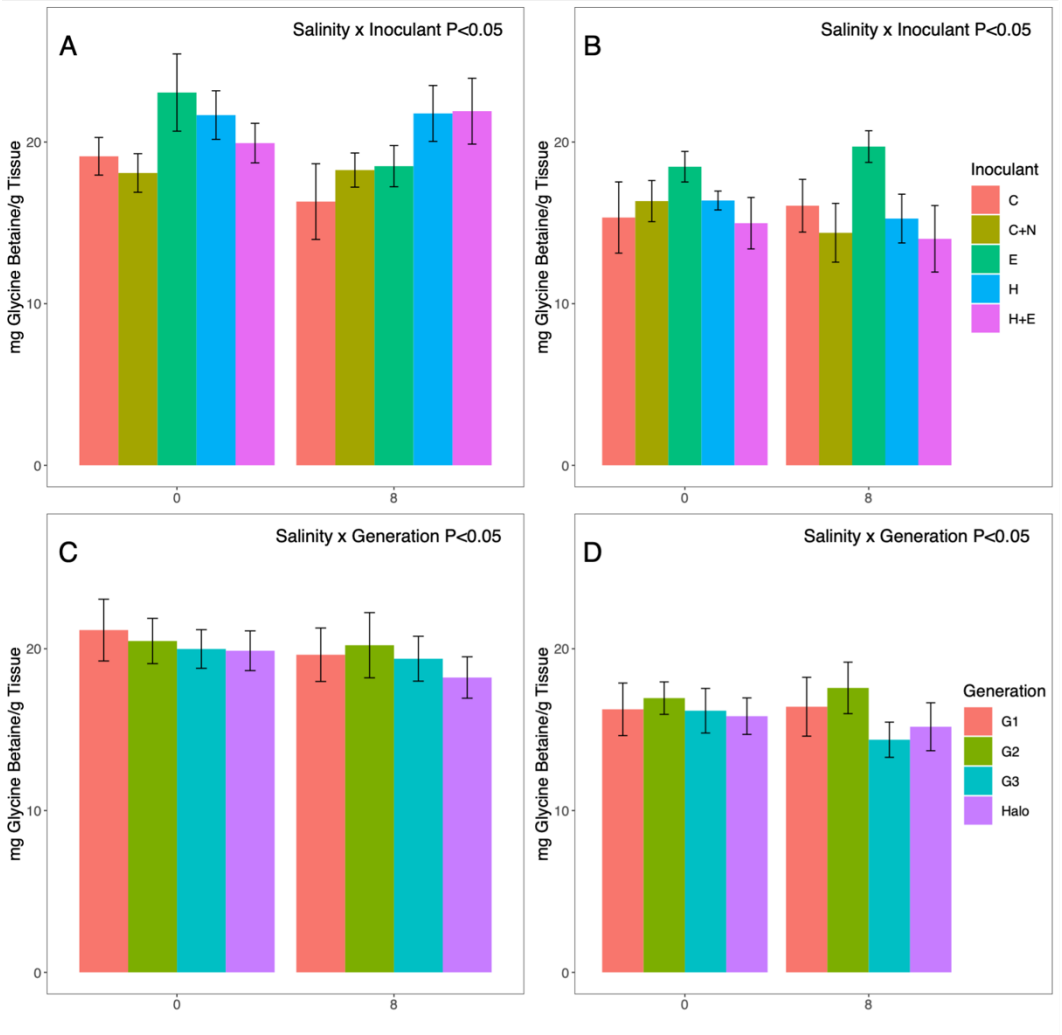

Fig. 3.4. The response of shoot (A, C) and root (B, D) glycine betaine content to salinity x inoculation (A, B) and salinity x generation (C, D) treatments. C=Control, C+N=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura,* H+E=H+E. CLD not shown due to lack of significant effects. Different lower-case letters represent difference between treatment at P = 0.05.

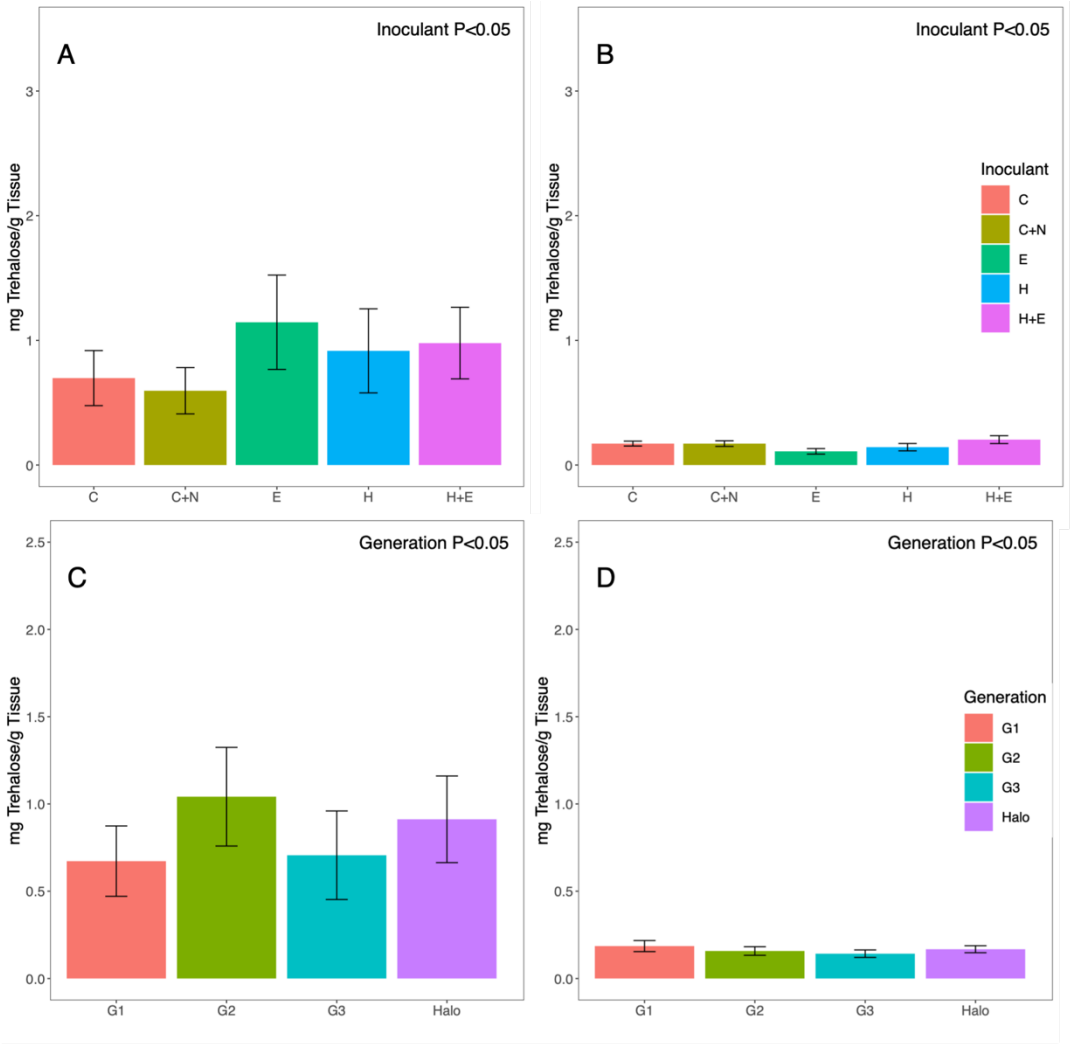

Fig. 3.5. The response of shoot (A, C) and root (B, D) trehalose content to inoculation (A, B) and generation (C, D) treatments. SE shown, N=3. C=Control, C+N=60 kg/ha nitrogen amendment, E=*Ensifer meliloti,* H=*Halomonas maura,* H+E=H+E. Different lower-case letters represent difference between treatment at P = 0.05.

3.5 Discussion

This study aimed to determine whether gains in salinity tolerance of alfalfa due to recurrent selection under salt stress could be further supplemented by the addition of tolerant or non-tolerant mutualistic soil bacteria. No treatment was capable of mediating 8 dS/m or 16 dS/m salinity on biomass for 120 days, although the G2 alfalfa treated with N fertilizer displaying higher shoot biomass at the 1st cut (Fig. 4A). No plants in the 16 dS/m treatment were able to

survive until 120 days. Plant height and chlorophyll were affected by alfalfa selection, for example the G3 alfalfa grew taller than any other generation regardless of salinity level and where G2 generation had the highest chlorophyll content in all salinity levels from 0-60 days. While selection did provide some advantages in salinity (e.g., increased chlorophyll content, height, biomass, and osmoprotectants), G3 was generally less fit than G2, suggesting that intense recurrent selection may lead to inbreeding depression due to a low genetic diversity. Surprisingly, a simple nitrogen amendment appeared to be more effective in alleviating salt stress than inoculating with salt-tolerant microbes.

3.5.1 Effect of alfalfa plant selection

Selection of alfalfa populations for salt tolerance over multiple cycles primarily caused taller plants with increased chlorophyl content in our study. Alfalfa populations used for this experiment were selected under salinity stress from the germination to maturity growth stages for three generations (G1-G3). While the G3 alfalfa population exhibited the greatest height and stem number irrespective of salinity level, G2 otherwise performed the best in relation to growth attributes such as chlorophyl content, biomass, and osmoprotectant accumulation. These results agree with those of Sandhu et al., (2017) who also found tolerant alfalfa genotypes to grow taller than non-tolerant genotypes in 16.6 dS/m. The increase in height of G2 and G3 populations in this experiment may be partly responsible for the numerical increase in yield, especially in the N amended treatment in 8 dS/m salinity treatment at 60 days. Bertrand et al., (2020) reported 20% and 44% yield gains in two alfalfa populations, that underwent 3 cycles of recurrent selection under 12 dS/m salinity stress for height and biomass in a hydroponic flood tank system. Although we did not see a dramatic yield increase in the greenhouse, we speculate that the small yield increase of individual plant (e.g., in the G2 population) may result in a significant yield advantage if tested in a large-scale field trial, although this yield gain may be predominantly non-digestible as increased yield appeared to be from taller/greater number of stems rather than increased number of leaves.

Alfalfa selection for salt tolerance increased chlorophyl content when exposed to salinity, with the G2 generation consistently exhibiting the highest chlorophyl content in all salinity levels until 60 days. The increased chlorophyl content in salinity treatments is also consistent with Sandhu et al., (2017) and Bhattarai et al., (2022) who found increased chlorophyl content in 16.6

dS/m and 12 dS/m salinity stress, respectively. Ashrafi et al., (2014) found chlorophyl content under salt stress to be associated with salinity tolerance, similar to Bhattarai et al., (2022) who also found chlorophyl content to be associated with improved biomass in salinity, suggesting that selecting plants with improved chlorophyl content under salinity stress may produce genotypes which produce higher biomass under salinity stress. In relation to osmoprotectant production in our study, the G2 population consistently produced the numerically highest concentrations of glycine betaine, proline, and trehalose. However, Bertrand et al., (2020) did not find significant effects of alfalfa selection on proline content.

Our results suggest that recurrent selection in alfalfa may increase certain characteristics associated with salinity tolerance (i.e., increased chlorophyl content and plant height). Furthermore, G2 appeared to display increased performance in regard to multiple growth parameters (chlorophyl content, yield, osmoprotectant levels) as compared to alfalfa populations G1 and G3. The fact that performance did not necessarily increase in a linear fashion from G1 to G3 suggests that there mightay either be differential responses to selection between the mixed-salt solution used for selecting G1 and G2, and the NaCl solution used for G3, or there may be an inbreeding depression after several cycles of intensive selection (selection intensity ranging from 2-3% in our study). Thus, it is also important to consider genetic diversity as selection progresses given that alfalfa is an outcrossing species (Annicchiarico et al., 2015). Based on our study, intense recurrent selection over multiple generations might not be an ideal method for improving salt tolerance in alfalfa.

3.5.2 Inoculation Effects

The N fertilizer increased both plant height and biomass in the 8 dS/m salinity level at the first cut. However, the magnitude of the mineral N effect on plant height and biomass in 8 dS/m decreased dramatically after the first cut, likely because of the finite N pool being used up during the period of first growth. As expected, *E. meliloti* inoculation caused consistent increases in biomass of alfalfa plants at 0 dS/m across all cuts, especially at the 2nd and 3rd cut. At 8 dS/m salinity, *E. meliloti* or *E. meliloti* + *H. maura* treatments did stimulate the biomass and height at the 2nd and 3rd cuts, which may have been the result of biological nitrogen fixation as the lone *H. maura* treatment did not benefit alfalfa growth in a noticeable manner the nitrogen fixation was likely done by *E. meliloti*. The reduction in shoot biomass in 8 dS/m salinity relative to 0 dS/m

salinity may be associated with a decrease in nodulation. Previous work has shown that the host legume can control the degree of nodulation it experiences by cutting off the supply of essential nutrients (such as branched chain amino acids and sugars) to the bacteroids (Prell et al., 2009; Ferguson et al., 2019).

While *E. meliloti* and *E. meliloti* + *H. maura* treatments had positive effects on growth, *H. maura* treatment did not increase alfalfa performance under salt stress. This result was unexpected and runs contrary to the findings of Martínez et al., (2015) who used the same species to ameliorate extreme (>20 dS/m) salinity stress in alfalfa. In their study, *H. maura* treated plants grew greater biomass, had higher leaf water potential, and higher leghemoglobin contents than other treatments. The disparity between this study and that of Martinez et al., (2015) may either be because of a different strain used (S-31 for this study and S-30 for their study), because for unknown reasons it may not have survived in the soils used in this project, or because of the much higher salinity levels utilized in their study. *H. maura* is an obligate halophile and performs best in high salinity (Bouchotroch et al., 2001). Regardless, the *H. maura* treatment proved incapable of mediating either 8 or 16 dS/m salinity stress in this study. Future work may benefit from RNA-sequencing of the soil post-project to determine if the inocula used are still present in the soil.

3.5.3 Mineral N Effects

The most noticeable interaction was due to G2 (and to a lesser degree G3) interacting with the N fertilizer treatment to increase shoot biomass in 8 dS/m. The interaction indicates the importance of N supply in salinity tolerance. Nitrogen is essential for the production of osmoprotectants such as proline and glycine betaine, and their levels increased with N application. The lack of significant effect of *E. meliloti* at the first 60 days could be due to a lack of active root nodules during the first two months or because of a differential response of the plant to various forms of nitrogen. De Almeida Paula Figueira and Nunes Caldeira (2005) found that the response of *Pisum sativum* to salinity depended on the form of N fertilizer (NH_4NO_3 vs NO_3^-). The authors found that plants experienced less salt toxicity when fertilized with NO_3^-, however grew larger in non-saline conditions with NH_4NO_3. In Hu and Schmidhalter's (2005) review, the authors report that NO_3^- competes with Cl^- for uptake by the plant, with a similar relationship existing between Na^+ and NH_4^+. Similarly, Tian et al., (2022) found that the legume *Sophora japonica* responded

preferentially to NH_4^+ relative to NO_3^- under salinity stress, such that NH_4^+ treated plants showed increased photosynthesis, higher root antioxidant levels, and increased root NH_4 levels. It may therefore be that the NH_4^+ component of the N fertilizer used in this experiment also provided these same beneficial effects on photosynthesis and ROS scavenging. It is not possible to determine if nitrogen form impacted the success of the G2 by N fertilizer interaction as only NH_4NO_3 was used in this experiment, however this appears to be a promising area for future research.

The timing of nitrogen application may also play a role in salinity tolerance. Mohammadi et al., (2008) report that regrowth in 1.2, 7, and 12 dS/m salinity stress in their study was highly correlated with root N remobilization rates. The authors theorize that previously acquired N may be the key to surviving future salinity as N uptake may be inhibited by salinity stress. While the current study did not combine rhizobium and mineral nitrogen amendments, Elgharably and Benes (2021) conducted such an experiment and found that not only did mineral N stimulate biomass production even in highly saline 15 dS/m soil, but that low concentrations of mineral NH_4NO_3 (30mg/kg soil) actually stimulated rather than retarded bacterial nitrogen fixation (BNF) in 5, 10, or 15 dS/m saline soil; especially in the first of 3 cuts. After the 1st cut bacterial sourced nitrogen played a larger role in plant growth, which matches a non-significant trend in this study where rhizobium played an increasingly important role in 8 dS/m biomass production at 120 vs 60 days relative to mineral N amendments. The authors theorize that soil available N may be key in metabolizing the necessary machinery required for BNF. Both of these works suggest that early N amendments (as was done in the current experiment) may be key in mediating salinity stress.

3.5.4 Osmoprotectants

In our study, proline content in both shoot and root increased with increasing salt stress and the application of *E. meliloti* under non-saline conditions. We did not detect significant glycine betaine, and trehalose changes in response to any treatments. While there were no different osmoprotectant concentrations among alfalfa populations, a significant proline increase was detected in shoot and root tissue of the G2 alfalfa population after inoculating with *E. meliloti*. We also detected similar elevated trends for glycine betaine and trehalose in shoot of G2 alfalfa under *E. meliloti* inoculation. The G2 alfalfa population was selected for an improved salinity

tolerance, and these favorable salt resistant responses in related to *E. meliloti* inoculation is highly desirable. This suggested that salt-tolerant alfalfa population could also enhance its relationship with *E. meliloti* to cope with salt stress. The increased abundance of osmoprotectants associated with *E. meliloti* is also reported in alfalfa nodules by López-Gómez et al., (2014), promoting an increased ability to maintain N-dense enzymes under salinity stress. The increased proline production may link to gene expression change of the host plants as reported by Hidalgo-Castellanos et al., (2019) who found the oat *adc* gene to stimulate proline production. Our study only sampled glycine betaine and trehalose after 120d growth, it might provide certain insights if it has been sampled at various time points throughout the study.

3.6 Conclusions

Three cycles of recurrent selection for salinity tolerance had many interesting effects on growth parameters of alfalfa in our study. Plant selection clearly improved chlorophyll content, stem count, and height under salt stressed. However, three cycles of selection appeared incapable of ameliorating the effects of moderate or severe salinity stress on forage biomass yield over 3 cuts and 120 days of growth. Furthermore, the multiple selection cycles in a single population appeared to cause an inbreeding depression, as G2 population had higher biomass and higher levels of osmoprotectants than did G3. The halophilic bacterium *H. maura* did not increase the survival and salt tolerance of alfalfa populations at 8 dS/m. An application of 60 kg/ha N fertilizer increased alfalfa shoot biomass at the first cut, while the N-fixing rhizobium *E. meliloti* has more positive effects on biomass during regrowth. In our study, the combination of alfalfa G2/G3 populations with 60 kg/ha N was the most effective combination in 8 dS/m salinity, suggesting that N management may be important for salinity tolerance in alfalfa. Osmoprotectant levels increased the most in the *E. meliloti* treatment followed by the 60 kg/ha N treatment at 8 dS/m salinity, with this effect being greatest in the roots for proline and in the shoots for trehalose. This study concludes that while recurrent selection does improve some measures of salinity tolerance, care must be taken that intense selection pressure does not lead to inbreeding depressions.

4. DIFFERENTIAL EXPRESSION OF SALT-TOLERANT ALFALFA IN RESPONSE TO SALINITY *AND ENSIFER MELILOTI* INOCULATION UNDER LONG-TERM SALINITY STRESS

4.1 Abstract

The effects of recurrent selection on the salt-stressed relationship between alfalfa (*Medicago sativa*) and the rhizobium species *Ensifer meliloti* have not been determined on the transcriptional level. This study leveraged a salt-adapted alfalfa population selected by two cycles of recurrent selection either grown in 0 or 8 dS/m salt stress with or without inoculation by *E. meliloti*. RNA extractions were made from eight plants per treatment and sequenced using NoveSeq 6000. Results showed 176 differentially expressed genes (DEGs) either up- or downregulated in response to inoculation of salt-stressed plants. Notable KEGG and NR annotations of DEGs in plants under 8 dS/m stress included V-type ATPases, a chloride anion channel, and a gene involved in the TOR signaling pathway. The only significantly enriched GO term related to inoculated 8 dS/m stressed plants was GO:0000103 which coded for 'sulfate assimilation'. While inoculation did differentially upregulate two genes, AGXT and gcvH, involved in the metabolism of the osmoprotectants proline and glycine betaine, respectively, several other genes possibly responsible for successful alfalfa-rhizobium mutualism were downregulated. This study concludes that while rhizobium may confer a degree of salinity tolerance to salt-adapted alfalfa, these gains may be insufficiently beneficial as the host plant upregulated several genes related to pathogen defense. Future research should focus on the pathogen-defense related genes found in this study to determine their transcriptional patterns in roots.

4.2 Introduction

One of the greatest impacts of salinity stress on legumes including alfalfa (*Medicago sativa* L.*)* is the abortion of bacterial nodulation by the host plant (i.e., rhizobium). As a legume, alfalfa associates with rhizobium in a mutualistic relationship which involves the exchange of fixed carbon from the plant for fixed nitrogen from the bacteroid. Under ambient environmental conditions this exchange is mutually beneficial, however in periods of environmental stress, such as salinity stress, the bacteroids may transition from being net-assets to net-parasites as they drain the plant of carbohydrates (Ferguson et al., 2019). Under these conditions, the host plant will commonly terminate the relationship as it attempts to save valuable carbohydrates for its

own usage, rather than exchanging them for fixed nitrogen (Prell et al., 2009; Ferguson et al., 2019; Chakraborty and Harris, 2022). In effect, the plant's susceptibility to stress has the add-on effect of depriving it of the most essential macronutrient which weakens it even further.

There have been many studies conducted attempting to increase the salinity tolerance of the alfalfa-rhizobium complex by inoculating with salt-tolerant plant-growth-promoting bacteria or rhizobium (Martínez et al., 2015; Noori et al., 2018; Bertrand et al., 2020a). Inoculation can provide such benefits as increased nodulation, yield, and nitrogen content. While inoculation with salt-tolerant bacteria often provides benefits, the already superior tolerance of *E. meliloti* and *E. medicae* to salinity relative to alfalfa (Talebi et al., 2008) suggests that it may be more effective to target improvement of the host plant. It is possible that conventional breeding could provide this necessary improvement in fitness.

While conventional breeding could benefit the legume-rhizobium complex as a whole under salinity stress, the complex relationship between legume and bacteria could hinder elucidating which genes contribute to the improvement. Not only does the frequency of alleles change in alfalfa in response to selection, but the inoculants themselves can differentially stimulate up- or downregulation of Differentially Expressed-various Genes (DEGs) (Defez et al., 2017). This, in addition to the already altered transcription resultant from salinity stress, necessitates an "-omics" approach such as RNA-sequencing.

RNA-seq technique can be invaluable in identifying differentially expressed genes (DEGs) of genotypes in response to complex environmental conditions. Many transcriptome studies over the past decade have targeted salinity tolerance in alfalfa, highlighting large numbers of DEGs contributing to overall salinity tolerance (Bhattarai et al., 2021; Lei et al., 2018; Gruber et al., 2017; Postnikova et al., 2013). Findings showed that tolerant genotypes displayed increases in transcription of genes related to ROS scavenging, Ca^{2+} signaling, ROS scavengers, Na^{+} /K^{+} transport, and plasma membrane protection. Increased transcription of the Ca^{2+} signaling pathways, including the Salt Overly Sensitive (SOS) series of pathways (i.e., SOS1, SOS2, & SOS3) which stimulate many stress-related pathways including the SOS1 Na^{+} /K^{+} antiporter was also a sign of salinity tolerance. Tolerant genotypes also showed increased transcription for various ROS scavengers, such as superoxide dismutase and increased transcription for K^{+} transporters which would decrease K^{+} deficiencies resultant from Na^{+} buildup. While there have

been many studies examining the effects of breeding on RNA-transcription, few have examined the effects of inoculation on salt-adapted genotypes.

To begin addressing this knowledge gap we conducted an experiment whereby an improved population of alfalfa was grown in non-saline or moderately (8 dS/m) saline conditions with or without inoculation with *Ensifer meliloti*. We predicted that inoculation would stimulate altered transcription of genes related to both nitrogen metabolism as well as stress-related DEGs (i.e., ROS scavengers, osmoprotectants, calcium signaling, etc.). The objectives of this project were thus to 1) determine the effect on mRNA transcription of an improved alfalfa generation exposed to 8 dS/m salinity vs non-saline conditions, and 2) determine the gene expression profile of alfalfa in response to rhizobium inoculation at 8 dS/m salinity.

4.3 Materials and Methods

4.3.1 Experimental Design

The experimental set-up is described in Chapter 3. Briefly, it included three factors: salinity level, alfalfa generation, and bacterial inoculation. The experiment followed a split-plot design with three replications and the entire experiment was replicated twice. Bacterial treatment was the main grouping factor with 5 levels (control, control + nitrogen, rhizobium (*Ensifer meliloti*), a halophytic plant-growth promoting rhizobacteria (PGPR, *Halomonas maura*), and a combination of rhizobium/PGPR), while three salinity level (0 dS/m, 8 dS/m, and 16dS/m) and three alfalfa generations (generation 1, 2, and 3) along with check cultivar 'Halo' were fully randomized within the main factor. The alfalfa generations consisted of the S906 breeding lines selected sequentially by three breeding cycles.

4.3.2 Bacterial Material

The rhizobium treatment was as described in chapter 3. It consisted of *Ensifer meliloti* strain rm1021. Bacteria were stored in a fridge at 4 ^{o}C until propagation. Bacteria were propagated on yeast-mannitol agar plates and in yeast-mannitol growth broth. All handling of bacterial material was done aseptically in a laminar flow hood (NuAire Nu475 400 Biosafety Cabinet, Plymouth, MN, USA). Before inoculation of plant material, both strains were grown in growth broth to a density of at least 1x 10^{8} colony forming units (CFUs) as determined through plate counts.

4.3.3 Plant Material

Plant material consisted of the population resultant from the 2nd selection cycle, already described in chapter 3. Briefly, alfalfa germplasm with a diverse genetic background were assembled for initial screening at a salt level of 16 dS/m (NaCl solution) to develop alfalfa breeding line S906(1) [Generation 1 (G1)]. In the second cycle, ~1200 genotypes of half-sib progenies of S906 (1) were seeded in the greenhouse and screened at 20 dS/m. The superior performing genotypes were crossed using bumble bees in the greenhouse to develop S906 (2) (G2). All seedlings were watered by salt solution twice per day using automatic pumping system for 15min/period. The growth stages for evaluation include seed germination, and seedling to mature growth stages. The vigorous, surviving genotypes at the end of the screening process were recovered from salt stress and crossed using bumble bees. Plant selection was based on plant injury score, survival rate of half-sib family, seedling vigor, plant height, and plant biomass. Seeds were collected from all generations and stored at -20C.

4.3.4 RNA Isolation, Library Preparation and Sequencing

Plant tissue samples were collected at the time of harvest, 120 days after sowing from and leaves. They were from 0 and 8 dS/m treatments and control & rhizobium treatments within Generation 2, as Generation 2 displayed the highest levels of saline-tolerant traits (See Chapter 3). Samples were lyophilized in liquid nitrogen before being stored in a -80 C freezer until analysis. A Qiagen RNeasy Plant mini kit ()Qiagen, Hilden, Germany) was used to extract RNA as per the manufacturer's instructions. Briefly, plant tissues were ground in liquid nitrogen and homogenized with a QIAshredder during centrifugation. Ethanol was added to bind with RNA. The resulting solid was washed 3x in the buffers provided and the RNA eluted in RNA free water. After collection, 40 uL of resulting suspended mRNA was sent on ice to Novogene (California, USA) for sequencing on an illumina Novaseq 600 platform.

4.3.5 Mapping and Differential Expression Analysis

The file quality of raw reads was checked against the MD5 checksums. Reads were cleaned and analyzed through the Digital Research Alliance of Canada, Narval Node. The raw reads were cleaned of adapters using Trimmomatic v.0.36 software (Bolger et al., 2014), with threads=4,

phred33, and default quality cut-off values. Once prepared, trimmed reads were matched to the reference genome available at (https://doi.org/10.6084/m9.figshare.12327602.v3) (Zeng, 2020) using STAR (v2.6.1a)(Dobin et al., 2013) from within the quantification software 'RSEM'. Read counts were conducted using the software 'RSEM' (Li and Dewey, 2011). Differential expression analysis was conducted using the R package 'DESeq2' (Love et al., 2014), with DEGs identified using a Log fold change (Log2FC)>2 and a false discovery rate (FDR)/adjusted P<0.001. P values are adjusted in DESeq2 using the Benjamini and Hochberg method. Significant DEGs were calculated separately for both salinity (8 vs 0 dS/m) and inoculant (inoculated vs non-inoculated) treatments as well as together with an interaction term (inoculated 8 dS/m plants vs non-inoculated 8 dS/m plants).

4.3.6 Functional Annotation and Analysis

Functional annotations were retrieved for significant DEGs from the functional annotations previously done by Zeng et al., (2020)), gene ontology (GO) terms, KEGG KO terms, including NR BLASTp. KASS analysis was also conducted with DEG associated protein sequences (supplied by Zeng et al., 2020) to elucidate relationships between DEGs in KEGG metabolic pathways. Furthermore, gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment tests were conducted. GO enrichment analyses were conducted using the R package 'topgo'; GO terms were tested using Fishers exact test which utilizes a hypergeometric distribution and considered enriched with a P<0.05. KEGG enrichment was also done in R with a hypergeometric calculation and with significance determined at P<0.05.

4.4 Results

4.4.1 High Throughput Sequencing and Assembly

The NovaSeq 6000 Illumina system generated 173,023,874 total paired end raw reads for the 8 samples sequenced. Of the paired-end read, 171,165,017 (98.9%) remained after trimming. 94.91% of trimmed reads were mapped to the reference genome using STAR (v2.7.9). Samples ranged from 91.96%-95.97% of reads mapped to the reference genome. For information regarding raw and trimmed read counts and lengths, refer to table A2.

4.4.2 Identification of DEGs

Among all treatments there were many up- and down-regulated DEGs identified after mapping to the reference genome. Inoculated plants displayed 364 DEGs which were up- or downregulated relative to non-inoculated plants. These included 173 (47.5%) upregulated and 191 (52.5%) down-regulated DEGs. Between 8 and 0 dS/m there were 320 DEGs with 158 (49.4%) upregulated and 162 (50.6%) downregulated in 8 dS/m vs 0 dS/m treated plants. In the interaction between Salinity x Bacteria (interaction DEG pool), there were 179 DEGs with 76 (42.5%) up-regulated and 103 (57.5%) down-regulated in inoculated 8 dS/m treated plants vs non-inoculated 8 dS/m plants. For comparisons of DEG pool sizes, see Fig 4.1.

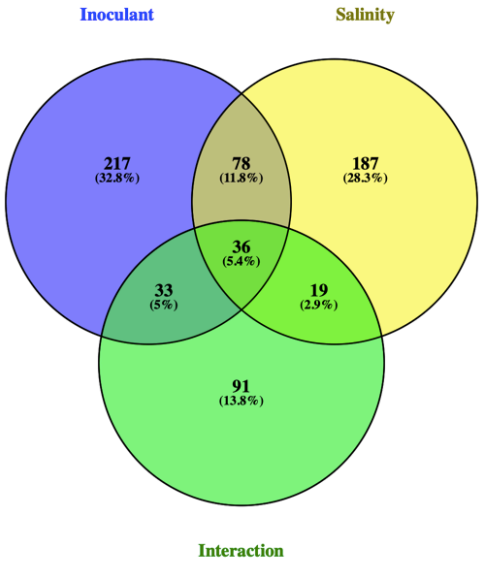

Fig. 4.1. Venn diagram showing the overlap between different pools of DEGs, made using the Venny tool (Oliveros, 2015).

4.4.3 GO Enrichment

Annotated genes were tested for significant enrichment in the three GO categories, Molecular Function (MF), Biological Process (BP), and Cellular Component (CC). There was a total of 12 enriched GO terms across all categories. Within the MF category there were no enriched GO terms annotated to salinity, inoculant, or interaction DEGs. Within BP there were 2 significant GO terms; GO:0001505 (regulation of neurotransmitters) which was annotated to 2 significant DEGs within the salinity DEG pool, and GO:0000103 (sulfate assimilation) which was annotated

to 1 gene in the interaction DEG pool (see Table. 4.3 for transcript IDs). Within the CC category there were 10 enriched GO terms, all within the salinity DEG pool and which are listed in table 3. For the proportion of total DEGs assigned to a GO category, refer to Table 1. Significant genes annotated to GO terms are given in Table 4.1.

Table 4.1. Showing the number of total DEGs as well as the number and percent annotated with GO categories. MF-Molecular Function, BP-Biological Processes, CC-Cellular Component.

Pool	Category	GO Annotated DEGs	Total DEGs	Percent of Total
Salinity	MF	226	320	70.6
	BP	15	320	4.7
	CC	10	320	3.1
Inoculant	MF	230	364	63.2
	BP	14	364	3.8
	CC	16	364	4.4
Interaction	MF	126	179	70.4
	BP	5	179	2.8
	CC	5	179	2.8

Table 4.2. Genes associated with all enriched GO terms, as well as KEGG or NR annotations (if present).

Gene	GO	KEGG	NR	GO Term
MS.gene21474.t1	BP	K02437	XP_003611464.1	GO:0001505
MS.gene012657.t1	BP		XP_003610457.2	GO:0001505
MS.gene006381.t1	BP		XP_013469727.1	GO:0000103
MS.gene067465.t1	CC		KEH39627.1	GO:0098588:GO:0031090:GO:0043227:GO:0043231
MS.gene50737.t1	CC	K02154	XP_003607000.1	GO:0098588GO:0000220GO:0033179GO:0031090GO:0005773GO:0005774 GO:0016471GO:0033176GO:0043227GO:0043231

Table 4.3. significantly enriched GO terms in the Cellular Component category within the salinity pool of differentially expressed genes. Total and significant genes annotated to a term are given.

Salinity	GO.ID	Term	Annotated	Significant	Expected	Fisher
	GO:0098588	bounding membrane of organelle	40	2	0.2	0.016
	GO:0000220	vacuolar proton-transporting V-type ATPase, V0 domain	5	1	0.03	0.025
	GO:0033179	proton-transporting V-type ATPase, V0 domain	5	1	0.03	0.025
	GO:0031090	organelle membrane	52	2	0.26	0.027
	GO:0005773	vacuole	9	1	0.05	0.045
	GO:0005774	vacuolar membrane	9	1	0.05	0.045
	GO:0016471	vacuolar proton-transporting V-type ATPase complex	9	1	0.05	0.045
	GO:0033176	proton-transporting V-type ATPase complex	9	1	0.05	0.045
	GO:0043227	membrane-bounded organelle	71	2	0.36	0.047
	GO:0043231	intracellular membrane-bounded organelle	71	2	0.36	0.047

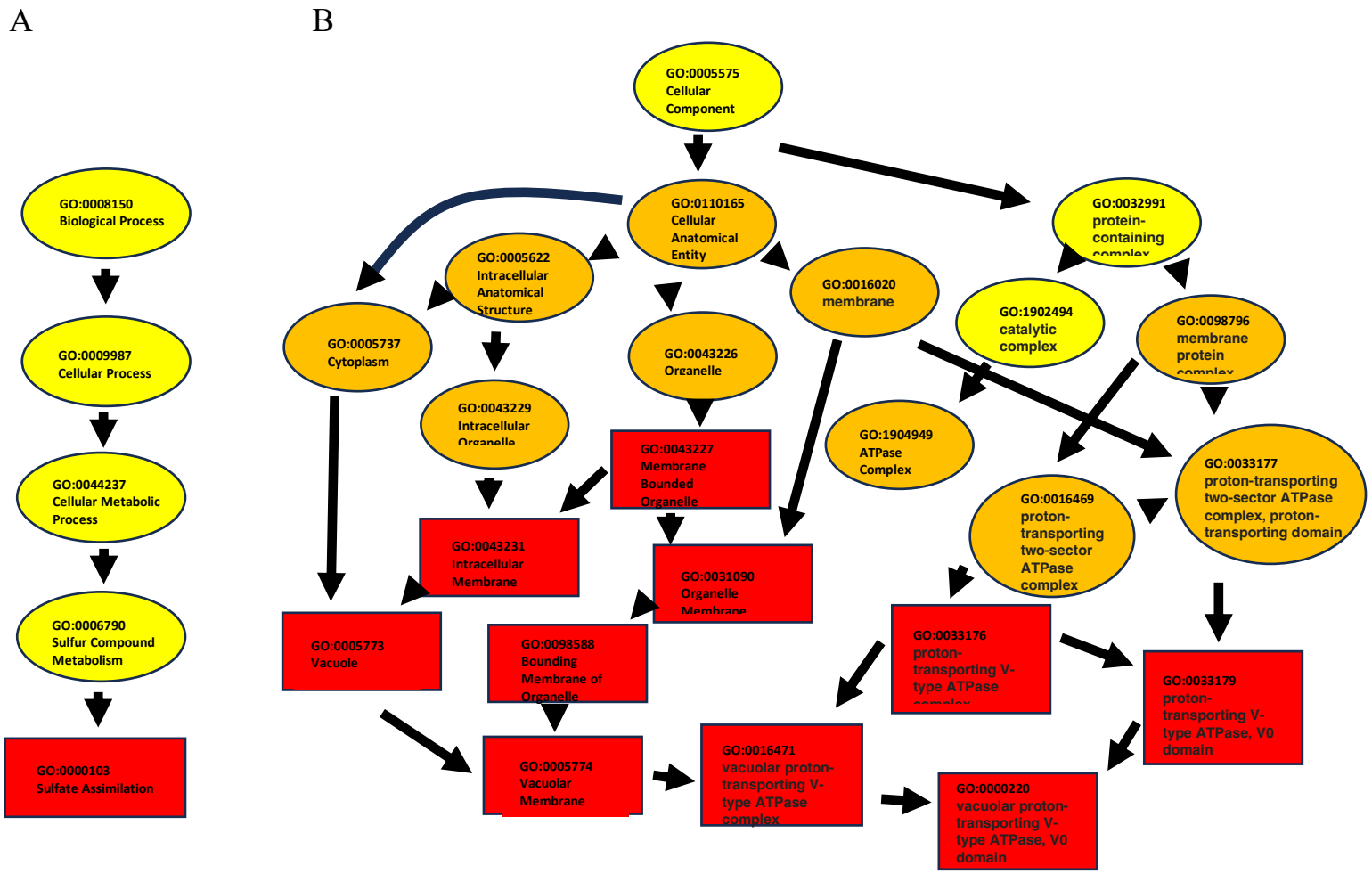

Fig. 4.2. Biological process GO graph (A) of enriched terms annotated to inoculation:salinity DEGs and Cellular Component GO graph (B) annotated to salinity DEGs. Boxes represent significant GO terms. Red-orange-yellow represents most-least important terms by P value (not shown). P values and the number of significant/total annotations given in each box. Enrichment based on Fishers exact test.

4.4.4 KEGG Enrichment/KASS

Total DEGs were assigned to 154 unique KEGG terms (Table S1). Of the 320 salinity-related DEGs, 85 (26.6%) were assigned to KO terms. Of the 364 inoculant-related DEGs, 90 (24.7%) were assigned KO terms. Of the 179 DEGs expressed in inoculation:salinity interaction pool, 46 (25.7%) were assigned KO term. There were no significantly enriched KO terms. For KEGG ontology terms and the associated number of DEGs in a given DEG pool refer to Table S1.

The most common KEGG term in the Salinity pool (annotated to 3 DEGs) was K17606, coding for the TAP46 protein. Also, of note was K05016, coding for chloride channel 7 (annotated to 2 DEGs), K09489 coding for the heat shock 70 kDA protein 4 (annotated to 2 DEGs), and K09489 the coding for the enhanced disease susceptibility 1 protein. The most common KEGG terms (annotated to 2 DEGs each) in the Bacteria pool were K18875 (EDS1 enhanced disease susceptibility 1 protein), K10523 (SPOP speckled-type POZ protein), K17302 (COPB2/SEC27 protein coatomer subunit beta), K00966 (GMPP protein, mannose-1-phosphate guanylytransferase), K11253 (H3, histone H3), and K22520 (LQY1, protein disulfide isomerase).

There were no KEGG terms annotated to more than 1 DEG in the interaction gene pool; for all interaction KEGG terms refer to table S1. K00830 (annotated to 1 DEG in the interaction pool) is of note as it is associated with the glycine, serine and threonine metabolism similar to K02437.

Of the DEGs associated with enriched GO terms, MS.gene21474.t1 was also associated with K02437, or the “glycine cleavage system H protein” which is involved the “Glycine, serine and threonine metabolism” pathway which produces glycine betaine. KASS additionally identified a matching gcvP protein. The KEGG term associated with GO enriched gene MS.gene50737.t1 was K02154 or “V-type H+-transporting ATPase subunit a” responsible for solute levels.

4.5 Discussion

This experiment was run to determine if there were any differential gene expression effects induced by the inoculant *Ensifer meliloti* on a salt-tolerant alfalfa population grown in saline (8 dS/m) or non-saline (0 dS/m) conditions. Many DEGs related to salinity stress (320 DEGs) and

inoculation (364 DEGs) were identified, however there were fewer for the interaction between the two (179 DEGs). Of these DEGs, it was possible to match 78.4% of salinity-related DEGs to GO terms, 71.4% of inoculant-related DEGs to GO terms, and 76% of interaction-related DEGs to GO terms. Of the 12 significantly enriched GO terms, 10 were associated Cellular Component in the 8 dS/m vs 0 dS/m gene pool. These GO terms all referenced membranes, vacuoles, and/or proton pumping. The remaining 2 enriched GO terms were associated with BP, with one related to 8 dS/m vs 0 dS/m salinity, and one related to the inoculant:salinity interaction gene pool. The former referenced "regulation of neurotransmitters", and the latter "sulfate assimilation". Enriched GO terms were associated with 5 significant DEGs, 2 of which were associated with KEGG terms: K02437 and K02154. K02437 codes for the "glycine cleavage system H protein" while K02154 codes for the "V-type H+-transporting ATPase subunit A".

4.5.1 Salinity Effects

Salinity was associated with 320 genes which were differentially expressed in 8 dS/m vs 0 dS/m affected plants. There were 11 enriched GO terms in the salinity DEG pool. These included GO:0001505, coding for the now obsolete "regulation of neurotransmitter levels" as well as the 10 GO terms listed in Table 1. All of the enriched CC GO terms were associated with two genes; MS.gene067465.t1 and MS.gene50737.t1. All of the ten CC GO terms referenced cell membranes, intracellular organelles (including vacuoles), or membrane transporters. Previous transcriptomic studies confirm this finding, with many (Postnikova et al., 2013; Gruber et al., 2017; Lei et al., 2018; Bhattarai et al., 2021; Li et al., 2022) reporting genes related to ion transport and storage being vital to the salt-response of tolerant alfalfa.

4.5.1.1 Ion Transport/Storage

MS.gene50737.t1, annotated to the GO term GO:0000220 "V-type ATPase" and the KEGG term K02154 which also codes for V-type H+ transporting ATPase subunit a. The V-type (V=vacuolar) transporting ATPase is an ATP powered H+ transporter which creates a proton gradient across membranes. This unit is made up of 12 subunits, ATPase A-H as well as ATPase -a, -c, -d, and -e (Kluge et al., 2003). V-type ATPases are important for the stress response of the plant whereby they are capable of compartmentalizing toxic ions (Koyro et al., 2014);Zhang and Blumwald, 2001). Bhattarai et al., (2021) also found altered transcription of the PMA1/PMA2 H

transporting ATPase in a salt-tolerant alfalfa cultivar, while Wang et al., (2022b) found downregulation of H^+ ATPases in root tips of 15 dS/m stressed and inoculated alfalfa, suggesting that the H^+ ATPase in this study is an important component of alfalfa's salt-stress response, and that it either is transcribed differentially in different genotypes or in response to differential salinity stress, while Postnikova et al., (2013) also identified DEGs related to H^+ ATPases in response to salt stress.

The upregulated gene MS.gene067465.t1 associated with GO:0043231 (intracellular membrane bounded organelle) had no associated KEGG term, however it was associated with the NR accession KEH39627 which codes for a SNAP receptor complex protein (aka SNARE protein). SNARE proteins are also necessary for transport and storage of toxic ions during salinity stress; while we did not find reference to SNARE proteins in alfalfa, they have been shown to be highly expressed in wild rice (*Oryza australiensis*) and asparagus bean (*Vigna unguiculata*) when experiencing ionic stress (Pan et al., 2019; Yichie et al., 2019).

In addition to the previous, 2 DEGs coding for chloride channels were also upregulated in 8 dS/m treated alfalfa. Chloride channels (CLCs) are anion transporters, which transport Cl- and NO3- (He et al., 2023). In this study, two genes were annotated with KEGG K05016 (Chloride Channel 7), however an NR protein database search returned two separate CLCs: CLC-a and CLC-c. CLC-a is a nitrate selective transporter while CLC-c is more analogous to the mammalian CLCN7, which is more chloride specific (Hechenberger et al., 1996;Wei et al., 2019). CLC channels are important for maintaining a healthy ratio of Cl^-:NO_3^-, as excess Cl^- can impede nitrate assimilation. In soybean, *Glycine max,* it was shown that CLC channel proteins were important for Cl^- management under salinity stress (Wei et al., 2019). Both of the DEGs associated with CLCN7 were upregulated under 8 dS/m salinity stress, indicating that the salt-tolerant population used in this study had an intrinsic mechanism to attenuate chloride stress. In the previous chapter it was reported that nitrogen appeared to be a key factor in mediating salinity stress in salt-adapted alfalfa. Since excess Cl- ions can negatively impact Cl:NO3- ratios, it could be that the upregulation of CLC-a channel protein favourably improved NO3- fluxes while CLC-c improved Cl-:NO3- ratios to facilitate plant growth even under Cl- stress.

A voltage dependant anion channel (VDAC) was also upregulated in salt-stressed plants. VDAC proteins are responsible for ion homeostasis across mitochondrial membranes as well as tRNA transport (Hemono et al., 2020). A recently conducted proteomic analysis of 15 dS/m

stressed alfalfa showed that VDAC proteins were upregulated in nodulated and non-nodulated alfalfa (Wang et al., 2022a). Conversely, in our study this VDAC protein was downregulated in inoculated 8 dS/m stressed alfalfa, suggesting a genotype x inoculant interaction not present in the inocula/genotypes used by Wang et. al.

4.5.1.2 Nutrient Management

Nutrient management is an important component of plants' stress response. Nutrient deprivation in salt-stressed conditions can occur when Na^+ ions outcompete with K^+ ions, or when Cl^- ions outcompete NO_3^- ions for uptake by the plant (Hu and Schmidhalter, 2005;Wakeel, 2013). Additionally, increased nutritional needs of stress-related metabolisms will cause a decrease in nutrition available for either growth or maintenance related functions (Munns and Gilliham, 2015). It is therefore critical for the plant to effectively manage both the uptake and processing of available nutrients during salinity stress. In this study, salt-adapted plants displayed 3 DEGs related to the TAP42/TAP46 protein, which responds downstream to the target of rapamycin (TOR) signalling pathway. The TOR pathway, when active, promotes translation, anabolism, as well as nutrient uptake and metabolism of nitrogen; however, when deactivated by rapamycin a complementary kinase complex, SnRK1 (Sucrose non-fermenting Related protein Kinase 1) is activated which can result in programmed cell death (PCD) and nutrient recycling being stimulated (Ahn et al., 2011; Deng et al., 2020;Rodriguez et al., 2019). The TAP42 protein is a downstream effector of the TOR complex, which in combination with protein phosphatase 2A (PP2A) modulates the TOR complex; Tap42- mutants deactivate the TOR pathway and cause major downstream effects including reduced activity of nitrogen reductase (NR) and other nitrogen metabolic genes (Ahn et al., 2011;Deng et al., 2020). Interestingly, one of the more prominent results in the previous chapter was the apparent increase in sensitivity to nitrogen that was displayed by the second generation of alfalfa under salinity stress. All three DEGs annotated with TAP42 were upregulated in 8 dS/m salinity, suggesting this pathway may be a component of the genotypes ability able to maintain nitrogen metabolism even under stress. Interestingly, one of the downstream effector proteins of the TOR pathway was also a DEG found in this study: the ATG1 (AuTophaGy 1) is responsible for autophagy. The ATG1 gene is modulated by the TOR complex, and is stimulated in times of nitrogen starvation (Wang and Hou, 2022). The gene related to ATG1 was downregulated in this study, further evidence that the TOR complex

played an important role in nitrogen sensitivity in chapter 3. This paper could not find previous transcriptomic studies reporting activity of the TOR pathway in salt-stressed alfalfa, suggesting this may be a pathway worth future study.

4.5.1.3 Drought Response

The salinity response of alfalfa is related to drought response as the two stresses bear many similarities. Ssalinity stress can result in effective drought conditions whereby unfavourable water potential gradients caused by soil salts prevent the plant from effectively absorbing water. An EDS1 gene was upregulated in inoculated 8 dS/m stressed plants; EDS1 is a protein required for the attenuation of disease pressure in plants, however research has suggested that it is also required for the functioning of specific drought-response genes in *Arabidopsis thaliana.* Chini et al., (2004) report that a gene roughly homologous to a serine/threonine protein kinase was responsible for an important drought response in *A. thaliana,* but that this gene was itself dependent upon the EDS1 gene for effectiveness. Both DEGs annotated with EDS1 in this study were highly upregulated (log2Foldchange of 19 and 23) in 8 dS/m stressed alfalfa, suggesting that a similar response may be present in salinity-stressed alfalfa. This study also found a DEG coding for a serine/threonine protein kinase (K22520), similar to Chini et al., however in this case the kinase was downregulated instead of upregulated suggesting it may be unrelated to the EDS1 gene.

There were 3 DEGs annotated to heat shock proteins, including HSP90 (1, K09487) and HSPA4 (2, K09489;a.k.a HSP70). Of the three proteins, only one of the HSP70 genes was upregulated, however this is congruent with the diverse nature of heat shock proteins. Heat shock proteins are not only metabolized in response to heat stress but are also produced or repressed in response to multiple other forms of abiotic stress including drought, cold, and salinity stress (Andrasi et al, 2021), with certain variations only responding to specific stresses. Additionally, previous transcriptomic works shows that HSPs appear to respond to salinity differentially depending on the alfalfa genotype (Sandhu et al., 2017). The downregulated HSPs reported here may therefore be induced by different stresses than salinity, or because of the specific genotypes used. Andrasi et al., further report that the upregulated HSP70 protein found in this study responds duly to either salinity or heat. Interestingly, HSP70 may be transcriptionally regulated by a heat shock factor HSFA4, which also interacts with the important calcium induced SOS1

pathway to induce ion homeostasis under salt-stress (Li et al., 2018). HSPs are reported in salt-stressed alfalfa by (Sandhu et al., 2017; Puri, 2019; Bhattarai et al., 2021).

4.5.2 Inoculant Effects on Salt-Stressed Plants

The results of the differential expression analysis showed that there were 179 genes which were differentially expressed in *E. meliloti* treated 8 dS/m treated alfalfa relative to non-inoculated 8 dS/m alfalfa. Of these 179 genes, 76 were upregulated, and 103 were downregulated. The *E. meliloti* treatment on 8 dS/m treated plants induced 46 genes with known KEGG proteins. Several of these were directly or indirectly associated with salinity tolerance in relation to nutrient uptake, osmoprotectants, or the mutualism between bacteroid and host.

4.5.2.1 Nutrient Uptake

The significantly enriched GO term GO:0000103, "sulfate assimilation", was associated with DEG MS.gene006381.t1 which was upregulated with a log2Fold change of 23.5 fold in *E. meliloti* treated plants in the 8 dS/m treatment relative to control 8 dS/m plants. The enzyme adenosine-5-phosphosulfate reductase (APS reductase, K13811) was annotated to the gene, and is one of the key enzymes controlling the assimilation of sulfate and the production of the plant's first sulfur containing product: cysteine (Gotor et al., 2015). Interestingly, the APS reductase enzyme requires an Fe-S cluster, the production of which is in-part governed by the sufC gene (Ming Xu et al., 2005); another significantly upregulated DEG found in this study. Sulfur metabolism, with cysteine being the organic building block, is vital for the stress response of plants as the important stress-signalling hormones abscisic acid, salicylate, and jasmonate are all sulfur based (Takahashi et al., 2011). Additionally, the vital ROS scavenger glutathione is sulfur based (Kwon et al., 2019), and previous alfalfa salt-stress transcriptomic studies have also identified sulfur-containing ("thiol") proteins with important roles in ROS defense (Bhattarai et al., 2021; Kaundal et al., 2021) including thioredoxins and thioredoxin-like proteins.

4.5.2.3 Inoculant Induced Osmoprotectant Changes

galA (K07407), the enzyme responsible for the interconversion of galactinol and D-myo-inositol was strongly (>30 Log2Foldchange) downregulated by inoculation of 8 dS/m stressed plants. Myo-inositol is an important osmoprotectant while galactinol may server as an ROS scavenger

(Nishizawa et al., 2008; Zhang et al., 2018) indicating a negative pressure on the pool of the respective compounds. Another interesting result was the dual downregulation of the alanine-glyoxylate transaminase (AGXT) enzyme. The AGXT enzyme is involved in the metabolism of the osmoprotectant glycine betaine. AGXT directly governs the reversible reaction creating glycine from serine glyoxylate (Fang et al., 2021). The Xaa-Pro peptidase (pepP) gene, related to the osmoprotectant proline, was downregulated in *E. meliloti* treated 8 dS/m plants. The pepP protein acts by cleaving off any amino acid off of the N-terminal of a peptide that is attached to a proline element (Chen et al., 2021). This metabolism can prepare proteins with an N-terminal proline to be acted upon by a proline aminopeptidase (PAP), which have been shown to increase proline levels in response to abiotic stress (Ghifari et al., 2022). Interestingly, the pepP gene was downregulated in *E. meliloti* inoculated 8 dS/m stressed plants, while it was instead upregulated in 8 dS/m plants vs 0 dS/m plants. Conversely, 8 dS/m plants displayed strongly downregulated transcription for ornithine amino transferase (OAT), an important enzyme governing proline production. The upregulation of pepP seemingly contradicts the downregulation of OAT in 8 dS/m plant as previous research has shown proline and related transcripts accumulating in times of salinity stress as it acts both to combat ROS accumulation and to stabilize osmotic potential across membranes (Bertrand et al., 2020;Fichman et al., 2015). Instead, this result suggests that salt-adapted plants had both positive- and negative inducements of proline producing genes (pepP vs OAT), but that inoculation by *E. meliloti* appeared to be associated with downregulation of proline-producing genes. This is despite results in the previous chapter showing inoculated 8 dS/m plants exhibiting significant increase in proline content. This dichotomy may be the result of confounding transcriptional influences, or it could be that elevated proline levels were resultant from increased nitrogen availability rather than any transcriptional control.

4.5.2.4 Photorespiration

A very interesting result was the down regulation of the glycine cleavage proteins in inoculated 8 dS/m plants. The glycine cleavage proteins are part of the glyoxylate decarboxylase complex (GDC) which is composed of glycine cleavage proteins P, L, T, and H. gcvH was strongly downregulated in inoculated 8 dS/m stressed plants, while gcvP protein identified by KASS analysis and could not be traced to a specific gene. The GDC complex is responsible for an

important step in the photorespiratory pathway in which phosphoglycolate is recycled into 3-phosphoglycerate after oxygen instead of CO_2 is fixed by the rubisco enzyme. The GDC complex is responsible for creating serine from glycine in a two-step reaction within the mitochondria (Maurino and Peterhansel, 2010) as a part of this process. During salinity stress stomata close to conserve water, which leads to lower concentrations of CO_2 in the cell and naturally higher rates of photorespiration. Plants in this trial exhibited elevated expression of constituents of the GDC complex under salinity stress, however depressed expression under combination inoculation/8 dS/m salinity stress showing that bacteria had a strong effect on transcription. VDAC proteins, already discussed, may play an additional role in this process; Che-Othman et al., (2017) review several studies which show mitochondrial transporters, including VDAC proteins, to be affected by salt stress and that this effect may be impactful on the plants photorespiratory ability. Recent studies (Taj and Challabathula, 2021; Ilangumaran et al., 2022) clearly demonstrate that mutualistic bacteria can reduce the rates of photorespiration in salt-stressed crops by overexpressing photorespiratory enzymes (including elements of the glycine cleavage system) and osmoprotectants including glycine betaine. While the role of photorespiration is understood to be important in salinity stress, there appear to be comparatively few references to this process in alfalfa transcriptomic/proteomic studies; Wang et al., (2022a) is the only study among those referenced by this paper. While inconclusive, the present results suggest that *E. meliloti* inoculation is intimately connected to alfalfa's photorespiratory system, and that and this could be an important area for future research and improvement efforts.

4.5.2.5 Effects on Disease Resistance/Mutualism

The relA gene was also downregulated in inoculated vs non-inoculated salt-stressed alfalfa. The relA gene codes for guanosine pentaphosphate/tetraphosphate (ppGpp). ppGpp is an important alarmone responsible for beginning the stringent response in bacteria (Irving et al., 2021;Pacios et al., 2020). The stringent response is a phenomenon whereby the bacterium reduces or halts the production of RNA after being signalled by ppGpp in times of nutrient deprivation. This response protects the bacterium from starvation and can increase pathogenicity through increasing attachment proteins and secretory system, both of which aid in bonding with the host (Irving et al., 2021). Furthermore, ppGpp facilitates bacterial success in times of environmental stress; for example, a double spoT-/ relA- mutant of *Xanthomonas campestris* was shown to be

unable to produce ppGpp in response to oligotrophy (induced by salinity), nutrient deprivation, or copper stress (Bai et al., 2021). Previous work by (Postnikova et al., 2013; Gao et al., 2019; Puri, 2019) report up to 15% of salt-responsive genes to be disease related, and the downregulation of inoculated alfalfa in 8 dS/m stress suggests that the plant is attempting to increase the strength of its immune system to preclude infection during a time of vulnerability brought on by salinity stress. It would be interesting in a future study to observe if the same effect occurred in the roots, as it may have the additional (whether intentional or not) effect of precluding mutualism by 1) reducing the fitness and resilience of *E. meliloti* in salinity stress, and 2) reducing the pathogenicity of *E. meliloti* by impeding its production of ppGpp. These results are in agreement with the review of (Ferguson et al., 2019) and (Chakraborty and Harris, 2022) who report that host legumes prevent/abort nodulation in times of salinity stress. Interestingly, this gene was upregulated both in 8 dS/m vs 0 dS/m and inoculated vs control, but downregulated in inoculated 8 dS/m plants relative to non-inoculated. This could suggest that the bacteria's attempts to infect the would-be host may stimulate the downregulation of the relA gene.

KASS revealed that inoculated 8 dS/m plants additionally exhibited altered transcription of several Ca^{2+} inducible disease resistance genes including CALM (calmodulin), VDAC which is involved in the NOD-like receptor pathway. Both proteins may be involved in pathogen resistance (Tateda et al., 2011; Aldon et al., 2018) through calcium and/or ROS signalling.

Interestingly, the GDC complex discussed under "Photorespiration" is present in rhizobium as well as legumes, and is at least in part responsible for the successful mutualism between the two. Lorio et al., (2010) report that a gcv- mutant of *Ensifer fredii* was able to successfully nodulate improved soybean (*Glycine max*) cultivars, which had previously been resistant to nodulation. While the current study only examined shoot tissue of the host plant, this interaction may be worth further study as it could indicate that salt-stressed legumes may attempt to avoid or improve nodulation under salt-stress partially through regulation of the gcv gene/GDC complex.

4.6 Conclusions

This study was conducted to determine the effects on transcription of a rhizobium inoculant on a salt-adapted population of alfalfa under 8 dS/m salinity stress. Results showed that salt-tolerant

alfalfa responded to 8 dS/m salinity by increasing production of ion transporters and by repressing PCD and nitrogen catabolism through the TOR signalling pathway. *E. meliloti* was associated with both advantageous and deleterious changes to gene expression levels of salt-stressed alfalfa, including both positive and negative modulations of DEGs possibly related to osmoprotectant metabolism. Additional advantageous changes included upregulation sulfate assimilation which leads to production of the important antioxidant glutathione, while deleterious changes included downregulation of a gene responsible for creating more free proline and possible inhibition of the photorespiratory pathway. An ambiguous result which may deserve future research was the differential effect of inoculation on the gcvH and relA genes which both may modulate plant-microbe relations, including rhizobium. While the salt-tolerant genotypes used in this study did derive some benefits from inoculation under salt stress, this study concludes that inoculation with *E. meliloti* appears to be associated with mixed effects on plant growth under salinity stress, indicating that the complex relationship needs to be detangled in order to more effectively breed alfalfa to successfully associate with rhizobium under salt stress.

5. General Discussion

The objectives of this work were to assess the effectiveness of conventional recurrent selection in improving salinity tolerance of alfalfa, as well as to assess any additive effects of several mutualistic soil inocula, and finally to attempt to ascertain potential mechanisms of any improvements obtained through the former objectives by RNA-sequencing. It was predicted that both recurrent selection as well as inoculation would increase various parameters of plant growth under salt-stress, including biomass production, chlorophyll content, height, stem counts, and the production of various osmoprotectants.

Results showed that selection provided substantial gains in plant height, stem count, and chlorophyll content, but only marginal gains in biomass production under moderate salinity stress. Additionally, the most advanced population displayed signs of diminishing improvements in relation to biomass and osmoprotectants, suggesting the onset of an inbreeding depression. The modest size of this project limits the conclusions that can be made, and future work could benefit from a large-scale field experiment. Such a project may find significant effects where this project saw non-significant trends. Nonetheless, within limits these results support the effectiveness of recurrent selection for improving salinity tolerance in alfalfa. At 60 days this study did see non-significant yet elevated yield gains of generation G2 and G3 relative to G1 in 8 dS/m salinity (~7%), and a more noticeable 14% and 10% increase in nitrogen treated generation G2 and G3 relative to G1 in 8 dS/m, which may have produced significant trends given a larger population size than the modest size in this study. Yet, this did not match the 20% or especially the 44% yield gains in 8 dS/m salinity realized by Bertrand et al., (2020) after a similar three cycles of recurrent selection. This differential result is likely because of the much higher selection pressure in this study (1-3% selection vs 10% selection of original population by Bertrand et. al.) which may have caused an inadvertent inbreeding depression. This result suggests that recurrent selection may be more effective in alfalfa breeding programs with a selection pressure closer to 10%.

Genes related to salinity tolerance associated with salt-adapted generation G2 in this study were related to ionic stress management and nutrient management. The up or downregulated in response to 8 dS/m stress in generation G2 included two chloride ion channels (CLCs), a voltage dependent ion channel (VDAC), a SNARE protein, and a vacuolar H^+ ATPase.

H^+ ATPases have repeatedly been identified in previous transcriptomic studies as important in the salt-stress response of alfalfa by transcriptional/proteomic studies (Postnikova et al., 2013; Puri, 2019; Bhattarai et al., 2021; Medina et al., 2021b). The remaining proteins have been identified as important in the salt response of other plants such as *A. thaliana* (Wei et al., 2019; Hemono et al., 2020; Kwon et al., 2020). Important genes related to nutrient uptake and management included three TAP42 genes involved in the TOR pathway, a sulfate uptake gene, and a nitrate transporter. Control of the TOR pathway has been shown to be essential for plant growth under stressful conditions (Ahn et al., 2011), uptake of the macronutrient sulfur is essential for both plant nutrition and salt stress responses (Takahashi et al., 2011), and chloride anion channels are vital for the transport of NO_3^- in the plant and maintaining healthy Cl^-: NO_3^- ratios (Wei et al., 2019; He et al., 2023). Although the TOR pathway is vital for growth and development in stressed and unstressed conditions, this paper could not find reference to it in similar transcriptomic studies, suggesting an avenue for future research.

The effects of inoculation were not as beneficial as had been hypothesized. Previous work suggested that the PGPR *Halomonas maura* in combination with *Ensifer meliloti* would be effective at ameliorating salinity stress up to 20 dS/m (Martinez et al., 2015), however this study found that yield was identical between inoculated and non-inoculated plants in 8 dS/m at 60 days. The results of Martinez et al., (2015) imply that *H. maura* is capable of providing complementing benefits both to *E. meliloti* as well as the host plant; a result which was not borne out in this study. In fact, the only significant beneficial effect of any inoculant in 8 dS/m salinity was the significant increase in proline production associated with inoculating 8 dS/m stressed plants with *E. meliloti*. Interestingly, the short-term effects on biomass of the 60 kg/ha nitrogen amendment appeared to benefit plants more in 8 dS/m salinity than inoculation. These results ran counter to this paper's hypothesis and perhaps suggests a genotype x bacterial strain interaction, whereby different alfalfa cultivars or genotypes may be differentially responsive to various inocula. If this is the case, this interaction should be an important area of focus for future studies.

In addition to the phenotypic effects mentioned, this paper found many transcriptional effects of both 8 dS/m salinity alone or in tandem with inoculation by *E. meliloti* on salt-adapted generation G2. 8 dS/m salinity caused the differential expression of many salt-stress related DEGs. This paper predicted that a combination of inocula and breeding could stimulate a larger pool of osmoprotectants, specifically proline, glycine betaine, and trehalose. This paper did find

significantly higher levels of proline in *E. meliloti* inoculated 8 dS/m plants, however this result was not matched by a clear upregulation of DEGs related to proline metabolism. Sandhu et al., (2017) found that the enzyme Pyroline-Carboxylase-5-Synthase (PC5S1) was upregulated in tolerant genotypes of alfalfa in response to 16.6 dS/m salt-stress. Conversely, in this study none of the primary genes responsible for proline catabolism (Proline Dehydrogenase 1 (PRODH1), PRODH2, or Pyroline-5-Carboxylate Dehydrogenase (PC5DH)) were downregulated, and neither PC5S1 or PC5S2 responsible for synthesis were affected by salt stress or inoculation. Only a single OAT gene was strongly downregulated by 8 dS/m salinity which is dichotomous with the large pool of proline found in 8 dS/m stressed plants. The pepP gene, responsible for exposing proline on pre-existing peptides (potentially for removal by a proline aminopeptidase to increase the pool of free proline) was upregulated in 8 dS/m plants relative to 0 dS/m plants, however, this effect was lost with inoculation suggesting that benefits of inoculation on proline production may be more related to increased nitrogen availability than to transcriptional regulation.

This paper had theorized that the rhizobium-alfalfa mutualism could be made more resilient in the face of salinity stress through plant selection. While there were no conclusive results supporting this hypothesis, several significant DEGs in 8 dS/m stressed inoculated plants hinted at the complex relationship between host and bacteria in salinity stress. These genes included the relA, CALM, VDAC, and gcvH genes. The downregulated relA gene could cause a decline in pathogenicity of gram negative bacteria, such as *E. meliloti,* through decreased production of ppGpp (Irving et al., 2021). Since both CALM (calmodulin) and VDACs are involved in the NOD receptor pathway, a pathway essential for successful nodulation, alterations in their transcription could have had similar effects on the mutualistic relationship. Lastly, the glycine decarboxylase system (GDC) present in both plants and bacteria was downregulated in the host plant; since this system can facilitate or inhibit nodulation of rhizobium in other legumes (Lorio et al., 2010) it could be that this system is a component of a regulatory pathway which restricts nodulation by *E. meliloti.*

5.1 Conclusions

This study set out to determine the efficacy of both recurrent selection and inoculation by mutualistic soil microbes in mediating salinity stress in alfalfa. The phenotypic and RNA-seq

data obtained from salt-stressed 120-day old populations with varying salinity tolerances and grown with various inocula revealed several patterns regarding legume-rhizobium dynamics under salinity stress. rhizobium were able to confer salinity tolerance to the host plant under 8 dS/m stress by stimulating the production of the osmoprotectant and ROS scavenger proline. However, neither the nitrogen-fixing nor the transcriptional effects of rhizobium were capable of creating the same short-term yield gains displayed by 8 dS/m stressed plants given a 60 kg/ha nitrogen amendment. It appears that plants benefited most strongly from receiving 'free' nitrogen for which they did not need to trade with bacteroids for valuable fixed carbon. In addition to the beneficial increases in proline, through RNA-seq this paper identified many genes related to ion transport/storage, and nutrient management. Notable genes coded for H^+ ATPases, SNARE proteins, and chloride ion channels important in ion transport and management. The TAP42 and TOR pathway responsible for growth and nutrient management in saline stress was upregulated in salt-tolerant generation G2, while *E. meliloti* provided both beneficial and seemingly deleterious effects to salt-stressed plants. These included upregulation of the APS enzyme, a key enzyme for sulfur uptake and associated ROS scavenger production, as well as downregulation of enzymes (OAT & pepP) which could create the important osmolyte proline. Furthermore, this paper reported several disease-related genes upregulated by *E. meliloti* inoculated plants under salt stress which may offer important avenues for future study on the complex relationship between the two organisms under salt stress. This paper concludes that alfalfa breeding programs may benefit by focusing on both responsiveness to mutualistic bacteria and macronutrient uptake traits in addition to ROS scavengers, osmoprotectants, and ionic management traits to mediate alfalfa's susceptibility to salinity stress.

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APPENDIX TABLES

Table A1. P value of Repeated Measures

	Chlorophyll	Plant height	Stem count
Salinity (S)	<0.001	<0.001	<0.001
Generation (G)	0.02	<0.001	0.02
Inoculant (I)	0.03	0.16	<0.001
I x S	0.08	0.01	<0.001
G x S	0.97	0.37	0.27
Time (T)	<0.001	<0.001	<0.001
S x T	<0.001	<0.001	<0.001
I x T	0.16	0.01	<0.001
S x I x T	0.18	0.20	<0.001
S x G x T	0.36	0.21	0.98

Table A2. P values of Analysis of Variance (ANOVA)

	Shoot Biomass (60 days)	Shoot Biomass (90 days)	Shoot Biomass (120 days)	Root Biomass (120 days)	R:S Ratio	Proline (Shoot)	Proline (Root)	Glycine Betaine (Shoot)	Glycine Betaine (root)	Trehalose (Shoot)	Trehalose (root)
Salinity (S)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.39	0.28	0.77	0.64
Generation (G)	0.10	0.56	0.99	0.91	0.83	0.80	0.22	0.85	0.24	0.32	0.90
Inoculant (I)	<0.001	<0.001	<0.001	<0.001	0.24	<0.001	0.02	0.76	0.70	0.71	0.07
I x S	0.23	<0.001	<0.001	<0.001	<0.001	0.49	0.80	0.91	0.92	0.33	0.97
G x S	0.65	0.75	0.62	0.45	0.67	0.16	0.15	0.26	0.20	0.95	0.57
I x G	0.58	1.00	0.99	0.39	0.24	0.94	0.02	0.48	0.75	0.94	0.44
I x G x S	0.47	0.78	0.93	0.39	0.32	0.98	0.97	0.77	0.65	0.26	0.05

Table A3. Paired end raw and mapped read data for all samples.

Sample	Forward Read	Reverse Read	Uniquely Mapped Reads	Multi-Mapped Reads	Total Mapped	% Mapped	Average Mapped Length
R5C02	21036054	21036054	7994126	12096115	20090241	95.5038478	294.16
R4C02	20482260	20482260	7782229	11053691	18835920	91.9621175	293.88
R6C82	21060149	21060149	7872397	12271725	20144122	95.6504249	293.56
R5C82	22760905	22760905	8668412	13010971	21679383	95.2483348	294.51
R5E02	21206181	21206181	7879943	11972722	19852665	93.6173515	294.01
R4E02	22664318	22664318	9003802	12747509	21751311	95.9716105	294.48
R5E82	21230232	21230232	8089400	12236557	20325957	95.7406259	294.52
R4E82	20724918	20724918	7738954	12035028	19773982	95.4116296	294.24