

ABSTRACT

JONES, ALENA POWELL. The Effect of External Potassium Availability on Salt Tolerance in Two Soybean Cultivars upon Symbiosis with Arbuscular Mycorrhizal Fungi and Nitrogen-Fixing Bacteria

(Under the direction of Dr. Kevin Garcia).

In North Carolina, salinity poses a significant threat to soybean growth and production due to sea level rising and saltwater intrusion. Therefore, it is vital for farmers to use sustainable methods to mitigate this stress. Potassium (K^+) is an essential macronutrient that plants need for physiological growth, reproduction, and development. Soil K^+ availability has an important role in salt tolerance in plants, however, when plants are in K^+ deficient conditions, physiological functions are negatively impacted. To mitigate these abiotic stresses, the use of beneficial microbes such as arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria (*Rhizobium*) can be recruited by plant roots. AM fungi improve water and nutrient acquisition and can alleviate various abiotic stresses, including salinity and K^+ deficiency. Nitrogen-fixing bacteria symbiotically interact with legume plants, resulting in the formation of root nodules that fix atmospheric dinitrogen. In return, the plant will provide these symbiotic microbes with carbon from photosynthesis. In this thesis, two soybean cultivars were colonized with an AM fungus or a salt-tolerant rhizobia bacteria and placed under various K^+ and salinity conditions. The dual impact of external K^+ availability and these symbiotic associations was investigated using physiological and stable isotopic approaches. First, soybean plants were placed in two-compartment systems and rubidium (Rb^+) was used as a proxy to track K^+ transport in AM symbiosis. Plants were inoculated with the AM fungus *Rhizophagus irregularis* DAOM 197198 and grew in various K^+ and sodium (Na^+) regimes. Root development parameters, biomass, colonization rate, and nutrient concentrations were assessed in AM and non-mycorrhizal plants. All NaCl treatments highly affected soybean root development, yet K^+ availability did not have many effects. Mainly under limiting K^+ conditions, AM significantly decreased from high salinity but it was still effective from the improved K^+ concentrations and it prevented Na^+ accumulation in inoculated plants. Lastly, Rb^+ transport only occurred when plants were in demand for K^+ but was inhibited by high salinity, showing some limitations in using Rb^+ as a proxy for K^+ . Second, soybean plants were inoculated with a salt-tolerant nitrogen-fixing bacterium, *Sinorhizobium fredii* USDA 208, and various K^+ and sodium

(Na⁺) regimes were applied. Nodule count, plant biomass, and shoot nutrient concentrations were analyzed in inoculated and non-inoculated plants. Nodule count, shoot and root dry weights, all decreased with increasing NaCl exposure. Nutrient concentrations resulted in varying responses to salinity. However, in no salinity and limited and sufficient K⁺ conditions, both inoculated and not-inoculated plants had lower shoot K⁺, Na⁺, and calcium concentrations compared to plants in NaCl treatments. These studies showed that symbiosis with AM fungi and nitrogen-fixing bacteria mitigates salt tolerance in soybeans depending on K⁺ availability.

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The Effect of External Potassium Availability on Salt Tolerance in Two Soybean Cultivars upon
Symbiosis with Arbuscular Mycorrhizal Fungi and Nitrogen-Fixing Bacteria

by
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DEDICATION

I would like to dedicate this to all my family and friends. I am eternally grateful for their love and support.

BIOGRAPHY

Alena Jones was born in Simferopol, Crimea, Ukraine and grew up in the foothills of North Carolina. In 2020, she graduated with her B.S. in Biochemistry from the University of North Carolina at Greensboro. Her undergraduate research was in Astrobotany; finding which *Arabidopsis thaliana* genotypes were resistant to microgravity. While searching for graduate programs, soil science caught her eye. She did not know what really went on in the soil. After taking a class, Alena knew this was what she wanted to study. In May of 2022, Alena joined the lab of Dr. Kevin Garcia. She studies the symbiosis of soybean and arbuscular mycorrhizal fungi and nitrogen-fixing bacteria under salt stress.

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CHAPTER ONE: The importance of arbuscular mycorrhizal fungi and nitrogen-fixing bacteria to help mitigate salt tolerance in soybean influenced by external potassium availability

Introduction

This thesis investigates the impact of external potassium (K^+) availability on the role of arbuscular mycorrhizal (AM) fungi and nitrogen-fixing bacteria in salinity tolerance in soybeans. This project shows that these microbes can help in nutrient uptake and mitigating abiotic stress such as salinity. In North Carolina, salinity is on the rise and poses detrimental effects to crops like soybeans. K^+ deficiency is another stress soybeans must overcome. Here, the interrelationship between K^+ , salinity, AM fungi, and soybean have been studied to help further our understanding.

Role and importance of potassium and sodium in plants

Importance of potassium in plants

K^+ is a vital macronutrient that has a diverse fundamental role in multiple physiological growth and developmental processes in plants. Since cytoplasmic K^+ concentration is approximately 0.1 M (Nieves-Cordones et al., 2016a), it is essential to maintain optimal cellular K^+ levels to ensure proper plant physiological and biochemical functions. Some of these functions are stomatal regulation, photosynthesis, water acquisition, uptake of other nutrients, and membrane potential maintenance (Sardans and Peñuelas 2021). For example, if K^+ impacts stomatal regulation, then photosynthesis is affected. Indeed, the concentration of K^+ controls guard cell turgor pressure of stomatal movement, making it vital to prevent water loss. Stomata opening depends on K^+

concentration, so if K^+ fluctuates, stomata opening or closing will vary, causing photosynthesis rate to either decrease or increase (Johnson et al., 2022).

Generally, K^+ is abundant in soils, however, the fraction of soil K^+ that is readily available for plants is approximately 1-2 % of total K^+ . Additionally, some soil-available K^+ can be lost through leaching which takes part in the scarceness of plant-available K^+ (Sardans and Peñuelas 2015). K^+ can also be withheld in the interlayer of some soil clay minerals such as kaolinite and vermiculite; these clay minerals have little to no expansion ability. This causes cations like K^+ to be fixed in the interlayer of the clay mineral (Fig. 1.1, Hafsi et al., 2014).

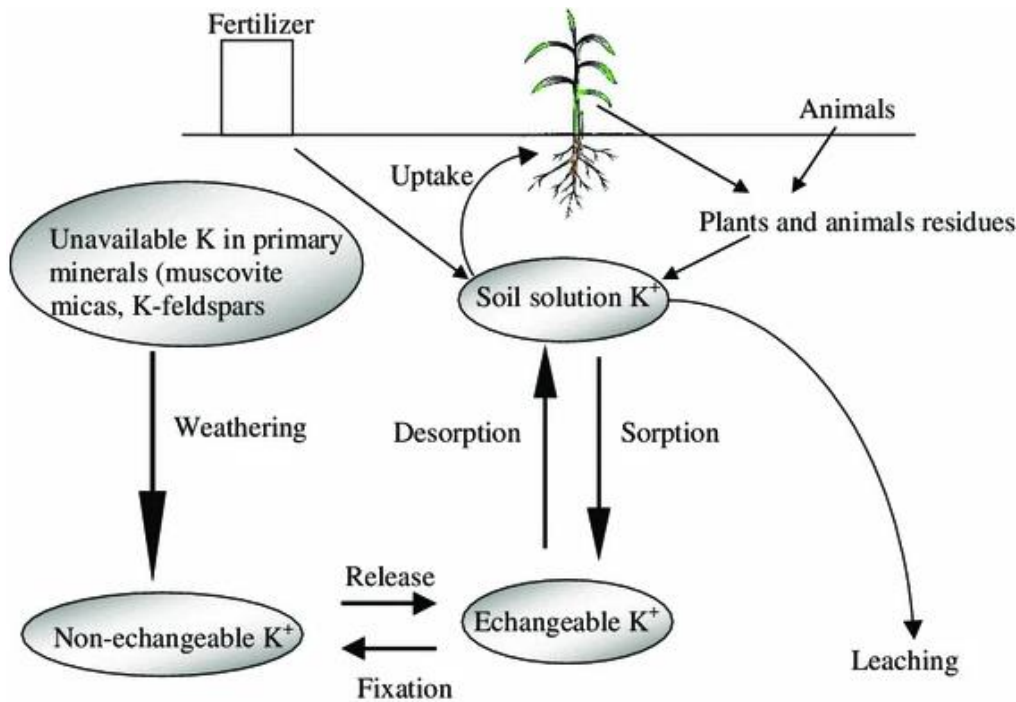


Figure 1.1: Potassium cycle in the soil. It is in four pools which include unavailable K^+ in primary minerals, non-exchangeable, exchangeable, and soil solution. K^+ can transform between different forms by fixation and release. The availability of K^+ depends on weathering, fertilizer application, and type of clays in the soil. K^+ is also lost through leaching (Hafsi et al., 2014).

In plants, K^+ is transported through a variety of high- and low-affinity transport systems (Morgan and Connolly 2013). To depict between the two, low-affinity transporters transport K^+ in

high concentrations of K^+ in the soil. High-affinity transporters transport K^+ in low concentrations of soil K^+ . Ion channels also mediate K^+ transport such as Shaker channels which are voltage gated K^+ channels (Sharma et al., 2013). These consist of four α -subunits which contain six transmembrane domains and one pore domain.

When enduring K^+ deficient conditions, plants have adapted with various mechanisms to survive the stressful environment. Without sufficient K^+ , plants can experience stunted growth, reduced photosynthesis, decreased turgor pressure, irregular stomatal regulation and more (Rawat et al., 2022). To cope in K^+ deficiency, plants respond by morphological, physiological, biochemical, and molecular adaptations.

Impacts of environmental salinity on plants

Salt accumulation in the soil is defined as salinity and there are two major sources of salinity, primary and secondary. Primary salinity is natural and is caused by water table fluctuation. Secondary salinity is caused by agricultural practices from changing out native perennial vegetation to crops and pastures. To be a saline soil, the saturation extraction in the root zone's electrical conductivity is greater than 4 dS m^{-1} ($\sim 40 \text{ mM NaCl}$) at $25 \text{ }^\circ\text{C}$ and exchangeable Na^+ of 15 % (Shrivastava and Kumar 2015). In the Southeastern U.S., salinity is caused by multiple factors such as storm surges, ocean level rise, and droughts (Tully et al., 2019). Salinity poses a major threat to crop yield and productivity and to our food security. It has detrimental effects on plants if not mitigated in sustainable management practices. One example is the incorrect use of irrigation which can lead to soil salinity. This practice can be changed by using partial root zone dry method and drip irrigation to optimize water use. Another way to mitigate this issue is bringing back perennial plants that grow continuously and use water in the non-annual crop plant season.

The balance between water use and rainfall can be restored which further can keep the water table from rising and reduce the salt from moving to the soil surface (Shrivastava and Kumar 2015).

High salinity can affect plants in ways such as growth, photosynthesis, protein synthesis, and energy metabolism (Parida and Das, 2005). In a study testing salinity on wild and cultivated soybean, it was observed a significant decrease in growth, photosynthesis rate, and gas exchange parameters from increasing salt concentrations as well as increased root sodium (Na^+) and chloride (Cl^-) ion concentrations (Ullah et al., 2019). Over time, plants have learned how to adapt to this abiotic stress with tolerant mechanisms to maintain control cell function and development. Plants heavily rely on signal perception, signal integration, and processing (Hanin et al., 2016). A way plants respond is by halotropism of the plant root. This process occurs at the root tip where asymmetries in auxin signaling are caused by phospholipid signaling. Asymmetries of cell elongation and bending of the root tip away from salinity is the result from varying auxin responses (Dinneny 2015).

In high salinity, plants can accumulate more Na^+ and Cl^- than other important nutrients. In order to cope with salt stress, plants can sense the hyperosmotic part of salt stress. At the beginning of NaCl exposure, cytosolic Ca^{2+} levels spike and it is thought that the hyperosmotic sensors are possibly linked with Ca^{2+} channels (Knight et al., 1997). Some plants have the ability to compartmentalize Na^+ in vacuoles which makes them more tolerant. These plants are called halophytes and are much more tolerant to salt stress compared to glycophytes. Halophytes generally have higher shoot Na^+ concentrations than glycophytes suggesting a larger vacuolar volume that can store more Na^+ (Tester and Davenport, 2003).

Transferring genes responsible for salinity tolerance from halophytic plants to glycophytic plants could be one method to increase salt tolerance in plants. The determination of these genes

can now be done by full genome, transcriptome, and proteome comparisons of related halophytes vs. glycophytes (Himabindu et al., 2016). For example, Guo et al. (2021) observed a physiological difference in salt tolerance between a model halophyte *Puccinellia tenuiflora* and glycophytic Gramineae plants. Root K^+ concentration was slightly higher in *P. tenuiflora* and greatly decreased in Gramineae plants. They also found that some salinity-tolerant K^+ uptake gene families (e.g. inward rectifier K^+ channel, voltage-gated K^+ channel, and K^+ transporter) from *P. tenuiflora* endured family expansion plus positive selection during evolutionary history. This caused an increase in K^+ uptake and decreased Na^+ accumulation and toxicity in the halophyte *P. tenuiflora*. This suggests that further studies can be done to better understand the difference in salt tolerance of halophytes and glycophytes so that we can find ways to mitigate salt stress in plants. Another method to help mitigate salinity is the use of symbiotic microbes, particularly mycorrhizal fungi as it will be discussed below.

Role of potassium in salt stress tolerance in plants

Salinity can also affect how nutrients are taken up by plant roots. When plants are exposed to high salinity, K^+ can have an impact on a plant's ability to obtain a high salt tolerance or not. Na^+ cannot replace K^+ in its role in the activation of more than 50 enzymes hence, high salinity can negatively impact protein synthesis which further impacts many processes (Tester and Davenport, 2003). K^+ has many important roles in maintaining a plant's salt tolerance, some include sustaining ion homeostasis and controlling the osmotic balance (Johnson et al., 2022)

Both K^+ and Na^+ cations are chemically similar; they are in the alkali metal group which are highly reactive, and they have a single positive charge. A few examples of K^+ transporters in the model plant *Arabidopsis thaliana* are AtHAK5 and AKT1 which are major transporters involved in K^+ transport (Nieves-Cordones et al., 2016b). The HAK5 type of K^+ transporters are

classified as high-affinity K^+ transporters that take part in K^+ transport at low concentrations. When K^+ levels increase, HAK5 and AKT1 both contribute and when K^+ levels exceed 200 μM , only AKT1 is the main way for low-affinity K^+ transport (Nieves-Cordones et al., 2016b). For Na^+ uptake, genes involved remain unclear. HKT and HAK transport families have been able to take part in high-affinity Na^+ transport. Low-affinity Na^+ transport can include Na^+ permeable channels such as glutamate-like receptors (GLRs) and cyclic nucleotide gated channels (CNGS) (Nieves-Cordones et al., 2016b).

It has been reported in peanut (Chakraborty et al., 2016; Shi et al., 2020), soybean (Adhikari et al., 2020), sugar beet (Merwad, 2016), and tomato (Amjad et al., 2016) that improving plant K^+ acquisition has a beneficial effect under salinity. In a study using soybean, Adhikari et al. (2020) compared two types of K^+ fertilizers (KCl and K_2SO_4) with foliar application at a medium K^+ level of 2.5 %. Plants were exposed early to medium (6 dSm^{-1}) and high (12 dSm^{-1}) salinity. They observed no effect of external K^+ application on plant height and shoot and root biomass. However, they did see positive effects of K_2SO_4 on antioxidant activities, flavonoid, carotenoid, polyphenol, and chlorophyll contents in the plants. Furthermore, it was found that it did not significantly differ from plants that did not receive fertilizer application. This suggests that there needs to be other methods to use to overcome salinity stress and K^+ deficiency. A promising way is the use of symbiotic fungi and other beneficial microbes so we would not have to heavily rely on K^+ fertilizers. This leads to the next section of using symbiotic microbes in salt tolerance of plants, specifically legumes.

Symbiotic microbes for Legumes

Arbuscular mycorrhizal symbiosis

Arbuscular mycorrhizal (AM) fungi have been present for more than 400 million years and helped plants to colonize lands (Diagne et al., 2020; Rich et al., 2021). These fungi are ubiquitous in the soil and can form mutualistic symbiosis with approximately 80 % of terrestrial plant species (Lanfranco et al., 2016). AM fungi are obligate symbionts, they belong to the subphylum Glomeromycotina (Spatafora et al., 2016), and they need to colonize a host plant to complete their life cycle. AM fungi rely on sugars and lipids from the plant and in return, the fungus provides the plant with water and nutrients obtained from the soil (Posta and Duc 2020). They develop a diversity of structures in the soil and within plant roots that includes arbuscules, sometimes vesicles, auxiliary cells, external/internal hyphae, and asexual spores (Stürmer, 2012). Interacting with these beneficial symbionts improves the host plant with multiple beneficial functions, including water and nutrient acquisition, abiotic stress tolerance, and protection from pathogenic microbes (Chen et al, 2018; Diagne et al., 2020; Tang et al., 2023).

Plant root and arbuscular mycorrhizal fungal association

This symbiosis begins when a plant root releases a hormone called strigalactone which stimulates the metabolism and hyphal growth and branching of AM fungi (Fig. 2, Bonfante and Genre 2010). The fungus releases signaling molecules called lipochitooligosaccharides (also known as Myc factors) to establish symbiosis with the plant. The perception of Myc factors by specific receptors expressed on the root surface triggers a cascade of signalization within the host root that results in increases and decreases of Ca^{2+} concentration within the cell nucleus. These rapid changes in Ca^{2+} concentration is called “ Ca^{2+} spiking” and lead to the activation of cellular and transcriptional

responses, resulting in the establishment of the symbiosis. Once the AM fungal hyphae attach the roots through a specific structure called hyphopodium, it triggers the formation of a “tunnel-like” structure from the plant cell called pre-penetration apparatus (Fig. 1.2; PPA). In the PPA, fungal hyphae will follow the paved path from the root epidermis to the inner cortex. This process continues further in the inner cortical cells for branching where arbuscules are formed. Arbuscules form highly ramified hyphae inside the plant cells and are the area where nutrients are exchanged between the two symbiotic partners (Fig 1.3; Garcia et al., 2016).

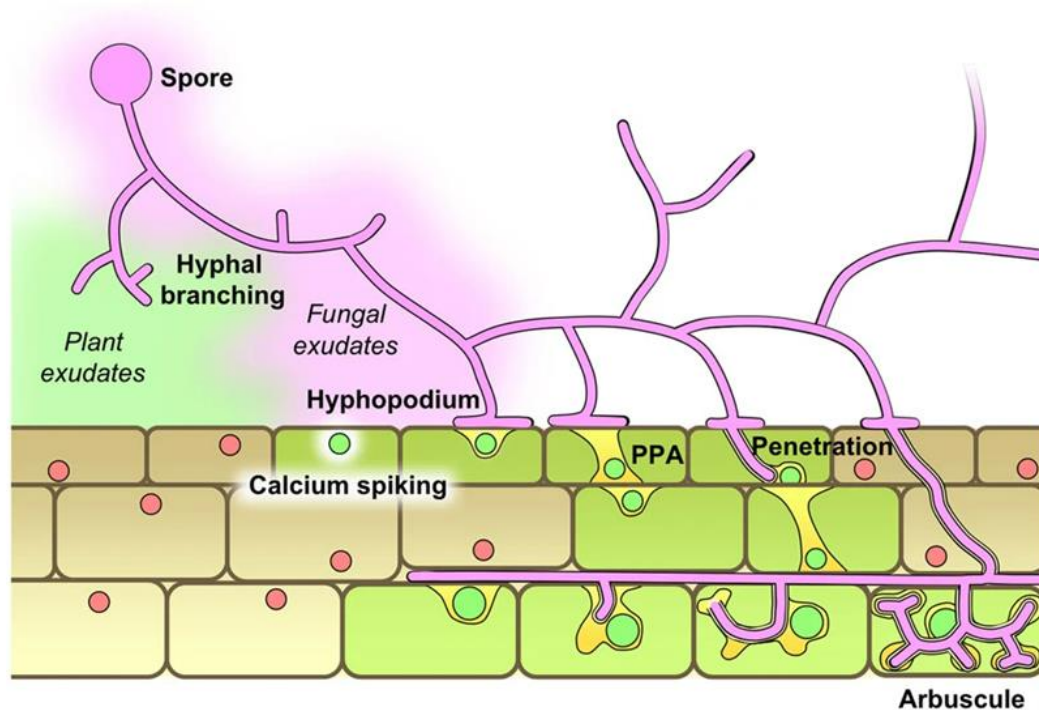


Figure 1.2: The initial association between plant root and AM fungi. Plant roots release a hormone called strigalactone which attracts Myc factors released by the fungus. Once attached to the root by a fungal structure called hyphopodium, growing hyphae penetrate the root guided by the pre-penetration apparatus (PPA, formed by the host plant), a tunnel-like structure that fungal colonization follows from the epidermis to the inner cortex. This mechanism continues up to the inner cortical cells for branching. When hyphae reach the inner cortex arbuscules are formed and nutrient exchanges between the plant and the fungus can start (Bonfante and Genre 2010).

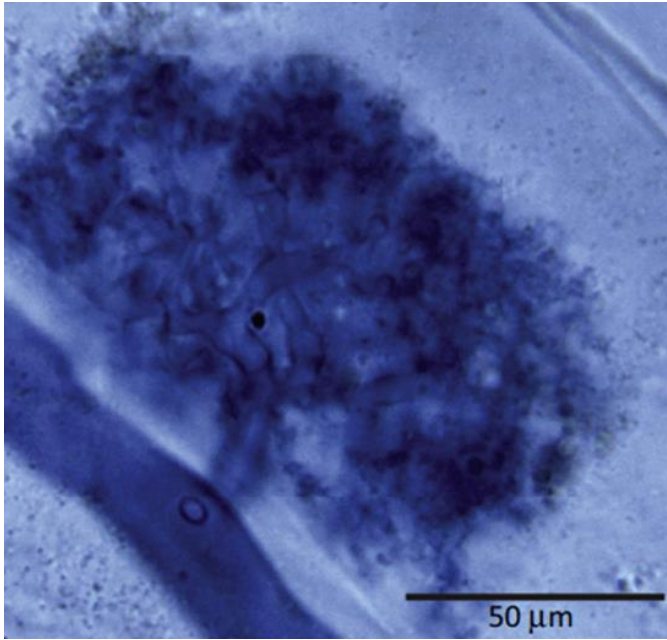


Figure 1.3: An arbuscule in the root cortical cell formed by fungus *Rhizophagus sp.* This is where nutrient exchange occurs between the plant and fungus (Garcia et al., 2016).

AM fungi take up macro- and micronutrients and water from the soil and transfer them to the roots. In return, the plant provides the fungus with photosynthate in the form of sugars and lipids (Fig. 1.4a; Lehmann and Rillig 2015). Since the arbuscules can be seen as “trading posts” for resources, specific fungal proteins need to be expressed to ensure transport from the soil to colonized roots (Casieri et al., 2013; Garcia et al., 2016). Therefore, multiple proteins are expressed in extraradical hyphae to take up nutrients and water from the soil; and others are expressed in the arbuscules to release nutrients and water into the symbiotic interface. Plants also express specific transport proteins in arbusculated cortical cells to take in the nutrients/water originating from the fungus, and also to release carbon into the symbiotic interface. Finally, AM fungi expresses transport proteins at the arbuscular membrane to take in carbon originating from the root (Fig. 1.4b; Garcia et al., 2016; Kafle et al., 2018).

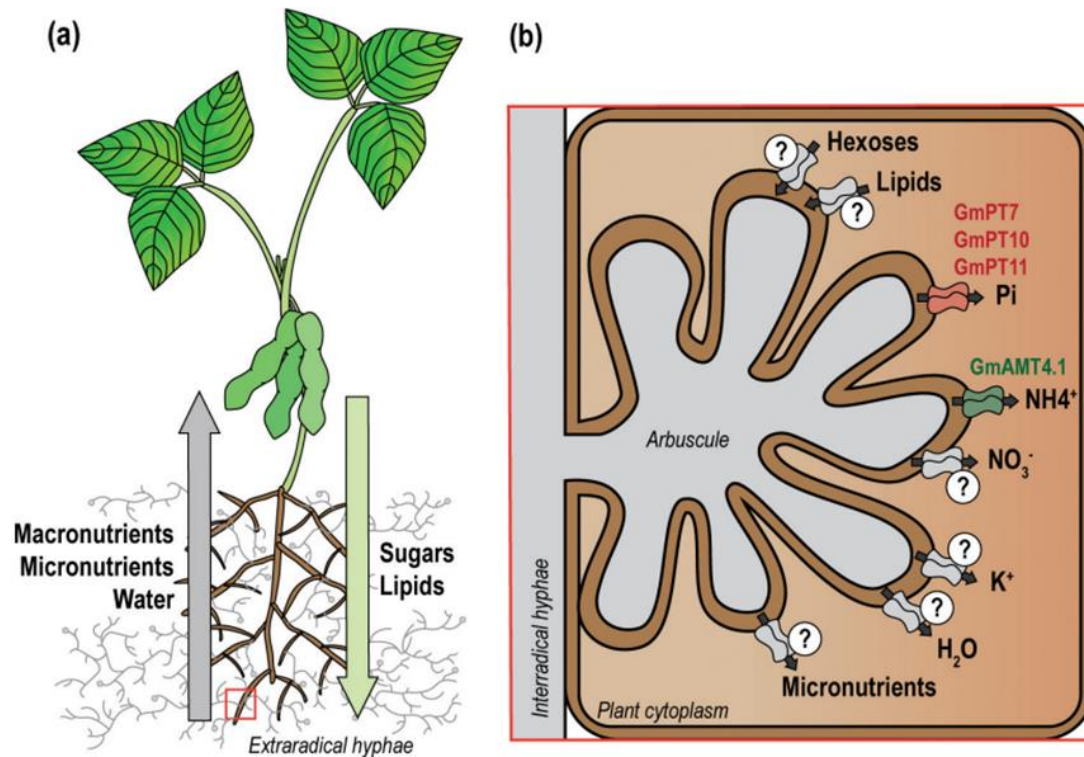


Figure 1.4: Diagram of the nutrient uptake pathway in AM roots of soybean plants. The fungus provides the plant with macronutrients, micronutrients, and water, while the plants transfer sugars and lipids to the symbiotic microbe (a). These exchanges take place at the symbiotic interface constituted from fungal arbuscules in the root cortical cell, surrounded by the periarbuscular membrane of the host (b). Mycorrhiza-specific transporters that play an essential role for the nutrient exchange are present in the fungal cell membrane and plant periarbuscular membrane in soybean (Kafle et al., 2018).

There has been extensive research on mycorrhiza-specific transport proteins in some model plants, such as the legume *Medicago truncatula*, but very little in crop plants like soybean. Indeed, there is limited information on these transport proteins for micronutrients, K^+ , and nitrogen in soybean while some have been characterized for phosphorus (P) and nitrogen (N) transport, such as *GmPT7* (Tamura et al., 2012) and *GmAMT4.1* (Kobae et al., 2010).

Importance of arbuscular mycorrhizal fungi for nutrient acquisition

Although AM fungi can probably transport all kinds of macro- and micronutrients towards colonized roots, they are mostly known for improving plant P and nitrogen (N) acquisition. P is a

limiting nutrient in the soil that is challenging for plant uptake due to its low mobility and solubility. Plants take up P in the form of inorganic phosphate (Pi), creating depletion zones around the root due to Pi absorption being greater than its diffusion rate in the soil (Ferrol et al., 2019). In alkali soils, applied P gets chemically fixed in the soil immediately, so high dosage of fertilizer is required (Dalshad et al., 2013). P is applied to the soil as mineral fertilizers or organic manure and can bind to the soil particles which can create a pool of residual P (Bindraban et al., 2020). It can also be lost from the soil by leaching, runoff, and erosion, leading to bodies of water and eutrophication. Extra P in the soil can also be sorped on metal (Fe, Al) hydroxides, carbonates (Ca), 1:1 phyllosilicates, and even bound to organic matter (Bindraban et al., 2020). Besides fertilization, AM fungi can also help mitigate this issue by increasing P acquisition in plants through the extension of their extraradical hyphae into the soil to obtain Pi past the depletion zones (Thioub et al., 2019; Adeyemi et al., 2021).

AM fungi has also been shown to enhance N acquisition in plants. However, N is more mobile in the soil compared to P so the need for AM fungi in N acquisition may not be as needed for the host plants. This could lead to possible competition between the plant and microbe for N. Plant roots normally take up soil N in inorganic forms such as nitrate (NO_3^-) and ammonium (NH_4^+). Roots can also uptake small amounts of soluble organic N such as amino acid and small peptides (Xie et al., 2022). AM fungal hyphae can take up N in the form of NH_4^+ and NO_3^- from the soil through specific transport proteins, such as GintAMT12/3 and GiNT, respectively (Xie et al., 2022; Garcia et al., 2016). Govindarajulu et al. (2005) also observed that AM fungi can acquire inorganic N, incorporating N into amino acids, translocating it as arginine, and transferring it to the plant.

The role of arbuscular mycorrhizal fungi in potassium acquisition

One mechanism a plant can use to help overcome K^+ deficiency is the use of soil microbes such as AM fungi to better uptake K^+ (Garcia and Zimmermann, 2014; Haro and Benito, 2019). This is indeed a promising mechanism to help endure K^+ deficiency. It is still a limited area of research but there has been more attention on this mechanism in recent years, particularly due to efforts from our group (Garcia et al., 2017; Lie et al., 2019; Kafle et al., 2022; Kafle and Garcia, 2022). Two mechanisms that soil microbes can help improve K^+ acquisition are solubilization and symbiotic associations with plants (Fig. 1.5; Haro and Benito 2019). Therefore, harnessing and preserving soil microbes could have the potential to reduce the use of K^+ fertilizers in agroecosystems. This could be a way to help improve the availability of K^+ since it is based on cation exchange reactions: K^+ is positively charged and soil's cation exchange capacity is negatively charged (Haro and Benito 2019). K^+ can be extracted from soil particles containing minerals with immobilized K^+ . This mechanism remains barely known but it is suspected that microbes could play a major role through the release of protons and organic acids into the soil to increase K^+ solubilization (Meena et al., 2014).

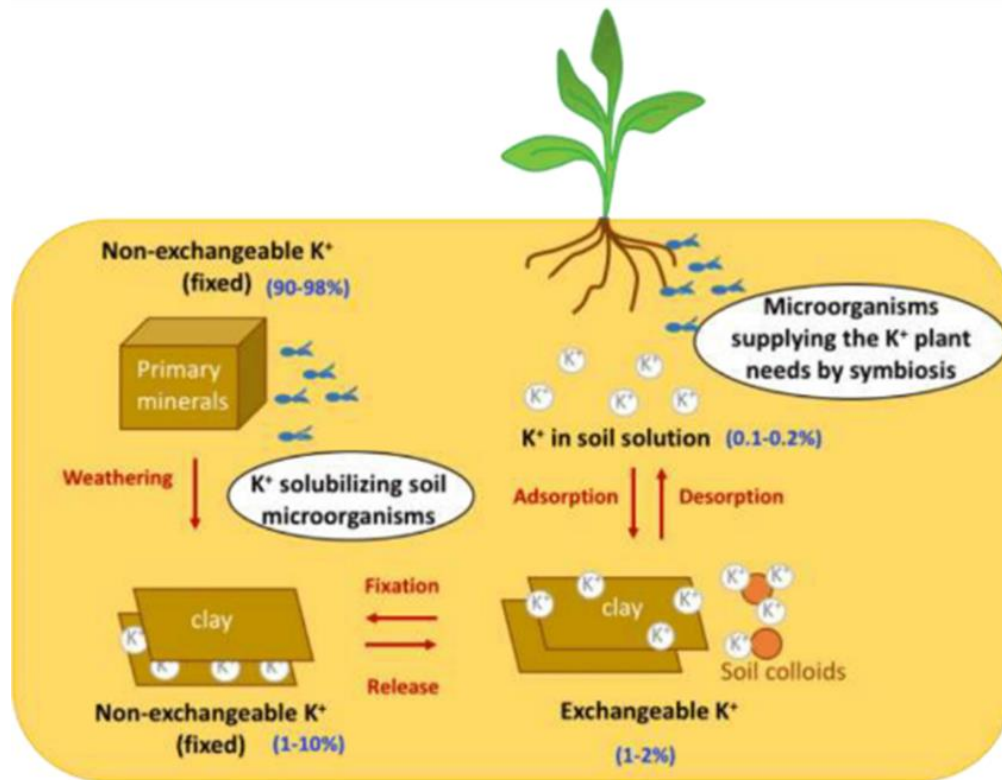


Figure 1.5: Mechanism of soil microbial enhancement of potassium. Potassium is found in four pools in the soil which are non-exchangeable of primary minerals (90-98 % of total soil K⁺), non-exchangeable of secondary minerals like clay 1-10 % of total soil K⁺, exchangeable (12 % of total soil K⁺), and the soil solution 0.1-0.2 % of total soil K⁺). Microbes can improve potassium availability by potassium solubilizing and symbiotic associations with plants (Haro and Benito 2019).

Tracking K⁺ transport in AM symbiosis has been challenging for a long time due to the difficulty to use K⁺ isotopes. Recently, the use of rubidium (Rb⁺), and to a lesser extent cesium (Cs⁺), has shown promising results to achieve this goal. Although Rb⁺ ions have a larger radius than K⁺ ones, they are transported through K⁺ transporters and channels since no specific Rb⁺ transport protein has been identified so far in any organism. Therefore, placing Rb⁺ in a compartment only available for the AM fungus and not for roots, and then being able to detect Rb⁺ in plant shoots would prove that the K⁺ transport pathway exists in AM symbiosis. Using this

approach, it has been recently demonstrated in the model legume *M. truncatula* where shoot Rb^+ concentrations were significantly higher in AM plants compared to non-mycorrhizal plants under limiting and sufficient K^+ conditions (Kafle et al., 2022). Positive correlations between shoot Rb^+ concentration, shoot K^+ concentration and AM colonization were observed which strongly suggests that Rb^+ was being transported to the plant from the fungus and there is a symbiotic K^+ transport pathway. This approach has also been used in loblolly pine (Frank and Garcia, 2021), but never to our knowledge in an AM crop, particularly soybeans.

Another recent study investigated the use of Cs^+ as a proxy for K^+ transport in AM symbiosis (Kafle and Garcia 2022). Plants were inoculated with AM fungi and placed in K^+ -deprivation or K^+ -sufficient conditions. Cesium chloride was added in four different quantities (0, 0.5, 1.5, and 3.75 mM) into the fungal compartment, so it was only accessible to the fungus. They did not observe any significant difference in shoot Cs^+ concentration between non-mycorrhizal and AM plants under K^+ -deprived conditions. With K^+ supply, there was only a significant difference of shoot Cs^+ concentration between NM and AM plants when 3.75 mM Cs^+ was added (Kafle and Garcia, 2022). Although it was concluded that Cs^+ could also be used as a proxy for K^+ transport, it seemed obvious that the use of Rb^+ was more conclusive than Cs^+ . This is the reason why in the following chapter, Rb^+ was used to track K^+ transport in mycorrhizal soybean under saline conditions, and not Cs^+ .

The role of AM fungi in plant salt tolerance

The impact of AM fungi in plant salt tolerance has received more attention; studies have shown that AM fungi can help alleviate salt stress in multiple crops such as wheat, cotton, maize, and soybean (Sarifi et al., 2007; Daei et al., 2009; Liu et al., 2016; Hashem et al., 2019). There are

multiple ways AM fungi can mitigate salt stress. For example, by enhancing nutrient uptake, photosynthesis, water-use efficiency, and accumulation of compatible solutes and enzymatic antioxidants (Abdel Latef and Chaoxing 2014). In a recent study, pepper plants inoculated with AM fungi and exposed to salt stress were more tolerant to salinity and increased their growth performance compared to non-mycorrhizal ones (Abdel Latef and Chaoxing 2014). However, AM fungi were negatively affected by increasing external salt concentrations. They also found that AM fungi had enhanced photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) significantly in inoculated plants compared to non-inoculated plants. AM fungi also relieved plants of malondialdehyde content in the roots and shoots compared to the NM plants. Altogether these results strongly support the ability of AM fungi to help their host plant cope with salt stress conditions.

In soybean (Sarifi et al., 2007), AM fungi alleviated salt stress by improving biomass, root proline, P, K, and zinc concentrations compared to non-inoculated plants. Also, shoot Na^+ concentrations were higher in non-inoculated plants compared to inoculated plants, supporting that AM fungi can reduce Na^+ accumulation in their host. Additionally, the AM fungus was pre-treated with NaCl, and plants inoculated with these fungi showed higher biomass, root proline, P, K, and zinc concentrations compared to soybeans inoculated with the non-pre-treated fungus. When pre-treated and suddenly exposed to salt, AM fungal efficiency increased. More recently, Hashem et al. (2019), inoculated two soybean cultivars (salt tolerant and salt sensitive genotypes) with AM fungi and exposed them to 200 mM of NaCl. Although AM colonization was reduced in both cultivars, the salt tolerant plant was significantly more colonized compared to the salt sensitive one. Other analyses tested were nodulation count, nitrogenase activity, leghemoglobin content, and auxin synthesis which were improved by AM fungi for both soybean genotypes when

compared to non-inoculated plants. This study showed that AM fungi enhanced salt tolerance in both cultivars by improving plant growth and symbiotic performance which was due to the stimulation of endogenous level of auxins. The auxins contribute towards better root systems and nutrient acquisition under salt stress.

Symbiosis with N-fixing bacteria rhizobia

N-fixing bacteria called rhizobia are beneficial microbes in the soil that infect the roots of some plant families, including legumes, resulting in the formation of nodules (Oldroyd and Downie 2008). Once in the nodules, rhizobia are able to fix atmospheric dinitrogen (N_2) into ammonia (NH_3). Root hairs release flavonoids into the soil, attracting surrounding bacteria, including N-fixing rhizobia. In the soil, free-living rhizobia proliferate and release Nod factors that plants can recognize, triggering a cascade of signalization and the formation of root nodules. In the presence of Nod factors, root hairs curl, infection threads are produced, and root cells divide. Infection threads develop from the root hairs to the root cortex and release rhizobia in the forming nodule where they differentiate into bacteroids (Fig. 1.6; Oldroyd 2013).

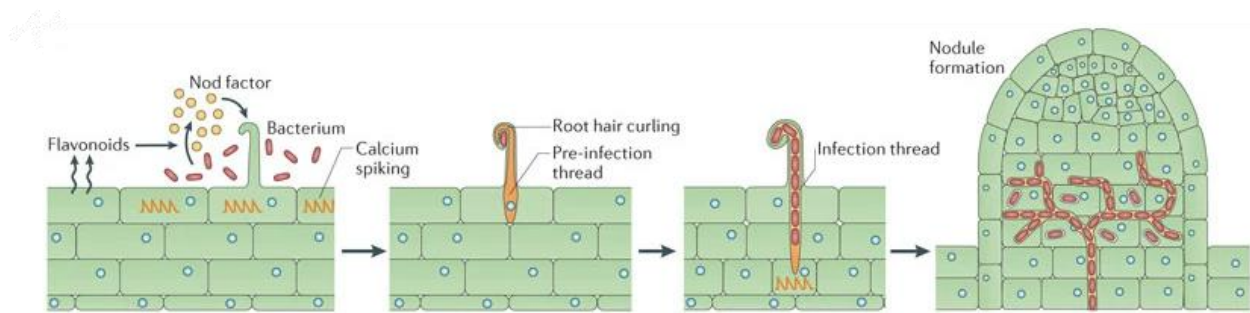
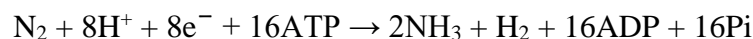


Figure 1.6: Establishment of root nodules by N-fixing bacteria. Plant roots exude flavonoids into the soil, resulting in the attraction of N-fixing rhizobia. The bacteria releases Nod factors that are perceived by the plant. Proliferation of bacteria will occur, root hairs begin to curl, and infection threads are created. The infection threads are formed from the root hairs to the cortex, guiding rhizobia. Bacterial cells are released from the infection thread and differentiate into bacteroids that can fix N. As bacteroids and cell division increases, a root nodule will be formed (Oldroyd 2013).

N fixation occurs in differentiated bacteroids: atmospheric N_2 is reduced into NH_3 by the nitrogenase enzyme. The bacterial genes coding for nitrogenase are grouped in a cluster and the complex enzyme is composed of a Fe protein (reductase) and a MoFe protein (catalytic). However, the reduction of N_2 to NH_3 is expensive for the host plant, particularly in P because 16 ATP are needed. Legume crops need P in large amounts for growth and N fixation, P deficiency can limit nodule formation (Devi et al., 2012).



Multiple genera of soybean-nodulating rhizobia have been tested for their salt tolerance. It has been shown that the genus *Bradyrhizobium* is mostly present in low acidity to neutral soils, while *Sinorhizobium fredii*, from the genera *Sinorhizobium* (*Ensifer*), can be found under arid/semi-arid climate and saline-alkaline soils (Nitawaki et al., 2021). One study investigated the impact of salinity on soybean growth and inoculation with seven species of rhizobia (Nitawaki et al., 2021). Although salt stress limited soybean growth and nodulation in most cases, only the

inoculation with *S. fredii* strains resulted in nodule number increase under salinity. However, up until 50 mM NaCl, inoculation with salt-sensitive bradyrhizobia resulted in higher symbiotic efficiency, soybean growth, and N-fixation ability compared to salt-tolerant sinorhizobia. That could partially be a cause of the pairing of soybean variety and inoculant. Above 50 mM NaCl, *S. fredii* proved to be a better inoculant for salt tolerance in soybean by increasing nodules and dominance. This is the reason why the nodulation experiments in this thesis were performed using *S. fredii* as an N-fixing microbial partner (see **Chapter 3**).

Soybean, a model crop to study plant-microbe interactions

Soybean (*Glycine max* L.) is one of the world's most important crops. It is a legume that is used in high-quality oil and protein for human and animal consumption (Ayilara et al., 2022). Between 2002 to 2019, soybean was the largest hectare row crop cultivated in North Carolina with an annual average of 637,200 planted hectares (Vann et al., 2021). The NC planting season for soybeans in 2023 was from March through July and soybean maturity groups (MG) selection can range from 000 of really early maturing to 10 of latest maturing although the early maturing varieties (<MG2) are not generally grown in this environment (USDA-NASS 2023; Vann Personal Communication). These are defined as areas from specific regions where a cultivar thrives optimally without suggesting MG-specific cultivars cannot be cultivated elsewhere (Mourtzinis and Conley 2017).

Factors that determine soybean MG selection are abiotic factors, temperature, photoperiod, and management considerations within a farming operation (Cober et al., 2001). In this thesis, one commonly planted soybean MG 5 and one MG 6 cultivars were used because MG 5 varieties have been reported to be best suited for most of the southern states and MG 6 were better suited for the southern parts of South Georgia and South Carolina (Fig. 1.7; Mourtzinis and Conley 2017).

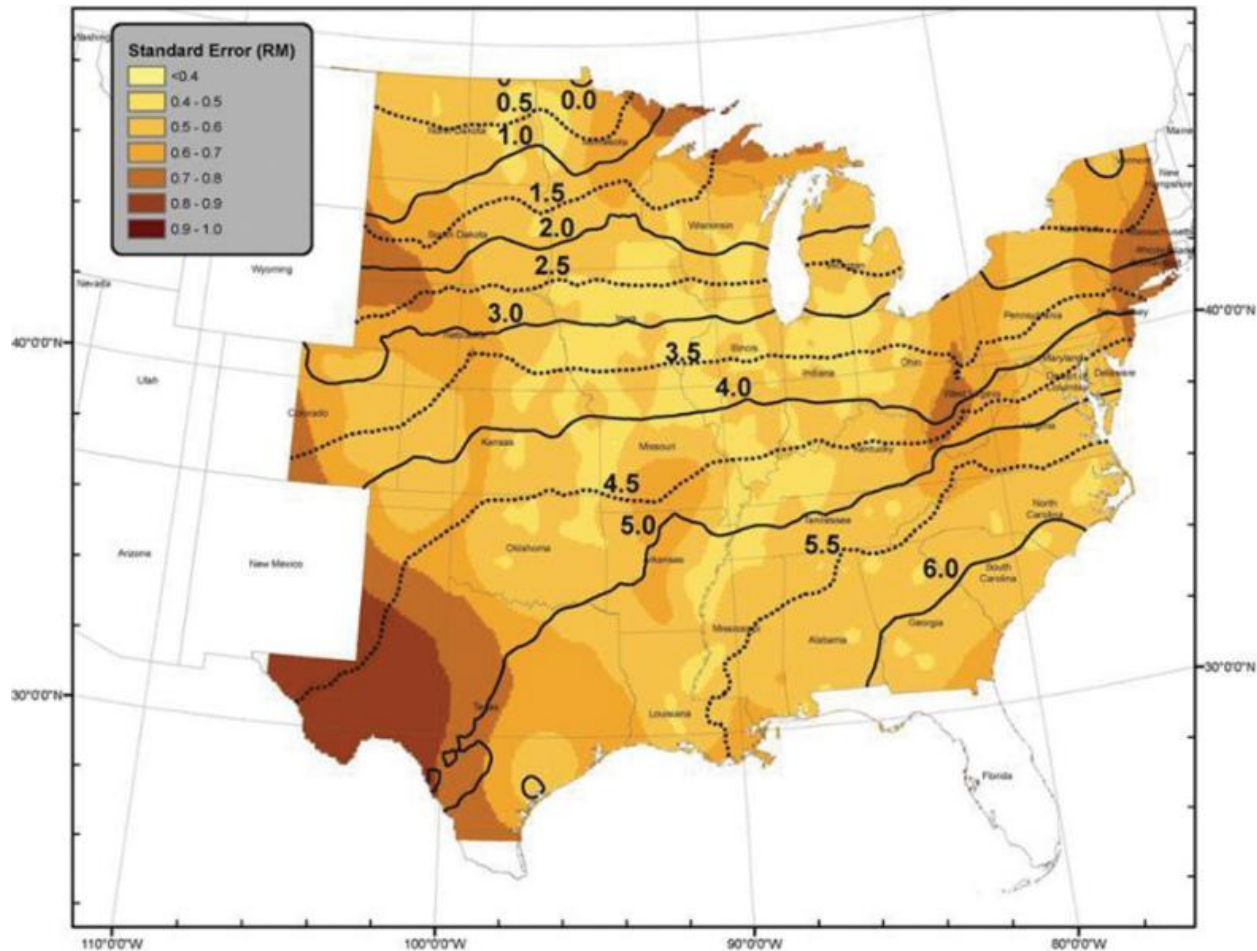


Figure 1.7: Map of soybean maturity group zones examined across the continental United States. A downward deflection of MG lines going from East to West is defined (Mourtzinis and Conley 2017).

Soybeans can also interact with microbes in their rhizosphere which are saprophytes and symbionts, including bacteria and fungi that are either beneficial, neutral, or detrimental to soybeans. Particularly, soybeans always interact with AM fungi and N-fixing bacteria that provide important nutritional benefits to their host plant (see above). The overuse of chemical fertilizer can cause problematic environmental consequences, so these symbiotic microbes can be an important alternative for agricultural application and ecosystem productivity, reducing the amount of fertilizer application (Meena et al., 2018). Most studies on these symbiotic associations have been done on model legumes such as *M. truncatula*, and knowledge on soybean remains rather limited

even if more attention has been earned in recent years (Kafle et al., 2018). Therefore, it is important to investigate the role these symbiotic partners have in environmental stresses such as nutrient deficiencies and salinity. Unveiling key genetic information can lead to new breeding strategies towards higher resistance to environmental stresses for a more sustainable production (Meena et al., 2018).

The dual impact of potassium availability and symbiotic interactions on salinity tolerance in soybean

The utilization of symbiotic microbes shows high potential for enhancing crop productivity and growth, potentially lessening the reliance on chemical fertilizer. However, there are still many challenges to fully understand plant-microbe-soil interactions. There are many factors that impact soil properties which influence plant and microbial properties, as well as their interaction. With salinity threatening soybean growth and yield, it is important to find alternative strategies to maintain or increase their salinity tolerance. Both AM symbiosis and K^+ application individually have major roles in mitigating salt tolerance in soybean. The model plant *M. truncatula* has been used extensively to study AM symbiosis in mitigating abiotic stress such as salinity. It has a small genome that has been fully sequenced so, it has had more attention in molecular studies involving AM fungi too. Soybean is an economically important crop that has multiple uses such as animal feed and as a high-quality oil. There is a great demand for soybeans, so it is essential to use biological methods to help mitigate abiotic stresses such as salinity and K^+ deprivation. The research in **Chapter 2** of this thesis aims at understanding the dual impact of external K^+ availability and AM inoculation on salt tolerance in soybean. Also, the use of Rb^+ as a proxy for K^+ in soybean under salinity was investigated.

As stated above, N-fixing bacteria are other beneficial microbes that legumes can form a symbiosis with. However, the impact of external K^+ on nodulation has been barely investigated. The research in **Chapter 3** of this thesis aims at investigating the impact of rhizobia on salt tolerance in soybeans under limiting or sufficient K^+ availability. Plant-microbe-soil interactions are essential to be understood so plants/crops can mitigate environmental abiotic and biotic stresses. Humans heavily rely on plants/crops in their diet. Today's world population is over 8 billion people, the demand for a healthy diet heavily depends on plants. With many types of threats to our plants/crops, we need to learn how to understand and maintain them in optimal conditions.

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CHAPTER TWO: Dual impact of external potassium availability and arbuscular mycorrhizal fungi on salinity tolerance in two soybean cultivars

Introduction

The objective of this chapter was to investigate the dual impact of external K^+ availability and AM symbiosis on two soybean cultivars: one from maturity group (MG) 5 and one from MG 6. The MG 5 is an excluder which excludes the uptake of chloride (Cl^-), and the MG 6 is an includer which includes the uptake of Cl^- from the soil. In two separate experiments, soybean plants were inoculated with *Rhizophagus irregularis* DAOM 197198 and grew in sufficient and limited potassium (K^+) and varying sodium (Na^+) regimes. Also, rubidium (Rb^+) was used as a proxy to track K^+ transport from the fungus to the colonized plants. Biomass, colonization rate, and shoot Rb^+ , K^+ , Na^+ , P, and Ca^{2+} concentrations were obtained. The experiment using soybean MG 5 is presented in **section 2.1** of this chapter, and it was submitted as a manuscript. However, the experiment using soybean MG 6 did not succeed due to little or no root AM colonization (**section 2.2**).

2.1: Manuscript as submitted in *Plant Science*

**The external potassium availability determines the effect of arbuscular mycorrhizal fungi
on the salinity tolerance of soybeans**

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Abstract

Arbuscular mycorrhizal (AM) fungi improve water and nutrient acquisition of most land plants. Additionally, they can help plants to alleviate abiotic stresses, such as salinity which causes a major threat for many crop species. Potassium (K^+) plays a major role in plant tolerance to salinity, and it has been recently demonstrated that AM fungi can improve plant K^+ uptake. Using two-compartment systems and rubidium (Rb^+) to track K^+ movements, we inoculated soybean plants with the AM fungus *Rhizophagus irregularis* and grew them in various K^+ and sodium (Na^+) regimes. Root development parameters, biomass, colonization rate, and nutrient concentrations were assessed in AM and non-mycorrhizal plants. Our results show that soybean root development was significantly affected by NaCl treatments, rather than K^+ availability. Additionally, although the AM symbiosis was drastically reduced by high salinity, it improved K^+ concentrations and prevented Na^+ accumulation in inoculated plants, mainly under limiting K^+ conditions. Finally, Rb^+ transport was observed only when the plants were in demand for K^+ but was inhibited by high salinity. This report shows the combined impact of K^+ availability and AM symbiosis on soybean tolerance to salinity, and the limitations of using Rb^+ as a proxy for K^+ .

Keywords

Arbuscular mycorrhizal fungi, *Glycine max*, Potassium nutrition, *Rhizophagus irregularis*, Rubidium, salinity.

Highlights

- Potassium has no impact on the reduction of soybean root development under salinity.
- AM colonization decreases upon salinity but still prevents shoot Na^+ accumulation.
- AM symbiosis improves K^+ uptake in soybean mainly under limiting K^+ conditions.
- As a proxy for K^+ , Rb^+ is transported from the fungus to the plant only at low K^+ .
- Fungus Rb^+ transport was inhibited under salinity, limiting its use in AM symbiosis.

Introduction

Arbuscular mycorrhizal (AM) fungi are beneficial soil microbes that form mutualistic symbioses with more than 80 % of terrestrial plant species [1]. The hyphae of these fungi explore the bulk soil and the rhizosphere of land plants to gather water and nutrients, and transfer these resources through structures called arbuscules to their host plant [2,3]. In return, the plant will provide the fungus with carbohydrates from photosynthesis in the form of lipids and sugars [4]. Besides their role in plant nutrition, AM fungi can also protect their host against pathogens by priming plant defenses, and significantly contribute to the tolerance against various abiotic stresses [5,6].

Although most previous studies have shown that AM fungi are crucial for plant nitrogen and phosphorus (P) acquisition [7–11], increasing efforts have been made to investigate the transport of other macro- and micronutrients by AM fungi [12–14]. Among them, the essential macronutrients potassium (K^+) is needed for a wide range of functions and processes in plants, including enzymatic and metabolic activity, stress tolerance, osmoregulation, and photosynthesis [15,16]. Therefore, its deprivation in the soil greatly affects plant growth and development [17,18].

Plants have evolved various strategies to overcome environmental K^+ limitations, including the interaction with AM fungi. Indeed, recent studies have shown that AM fungi can alleviate plant stress induced by K^+ deprivation by directly transporting K^+ ions towards colonized roots [19–22]. Evaluating K^+ transport in mycorrhizal symbioses has been understudied for a long time, but we recently used rubidium (Rb^+) as a proxy to track K^+ movements in a few symbiotic models [21,23].

In the southeastern US, salinity poses a major threat to crop yield and production, particularly in soybeans [24,25]. The overaccumulation of sodium (Na^+) ions in plant cells is toxic and negatively impacts plant photosynthesis, as well as cell growth and expansion [26]. Plant growth can become stunted, necrosis and chlorosis occur in leaves, and plants must turn to

physiological and biochemical coping mechanisms [27]. It has been described in various reports that AM fungi can improve the plant tolerance against salinity [28–30]. For example, AM plants showed a reduced Na^+ accumulation and higher $\text{K}^+:\text{Na}^+$ and calcium (Ca^{2+}): Na^+ ratios than uninoculated control plants [31]. However, the combined effect of external K^+ availability and AM symbiosis on salinity tolerance in soybean has so far not been studied.

Here, we used two-compartment systems and Rb^+ to track the transport of K^+ in AM soybean plants under increasing sodium chloride (NaCl) treatments and under limiting and sufficient K^+ conditions. Additionally, we investigated the impact of external K^+ availability on soybean root development in plants exposed to salinity.

Material and methods

Plant and fungal materials

Soybean seeds from a commonly planted maturity group 5 variety were surface sterilized in 0.6 % bleach for four minutes, rinsed five times with milli-Q water, and germinated in double-rinsed potting substrate (Safe T Sorb®; Ep Minerals®, Nevada, USA) for around 10 days in a growth chamber at 23°C/14 h and 18°C/10 h day/night cycle. *In vitro* axenic Ri T-DNA transformed carrot root organ cultures (*Daucus carota* clone DCI) grown on minimal medium were used to produce fungal inoculum of *Rhizophagus irregularis* DAOM 197198 [32]. The fungal spores were then extracted according to [33].

Potassium and sodium conditions

Plants were supplied with Long Ashton nutrient solution that was modified for multiple K^+ and Na^+ conditions (Table S2.1). Two treatments containing either limited (LK, 0.05 mM K^+) or

sufficient (SK, 3.75 mM K⁺) K⁺ levels were used, as well as four Na⁺ conditions with 0, 50, 100, and 200 mM Na⁺ in the form of NaCl. Both K⁺ and Na⁺ treatments were mixed to make a total of eight different treatment media, abbreviated below as LK+0/50/100/200Na or SK+0/50/100/200Na.

Impact of NaCl exposure on root development

Germinated soybean seeds were placed on large plates (26 cm x 26 cm; L x W) containing 250 ml of the LK and SK solutions supplemented with NaCl, as described above, and a piece of germination paper. Since a singular plate had five germinated seedlings, there were a total of eight conditions with fifteen replicates each. Tin foil was used to wrap the bottom part of the plate, to protect the roots from light. After 10 days of growth on a light shelf, plants were harvested, dry biomass was recorded, and the number and length of lateral roots, as well as the length of the primary root were determined using ImageJ software [34].

Two-compartment system

The two-compartment systems used in this study were previously custom designed by using plastic boxes (12 cm x 8 cm x 8 cm; L x H x W: from Carno A/S) in which a Magenta GA-7 Plant Culture Box (Bioworld) was placed [21,23,35]. The Magenta box formed a “fungal compartment” (FC) which only the fungus was able to reach, while the rest of the plastic box formed the “root compartment” (RC). On one side of the FC, 99 holes were drilled in and were covered on each side by two 52-microns nylon meshes through which only the fungal hyphae were able to pass through. The lowest hole layer was 2 cm above the Magenta box base to prevent any nutrient solution leakage from one compartment to the other, as we previously validated [23]. On the

bottom of the plastic box, six 5 mm holes were drilled in for watering. Both RCs and FCs were filled with 300 ml pre-washed potting substrate (Safe T Sorb[®]; Ep Minerals[®], Nevada, USA).

AMF symbiosis and rubidium supply

Germinated seedlings were transplanted to the RCs of the two-compartment systems and inoculated with 400 fungal spores and some colonized root segments (AM), or kept non-inoculated (NM). In a plastic tray (26 cm x 26 cm x 5 cm) six two-compartment systems were placed, corresponding to six replicates for each treatment. At the beginning of the experiment, 200 ml of LK+0Na or SK+0Na solutions were added to each tray every three days. After four weeks of co-culture, the Na⁺ concentrations were increased in the RCs every three days by 1/5 of the final Na⁺ concentration for each treatment to prevent osmotic shock (Table S2.2). The FCs did not have any holes at the base of the box, so the amount of nutrients given was controlled as follows: 30 ml of 1/10 dilution of the corresponding nutrient solution (LK+0Na or SK+0Na) was added to the FC once per week to avoid buildup of nutrients. Additionally, 30 ml of milli-Q water was also supplied weekly to the FC to maintain moisture. No increase in Na⁺ concentrations were applied to any FCs.

Ten days before harvest, 30 ml of a Rb⁺/K⁺ solution was added every three days in the FC of both AM and NM conditions. The solution consisted of RbCl (3.25 mM) and KCl (0.5 mM). We obtained a final Rb⁺/K⁺ concentration of 3.75 mM, matching the K⁺ concentration in the SK medium (see above). Plants were not watered on harvest day, and they were harvested eight weeks post-inoculation.

To determine the impact of the NaCl treatments on the availability of K⁺ and Rb⁺ in the potting substrate, pots filled with only the substrate were watered with each K⁺/Na⁺ solutions (200

ml in a tray filled with 6 pots) and 30 ml of 3.75 mM Rb⁺ solution added directly on top of each one of them. The substrates were collected after 1 day and extracted with 40 ml of 0.01 M CaCl₂ solution for three hours followed by filtration through filter paper (Whatman #42). Concentrations of K⁺, Na⁺, and Rb⁺ were then determined.

Determination of plant biomass, nutrient concentration, and AM colonization

After harvest, fresh shoot and root biomass were obtained, and the plants were dried for 5 days at 70°C to record the dry biomass. To quantify the AM colonization, a subsample of roots per plant was cleared with 10 % KOH (w/v) at 95°C for 8 minutes, rinsed with water, and stained with 5 % Pelikan ink vinegar (w/v) at 95°C for 8 minutes. Root colonization was quantified for each AM plant using the grid line intersection method [36], and all NM plants were checked for any signs of colonization.

Dried shoot samples were grinded, and digested in nitric acid to determine Rb⁺, K⁺, Na⁺, P, and Ca²⁺ concentrations by ICP-MS or ICP-OES by the Environmental and Agricultural Testing Service (EATS) at North Carolina State University.

Statistical analyses

All data analyses were based on 6 replicates per treatment. All box plots and regression scatterplots were made in R v.4.0.3 (R Core Team, 2020), using the ggplot2 package. Depending on the experiment, two-way or three-way analysis of variance (ANOVA) was conducted to determine the differences among means followed by Fisher's post-hoc LSD test using the agricolae package [37].

Results

High NaCl exposure negatively affects soybean root development

The impact of NaCl and K⁺ treatments on soybean root development was tested by placing germinated seedlings on large plates for 10 days under a light shelf (Fig. S2.1). Primary root length, as well as number and length of lateral roots were recorded (Fig. 2.1). In both LK and SK conditions, primary root length was not affected by increasing NaCl exposure. The only significant difference was between the LK+0Na and LK+50Na conditions, probably due to a couple of outliers (Fig. 2.1A). Additionally, the K⁺ availability had no effects on the primary root length. The number of lateral roots, however, significantly and gradually decreased under both LK and SK conditions with increasing NaCl concentrations (Fig. 2.1B). Only at +0Na, plants in SK had a significantly higher number of lateral roots than plants in LK (Fig. 2.1B). In both LK and SK conditions, the lateral root length and root biomass was not affected by an increase to +50Na but started to gradually decrease at +100Na and +200Na (Fig. 2.1C, S2.2).

Increasing salinity negatively affects AM colonization and soybean biomass

To assess the impact of salinity and K⁺ supply on AM symbiosis and biomass production in soybean, plants were inoculated with *R. irregularis* DAOM 197198 and compared to non-treated plants through placement in two-compartment systems under various NaCl and K⁺ regimes (Fig. S2.3; Table S2.1). Root colonization and plant dry weight were determined after eight weeks of co-culture overall but four weeks of NaCl treatments (Fig 2.2). In both LK and SK conditions, colonization was similar at +0Na and +50Na (~ 50%), but it drastically decreased to less than 20% on average at +100Na and +200Na (Fig. 2.2A), indicating a direct impact of salinity on AM symbiosis. Additionally, plants in LK+50Na had a significantly higher colonization rate than

plants in SK+50Na (Fig. 2.2A). At SK, shoot biomass of NM and AM plants remained similar during increasing NaCl exposure (Fig. 2.2B). Significant differences were observed in AM plants between +0Na and +50/+200Na treatments, but this was probably driven by one outlier in the SK+0Na conditions (Fig. 2.2B). At LK, a decrease trend in shoot biomass of AM and NM plants was observed, with plants growing in +200Na conditions being significantly different than those at +0Na. The only significant difference between AM and NM plants was reported in SK+200Na where NM plant shoots were bigger than AM ones (Fig. 2.2B). In SK conditions, root biomass of NM plants remained similar with increasing NaCl exposure, except at +100Na where it was significantly lower. However, root biomass of AM plants was significantly lower in all NaCl treatments compared to the +0Na conditions (Fig. 2.2C). At LK, root biomass of NM plants was similar amongst NaCl treatments, except at +200Na where it was significantly lower than under +0Na and +100Na conditions (Fig. 2.2C). Additionally, AM plants displayed significantly lower root biomass in all NaCl treatments compared to the +0Na control condition. When comparing AM and NM seedlings, we noted that NM plants growing at LK+50Na and LK+100Na had a significantly higher root biomass than AM plants (Fig. 2.2C).

Analysis of shoot rubidium, potassium, and sodium concentrations in soybean colonized by R. irregularis DAOM 197198

Plant shoots were then digested to determine Rb^+ , K^+ , and Na^+ concentrations by ICP-OES or ICP-MS, and $K^+ : Na^+$ ratios were calculated (Fig. 2.3). In the control conditions (*i.e.* +0Na), AM plants accumulated significantly more Rb^+ than NM plants only at LK (Fig. 2.3A), indicating a fungal transfer of Rb^+ exclusively under K^+ deficient conditions. This result was also observed in +50Na and +200Na treatments, but not at +100Na where no differences in shoot Rb^+ concentrations were spotted between NM and AM plants exposed to the same K^+ regime. Additionally, even if NM

plants did not have access to the Rb^+ added into the FCs, their shoot Rb^+ concentrations increased significantly at +100Na and +200Na in LK, and at +200Na in SK, compared to the control conditions (Fig. 2.3A). When seedlings were not exposed to NaCl, shoot K^+ concentrations of NM plants were higher in SK than LK conditions (Fig. 3B). However, when plants were colonized, more K^+ accumulated in LK than SK conditions, resulting in significantly more shoot K^+ in AM plants compared to NM ones only at LK. This difference in shoot K^+ concentrations between AM and NM plants was also observed under increasing NaCl exposure (Fig. 2.3B). The NM plants in LK had significantly lower shoot K^+ concentration than NM in SK, except at +200Na where no differences were spotted. In SK, shoot K^+ concentration in AM plants was significantly higher at +50Na and +200Na only compared to NM plants (Fig. 2.3B). Regarding shoot Na^+ accumulation, no differences between any plant were observed in +0Na conditions, and concentrations were significantly much lower than most other NaCl treatments (Fig. 2.3C). Additionally, NM plants at LK had greater shoot Na^+ concentrations than AM plants, and it was significant at +100Na and +200Na. In SK, NM plants also accumulated more Na^+ than AM ones only at +50Na (Fig. 2.3C). Shoot $\text{K}^+:\text{Na}^+$ ratios were also calculated for each plant in each treatment (Fig. 2.4). These ratios were significantly higher in AM plants than NM ones only under no NaCl exposure. Also, the $\text{K}^+:\text{Na}^+$ ratios in AM plants at LK+0Na were significantly greater than at SK+0Na, but this could be due to an outlier in AM plants of LK+0Na (Fig. 2.4).

Finally, analysis of substrates only (*i.e.* without plants or fungi) revealed that available K^+ , Na^+ , and Rb^+ increased proportionally with the addition of each NaCl solution, and that Rb^+ concentrations remained similar between LK and SK conditions (Fig. S2.4).

Analysis of shoot phosphorus and calcium concentrations in soybean colonized by R. irregularis
DAOM 197198

Since cellular P and Ca²⁺ concentrations are great indicators of a functional symbiosis and overall stress response, respectively, their accumulation in plant shoots was also obtained by ICP-OES (Fig. 2.5). In both LK and SK conditions, the shoot of AM plants accumulated significantly more P than NM plants at +0Na and +50Na only, and no difference was observed at higher NaCl exposure (Fig. 2.5A). Regarding shoot Ca²⁺ concentrations, no differences were observed between any of the plants unexposed to NaCl (Fig. 2.5B). However, Ca²⁺ concentrations of NM plants in LK significantly increased under increasing NaCl exposure and even became significantly higher than AM plants in these conditions. At SK, shoot Ca²⁺ concentrations remained low and not significantly different from the control plants, and no differences between AM and NM plants were spotted (Fig. 2.5B).

Salinity and potassium availability negatively affects shoot rubidium concentrations

Correlations of shoot Rb⁺ concentrations with shoot K⁺ concentrations, root colonization, and root biomass were determined in both LK and SK conditions. At both LK and SK, there were significant positive correlations in all NaCl treatments between shoot K⁺ and Rb⁺ concentrations (Fig. 2.6A,B). However, as NaCl treatments increased, R² values decreased, indicating that salinity had a negative effect on the ability to use Rb⁺ as a proxy for K⁺. Concerning correlations between root colonization and shoot Rb⁺ concentrations, only plants in LK showed positive correlations at +0Na and +50Na conditions. All other conditions show either no correlations or no significance (Fig. 2.6C,D). Finally, shoot Rb⁺ concentrations did not correlate with root dry weights in any conditions (Fig. 2.6E,F), indicating that plants with larger biomass did not accumulate more Rb⁺.

Discussion

Soil salinity poses a significant threat to crop growth and productivity that is attributed in part to improper irrigation methods, deforestation, and saltwater intrusion [38]. To help mitigate the impacts of soil salinity on plants, it is vital to understand how crops like soybeans can tolerate saline environments. An important factor for plants to tolerate high salinity conditions is the external K^+ availability. Indeed, improving the root access to K^+ helps in mitigating the cell accumulation and intercellular movement of Na^+ ions, and therefore improves the overall tolerance [39]. In this study, we observed that overall, soybean root development was strongly limited by NaCl exposure. Although the primary root length was not affected by the NaCl treatments, possibly to maintain the acquisition of other nutrients, a strong reduction in the number and length of lateral roots was observed. Similar observations were made in a recent study describing that soybean root biomass and growth were also negatively impacted by NaCl treatments [40]. Biological reasons for these responses may include the alteration of cellular division and elongation due to osmotic stress triggered by the internal Na^+ accumulation [40]. Here, we also report that there was absolutely no impact of K^+ availability on root development under any NaCl treatment in young seedlings. This indicates a limited effect of external K^+ availability on biomass production for this soybean cultivar to cope with NaCl exposure, at least at early developmental stages.

Multiple studies have shown the positive impact of AM fungi on salinity tolerance in various crops [41–46], including soybean [47,48], by preventing the accumulation of Na^+ ions in plant tissues. Here, we investigated the combined effect of both AM inoculation and external K^+ availability in soybean plants exposed to increasing NaCl treatments. First, we observed that although a slow decrease in shoot biomass can be observed with increasing salinity, no differences

occurred between AM and non-inoculated plants in both LK and SK conditions. On the other hand, the root biomass was similar between AM and NM plants unexposed to NaCl, but multiple differences appeared at higher salinity with more biomass produced in NM plants compared to AM ones. However, the external K^+ availability did not have a great influence on root weight either. Altogether, these data indicate that the symbiotic status of this soybean cultivar and the external K^+ availability had very limited impacts on the plant biomass production under moderate and high salinity conditions. In a study using salt tolerant and susceptible peanut plants, it reported that the application of K^+ ameliorated salt tolerance in the tolerant cultivar by excluding Na^+ [49]. Our results may suggest that the soybean cultivar we used was somehow tolerant to K^+ deprivation, at least in our experimental conditions. Interestingly, the AM colonization was negatively and strongly impacted by NaCl exposure: it gradually dropped from about 50 % in unexposed plants and those under moderate salinity (*i.e.* 50 mM of NaCl) to less than 20 % on average at high salinity (*i.e.* 100/200 mM of NaCl). Additionally, shoot P concentrations in NM and AM plants matched AM root colonization's response to increasing NaCl treatments. This could be due to the decrease of the number and length of lateral roots we observed in our short-term experiment (Fig. 1), making less colonization sites available for the fungus, and/or to the fungal inability to properly grow or form symbiosis. Overall, our results demonstrate that salinity does influence soybean root development, biomass, and AM colonization, but also show a more moderate impact of K^+ availability on these factors.

In AM symbiosis, tracking K^+ movements between fungi and plants is challenging because there are no stable or safe isotopes that can be easily used. Recently, we used for the first time Rb^+ and cesium as proxies for K^+ in mycorrhizal symbioses [21,23,35]. Although cesium did not show very compelling results, Rb^+ appeared to be more reliable. Since it is challenging to distinguish

whether nutrients are within the plant or the fungus in colonized roots, we only report here shoot nutrient concentrations. Soybean shoot Rb^+ and K^+ concentrations showed positive correlations, even if it declined with increasing salinity, indicating that Rb^+ is transported in a similar way as K^+ , and so can be used as a proxy in our experiments. Contrary to what we reported with the model legume *Medicago truncatula*, where more Rb^+ was recorded in AM plants under both LK and SK conditions [21], here, colonized plants displayed higher shoot Rb^+ concentrations in LK conditions only. This indicates that the AM fungus was able to provide soybean plants with Rb^+ only under K^+ -demanding conditions, and this transport was inhibited when plants had access to K^+ . This observation is corroborated by the positive correlation between shoot Rb^+ concentration and AM colonization at LK+0Na. However, the absence of correlation at higher salinity levels suggests limitations in the use of Rb^+ as a proxy for K^+ in these conditions. Altogether, our results showed that Rb^+ transport was more significant in plants in LK conditions and suggest a dual effect of AM symbiosis and external K^+ availability on Rb^+ transport upon salinity.

Additionally, we observed that colonized plants accumulated less Na^+ compared to NM plants in both LK and SK conditions. This observation was also made in high salinity conditions where a strong reduction of root colonization was recorded. It indicates that even if the fungus was not able to transport P to colonized plants, it has the capacity to prevent Na^+ ion accumulation in surrounding roots, probably by a sequestration mechanism in hyphae. Previously, Sharifi et al. (2007) also observed low root colonization under high salinity in soybean, but still had some significant differences in nutrient uptake with AM plants having more nutrients, similarly to what we observed here with K^+ . The positive impact of AM fungi in the prevention of Na^+ accumulation we described in soybean shoots was dependent on the external K^+ availability. Indeed, NM plants having access to K^+ did not accumulate more Na^+ ions than AM plants. This indicates that this

soybean cultivar was quite responsive to K^+ application and did not rely on the AM fungus to alleviate high salinity in high K^+ conditions. The maintenance of an elevated $K^+ : Na^+$ ratio in plant cells is crucial to tolerate environmental stresses such as K^+ deprivation and salt stress [50]. We observed that AM plants in both LK and SK conditions displayed a higher $K^+ : Na^+$ ratio than NM plants at no salt. The AM plants in LK resulted in a higher $K^+ : Na^+$ ratio than AM plants in SK.

This confirms that the AM fungus takes part in reducing Na^+ accumulation in the plant. This is corroborated by the shoot Ca^{2+} concentration records that also showed differences between NM and AM plants only at LK, indicating a better salinity tolerance under SK conditions. We also observed that AM plants had higher shoot K^+ concentrations compared to NM plants in all K^+ and Na^+ treatments, except at SK+100Na. However, shoot K^+ concentrations increased in all plants, including NM ones, with increasing salinity conditions. Although it could result from a classical plant response to alleviate salinity, the analysis of our substrate revealed that more K^+ was released under high Na^+ treatments (Fig. S2.4). Therefore, the observed increase in shoot K^+ concentrations may also be due to an increase in K^+ availability in the substrate triggered by the addition of NaCl. Similar observations were made with the Rb^+ contained in the medium (Fig. S2.4A), indicating that the substrate we used may not be optimal for salinity studies. Other substrates such as pure sand could be used in future experiments to continue investigating these questions.

Conclusion

In this study, we investigated the impact of NaCl exposure on soybean upon symbiosis with *R. irregularis* DAOM 198197 depending on external K^+ availability. Overall, our results show that 1) soybean root development was considerably reduced by salinity with no impact of K^+ availability, 2) AM root colonization decreased under high salinity even though the fungus presence prevented Na^+ uptake by soybean roots in an external K^+ availability-dependent manner,

and 3) AM-mediated Rb^+ transport was detected only under K^+ deficiency and was reduced upon salinity. Altogether our results show that both AM fungi and K^+ availability influence salinity tolerance in soybean and highlight some limitations for using Rb^+ as a proxy for K^+ in these conditions.

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Figure captions

Figure 2.1: Soybean seedlings were placed on large plates under various NaCl and K^+ regimes for ten days. Primary root length (A), number of lateral roots (B), and average lateral root length (C) were determined on ten-day-old soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and to either 0, 50, 100, or 200 mM of NaCl by using ImageJ software. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to two-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 15$.

Figure 2.2: Root colonization and plant biomass of soybean seedlings inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. (A) Root colonization was determined on eight-week-old soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl, using the grid line intersection method. Shoot (B) and root (C) dry weights were determined in eight-week-old inoculated (AM) or not inoculated (NM) soybean plants. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to two-way (A) and three-way (B and C) ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 6$.

Figure 2.3: Shoot rubidium, potassium, and sodium concentrations in soybean plants inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Rubidium (Rb⁺, A), potassium (K⁺, B), and sodium (Na⁺, C) concentrations were determined by ICP-OES or ICP-MS in the shoots of eight-week-old inoculated (AM) or not inoculated (NM) soybean plants grown limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 6$.

Figure 2.4: Shoot potassium:sodium ratio in soybean plants inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Ratios between shoot K^+ and Na^+ concentrations were calculated for each inoculated (AM) or not inoculated (NM) soybean plant grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 6$.

Figure 2.5: Shoot phosphorus and calcium concentrations in soybean plants inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Phosphorus (P, A) and calcium (Ca^{2+} , B) concentrations were determined by ICP-OES in the shoots of eight-week-old inoculated (AM) or not inoculated (NM) soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 6$.

Figure 2.6: Correlation between shoot rubidium concentrations and shoot potassium concentrations, root colonization, and root dry weights. Correlations were calculated between shoot Rb^+ and K^+ concentrations (A,B), root AM colonization (C,D), and root dry weights (E,F) in soybean plants inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of sodium chloride (Na^+ treatment).

Table S2.1: Recipe of Long Ashton solutions used in this study to prepare the limited and sufficient potassium conditions. All components were dissolved in milli-Q water. The first four components made up the major solution for limited (LK) and sufficient (SK) media, and the six following components made up the minor solution. The major solution was stored at $-20\text{ }^\circ\text{C}$ and the minor solution and Fe-EDTA-Na were stored at room temperature after autoclaving.

Table S2.2: Incremental increase of Na^+ treatment concentrations used in the eight-week-long AM symbiosis experiment.

Figure S2.1: Ten-day-old soybean seedlings grown in limited (top) or sufficient (bottom) potassium conditions at varying levels of sodium chloride exposure. These pictures illustrate the data presented in Figure 1.

Figure S2.2: Soybean seedlings were placed on large plates under various NaCl and K^+ regimes for ten days. Root dry weight was determined in ten-day-old soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and to either 0, 50, 100, or 200 mM of NaCl.

Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to two-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 15$.

Figure S2.3: Eight-week-old soybean plants inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Plants were inoculated (AM) or not inoculated (NM) and watered with limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ solutions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl. These pictures illustrate the data presented in Figure 2-6.

Figure S2.4: Available rubidium, potassium, and sodium elements in the substrate used in the mycorrhizal experiment. Rubidium (Rb^+ , A), potassium (K^+ , B), and sodium (Na^+ , C) concentrations were determined in our potting substrate by ICP-MS and ICP-OES. Limited (LK, 0.05 mM, 200ml) or sufficient (SK, 3.75 mM, 200ml) K^+ solutions and either 0, 50, 100, or 200 mM of NaCl treatments and $RbCl$ (3.75 mM, 30 ml) were added to the substrate. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 3$.

Figure 2.1

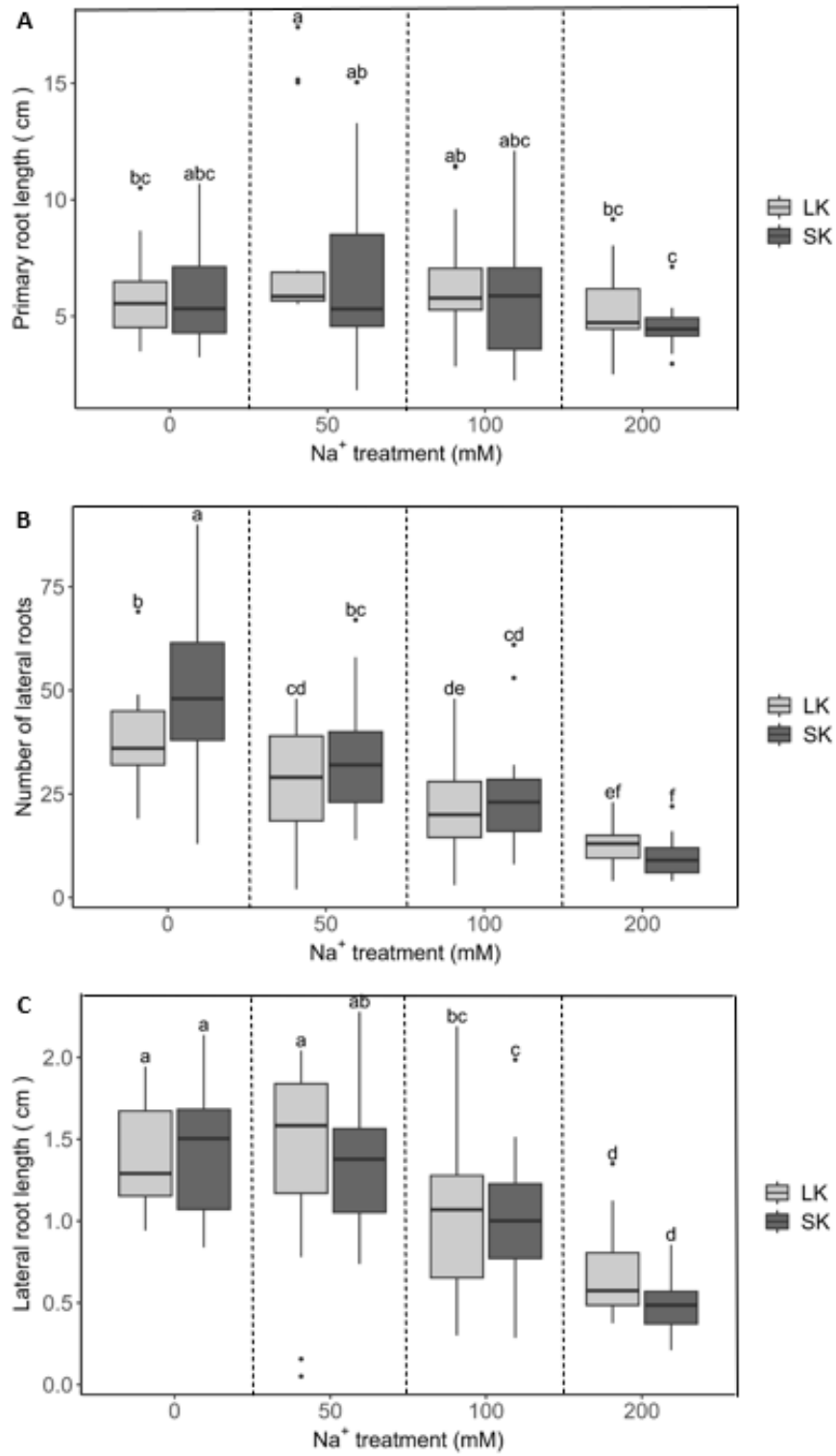


Figure 2.2

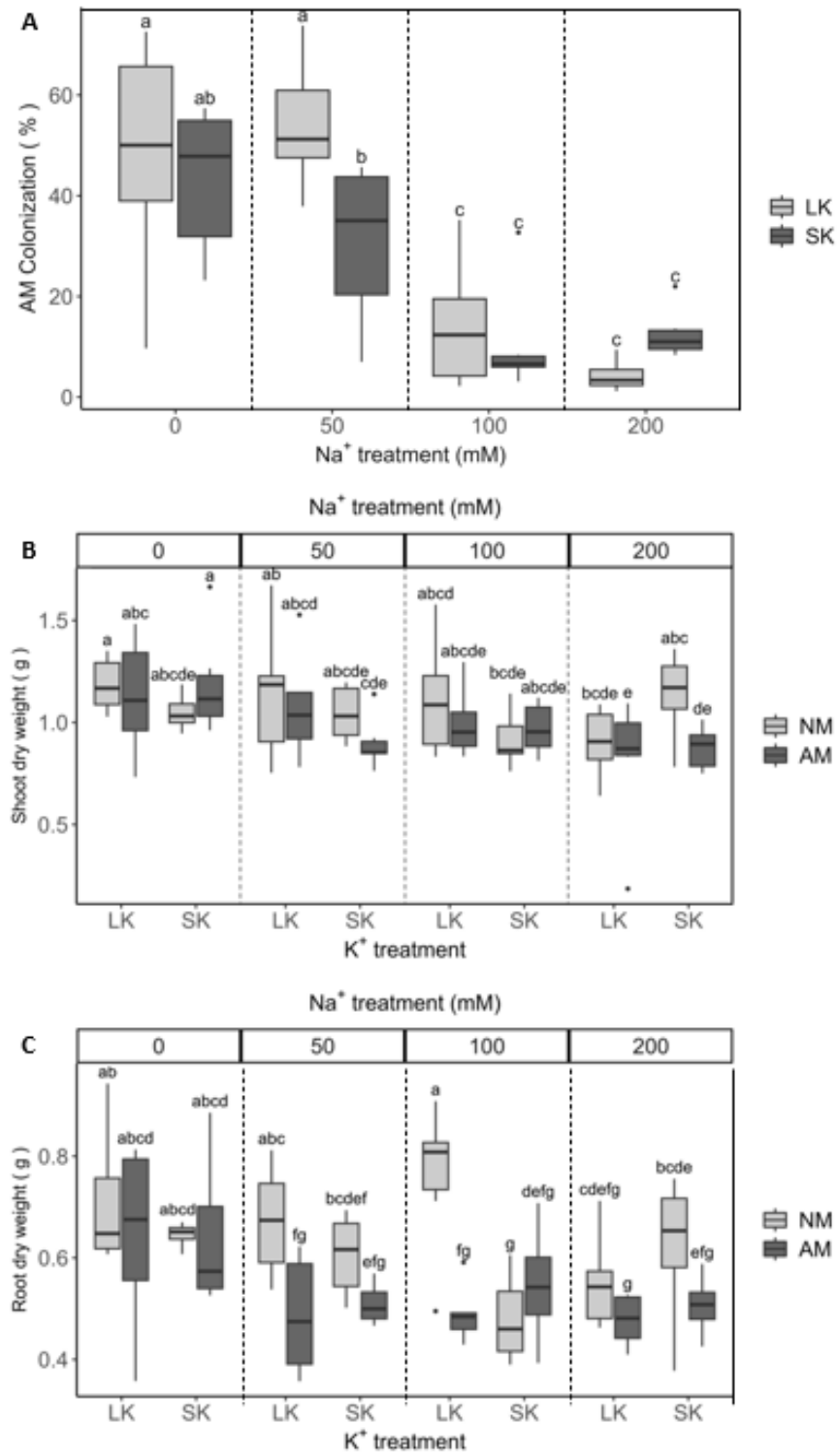


Figure 2.3

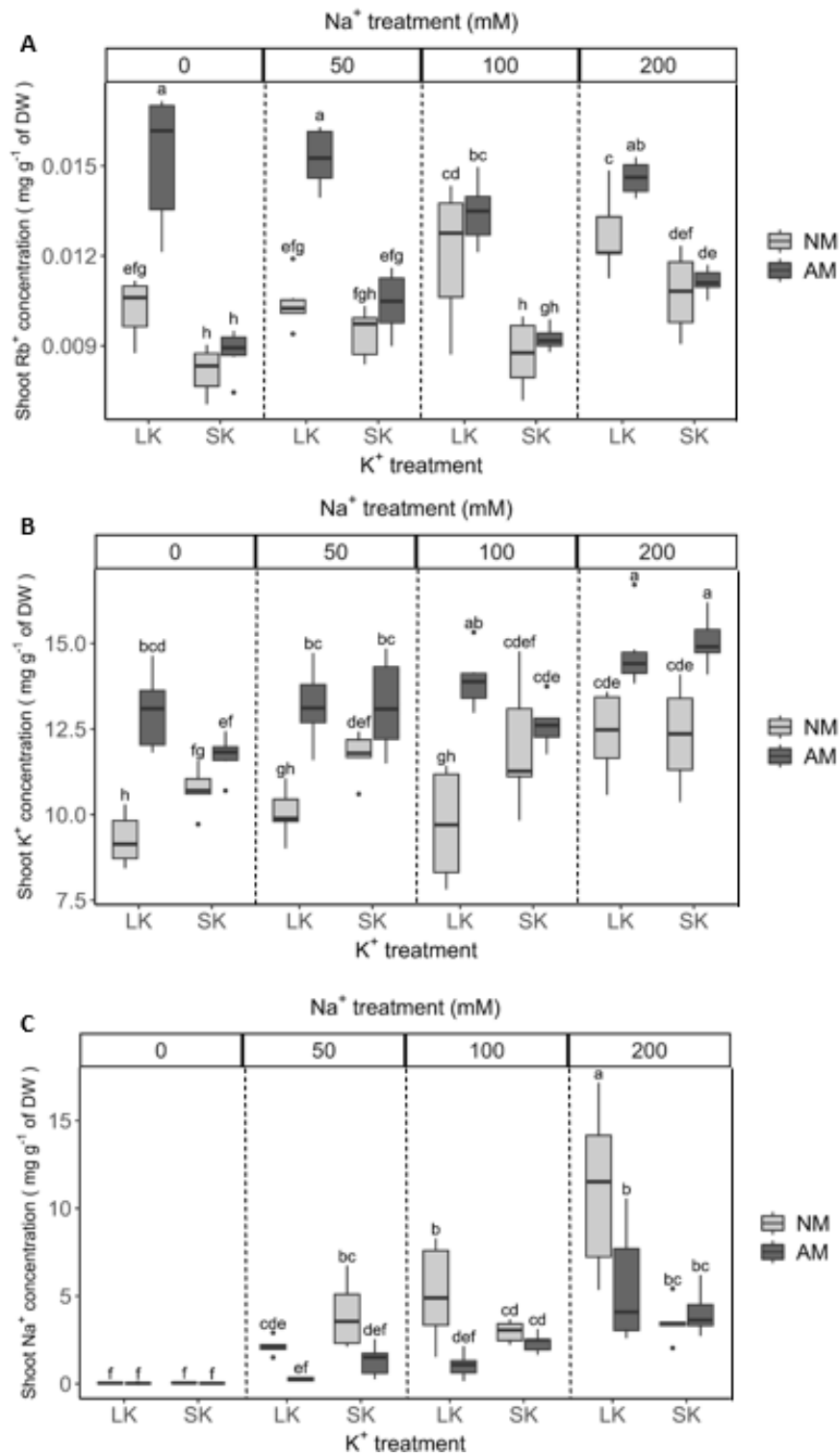


Figure 2.4

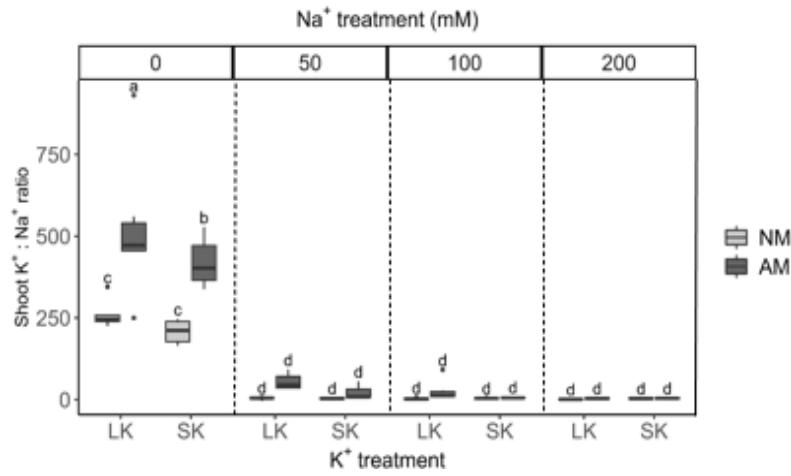


Figure 2.5

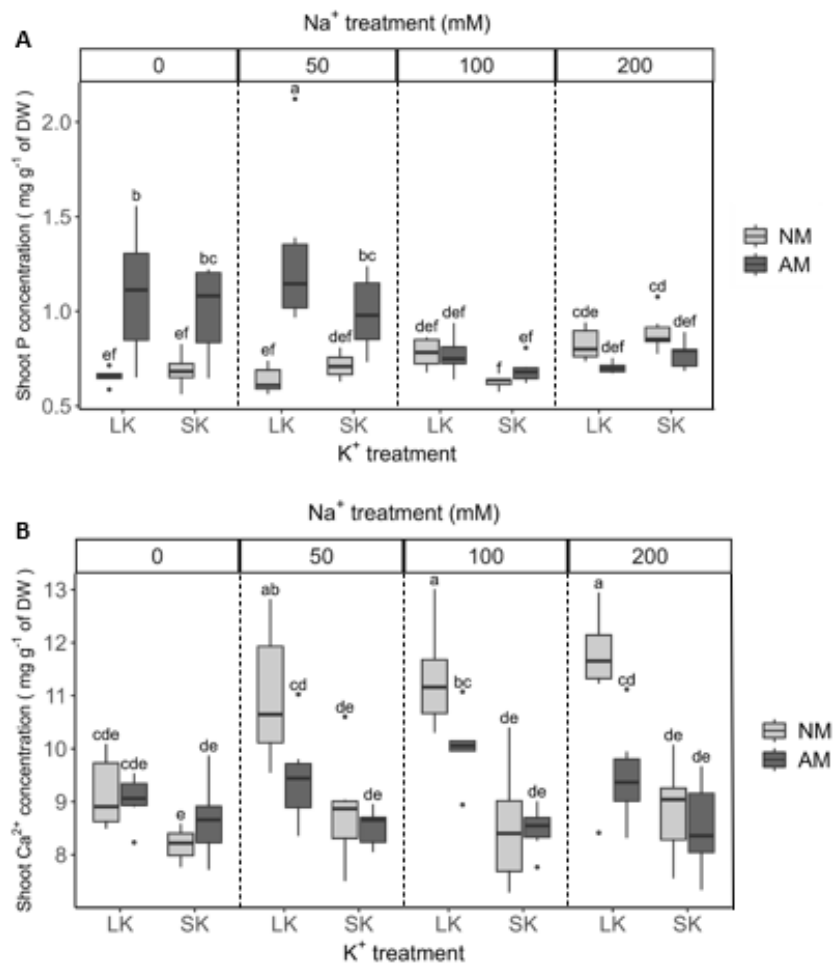


Figure 2.6

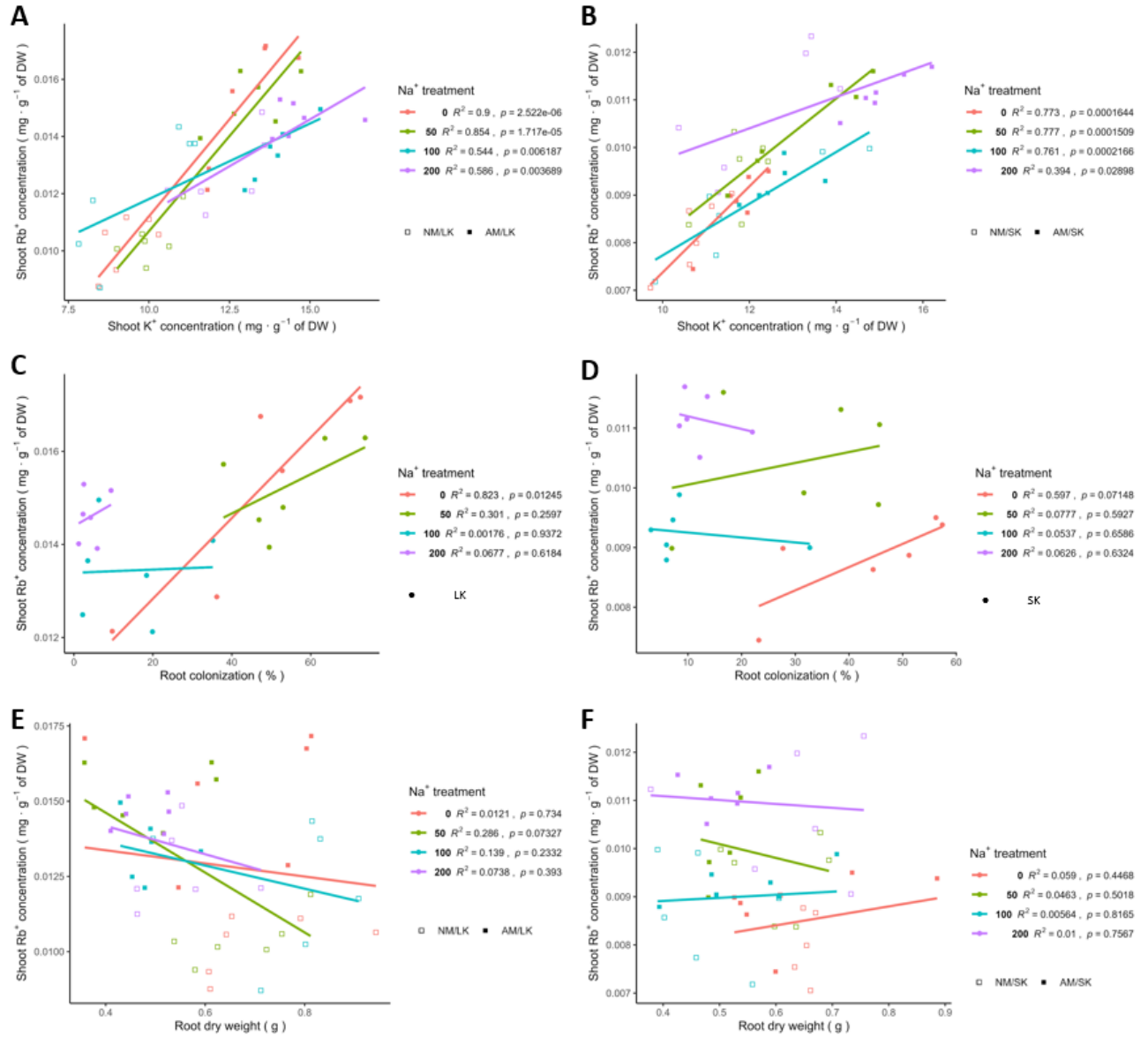


Table S2.1

Components	Concentration in LK solutions	Concentration in SK solutions
KNO ₃	1 mM	75mM
Ca(NO ₃) ₂ • 4H ₂ O	77mM	40 mM
NaH ₂ PO ₄	0.15 mM	0.15 mM
MgSO ₄ • 7H ₂ O	20 mM	20 mM
MnSO ₄ • H ₂ O		11.2 mM
CuSO ₄ • 5H ₂ O		1 mM
ZnSO ₄ • 7H ₂ O		1 mM
H ₃ BO ₃		48.5 mM
NaCl		85.6 mM
(NH ₄) ₆ Mo ₇ O ₂₄ • 4H ₂ O		0.07 mM
Fe-EDTA-Na		6 mM

Table S2.2

Final Na⁺ treatment	Na⁺ concentrations (mM) in Long Ashton solution at:					
	Day ≤ 25	Day 28	Day 31	Day 34	Day 37	Day ≥ 40
0 mM Na ⁺	0	0	0	0	0	0
50 mM Na ⁺	0	10	20	30	40	50
100 mM Na ⁺	0	20	40	60	80	100
200 mM Na ⁺	0	40	80	120	160	200

Figure S2.1

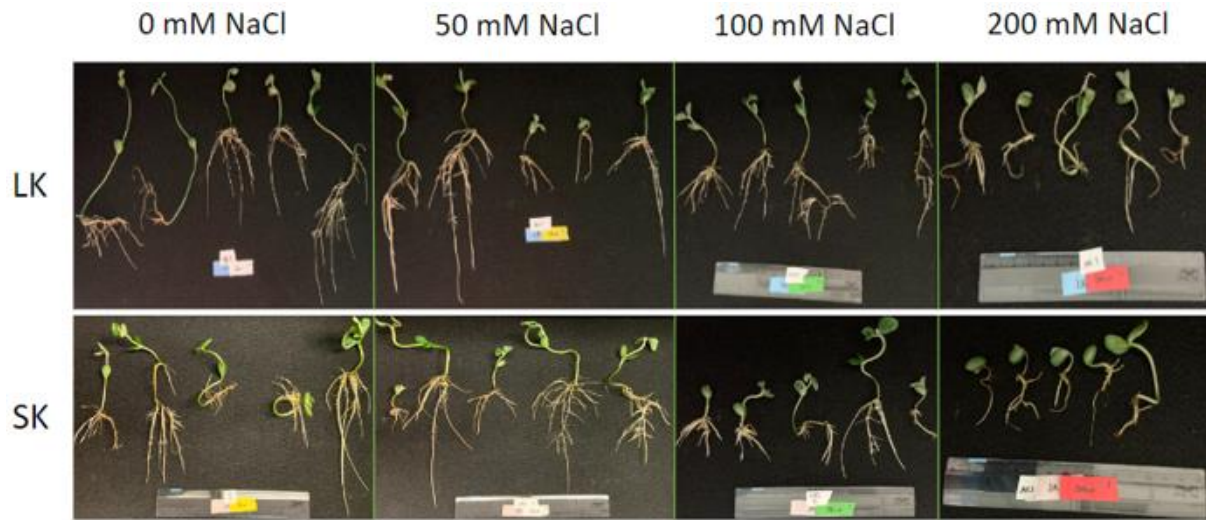


Figure S2.2

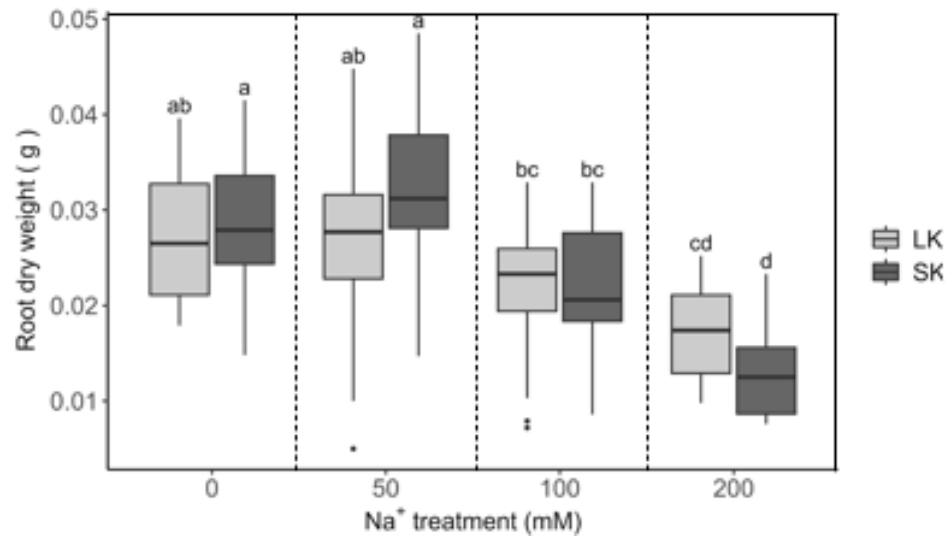


Figure S2.3

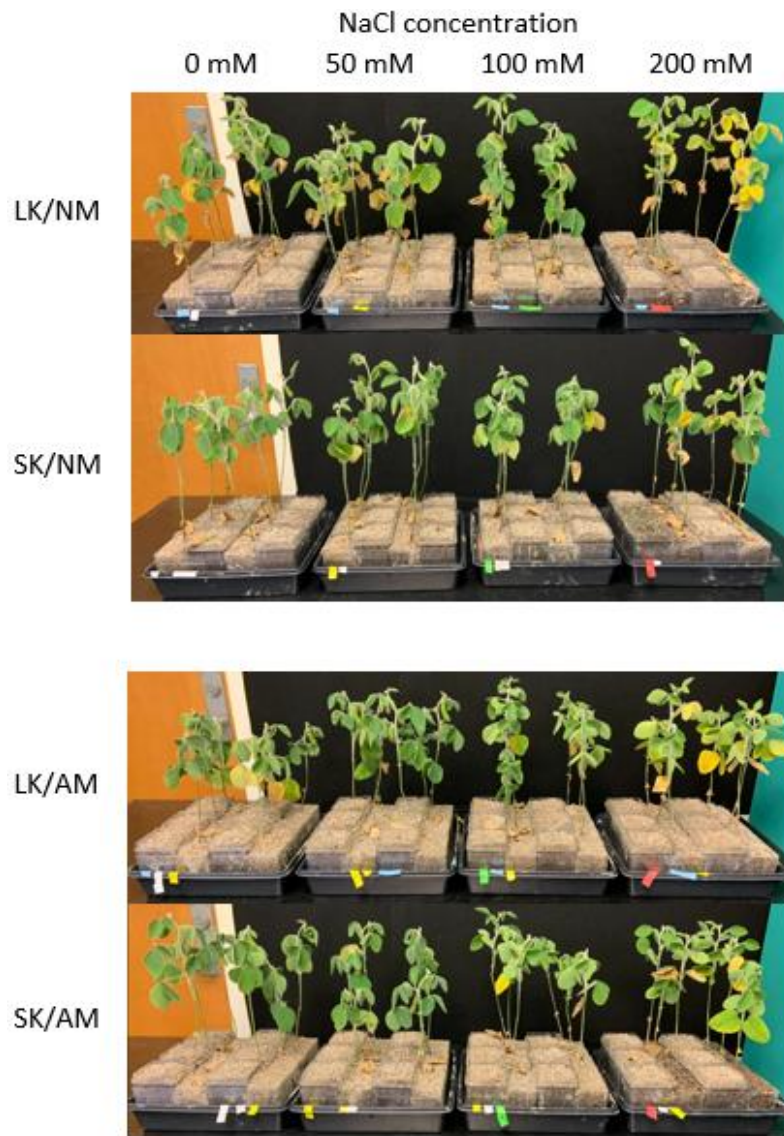
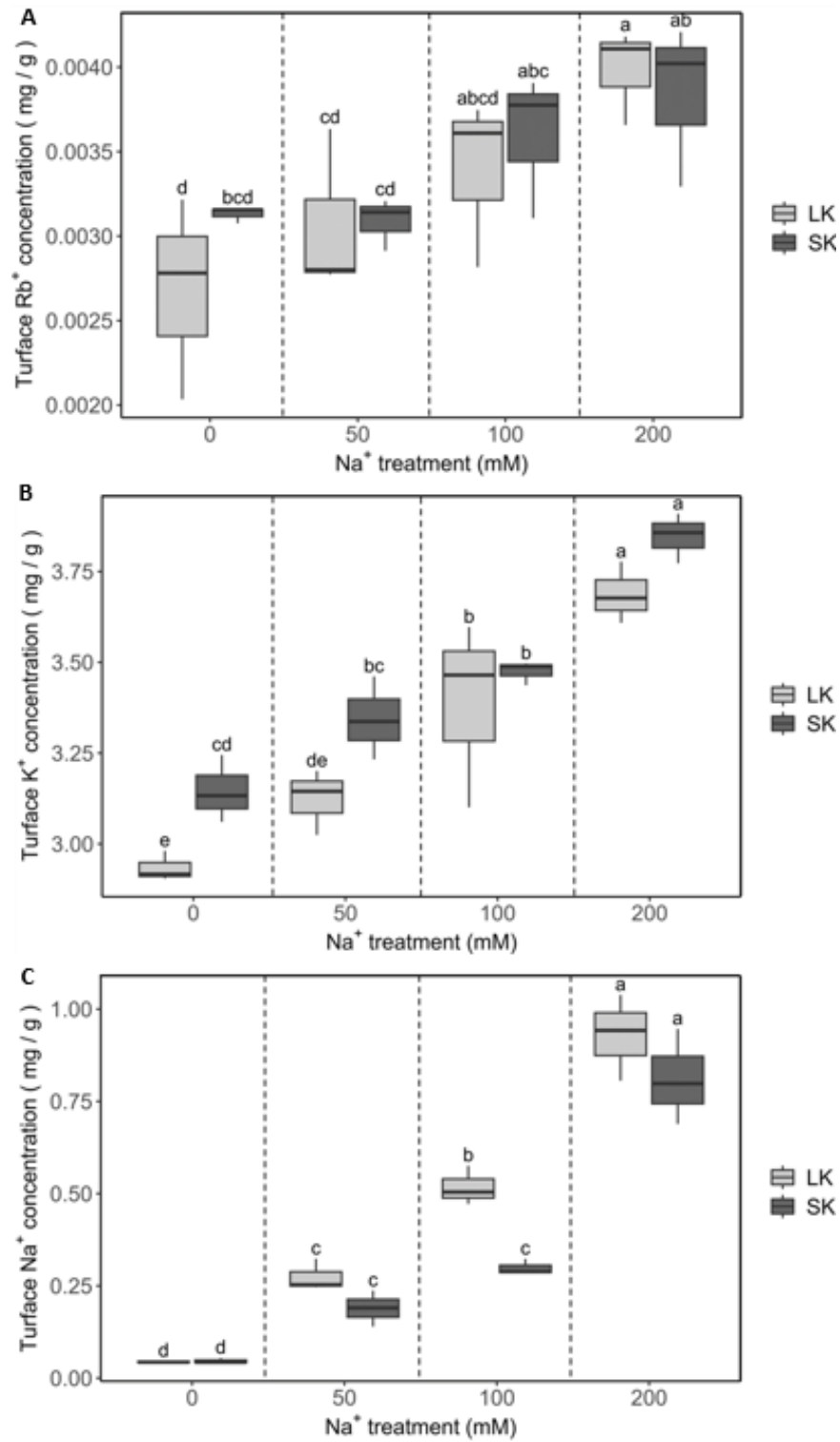


Figure S2.4



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Additional work: Dual impact of external potassium availability and AM symbiosis on soybean maturity group six in both limited and sufficient K⁺

The same experiment as described above was also completed with the other soybean cultivar from MG 6. The K⁺, NaCl, and growing conditions were the exact same. The experiment with cultivar MG 6 failed due to extremely low or no root AM colonization, especially in the sufficient-K⁺ of all NaCl treatments, indicating no establishment of AM symbiosis (Fig. 2.7). Also, colonization was very variable at LK-0Na, making it difficult to conclude anything. Therefore, this experiment was not analyzed more and may be repeated in the future.

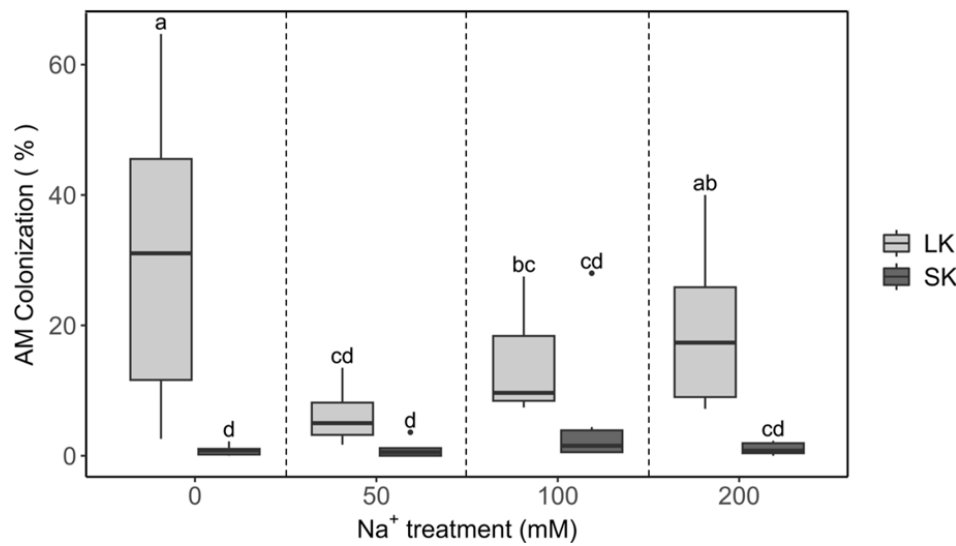


Figure 2.7: Root colonization of soybean (MG 6) seedlings inoculated with *Rhizophagus irregularis* DAOM 197198 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Root colonization was determined on eight-week-old soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and exposed for four weeks to either 0, 50, 100, or 200 mM of NaCl, using the grid line intersection method. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to two-way ANOVA followed by a Fisher's post-hoc LSD test (P < 0.05). n = 6.

Chapter THREE: Soybean nodulation by the salt-tolerant rhizobium *Sinorhizobium fredii* under salinity depends on external potassium availability

Introduction

Rhizobia are N-fixing bacteria in the soil that can form symbiosis with legumes such as soybean, by infecting the roots and forming nodules. Some rhizobia strains are highly specific about the plants they will interact with, but others have a broader host spectrum (Provorov and Tikhonovich 2003). In the nodule, differentiated bacteroids reduce atmospheric dinitrogen (N_2) into ammonia. This is an expensive process for the plant because it uses 16 ATP to reduce a single N_2 molecule. Therefore, plants allocate up to 30 % of their photosynthetic carbon to rhizobia (Provorov and Tikhonovich 2003), indicating that carbon allocation to nodules is a limiting factor in N_2 fixation.

For this thesis, the N-fixing bacteria *Sinorhizobium fredii* USDA 208 was chosen due to its quick growth and tolerance to salt. It is dominant in soils under arid/semi-arid climates and saline-alkaline soils (Nitawaki et al., 2021). In Elsheikh and Wood (1995), soybeans were grown in saline conditions and either inoculated with salt-tolerant *S. fredii* USDA 208 or salt-sensitive *Bradyrhizobium japonicum* RCR 3407. Under salinity, root biomass was the highest in plants inoculated with *S. fredii*. Even with decreased nodulation from salinity, the salt-tolerant rhizobia was able to fix more N_2 than the salt-sensitive strain. This is a great method to help mitigate soil salinity stress in soybeans and plants.

Soil salinity has a detrimental effect on plants threatening global food supply. Soybean is an important economical crop used all over the world with many uses such as a protein source, oil,

and animal feed (Jia et al., 2020). Salinity can negatively impact soybean's height, biomass, leaf size, number of branches, internodes, pods, and seed yield (Otie et al., 2021). Lenis et al. (2011) placed four accessions of soybean (*Glycine max*, *Glycine soja*, *Glycine tomentella*, and *Glycine argyrea*) under NaCl treatments (0, 50, 75, and 100 mM) to investigate their differential adaptation to salt tolerance. It was observed that lower leaf scorch occurred and a higher capacity to prevent Na⁺ and Cl⁻ transport from the soil solution to the plant was in tolerant genotypes compared to sensitive genotypes. Additionally, leaf Na⁺ concentrations contributed more to plant injury instead of leaf Cl⁻ concentrations. Also, in salt tolerant genotypes, leaf chlorophyll-meter readings were higher in tolerant plants than sensitive ones at all NaCl levels. Highly salt-tolerant accessions were *G. argyria* and *G. tomentella* compared to *G. soja* and *G. max*.

Another important factor alleviating salt tolerance in soybeans is external potassium (K⁺) availability. K⁺ is essential for multiple functions to help a plant's growth and development. When plants are K⁺-deprived, growth and development can be hindered. In a recent study, Thornburg et al. (2020) grew wheat in sufficient (2.5 mM) and limiting K⁺ (0.02 mM) conditions. It was observed that in K⁺ deficient conditions, wheat seedling growth and development was negatively impacted. It was apparent by decreased plant biomass and smaller plant size. All of these factors lead to the aim of this chapter which is to understand the dual impact of salinity and external K⁺ availability on nodule formation and nutrient accumulation in soybeans inoculated with *S. fredii*. Here, soybean plants were germinated and transplanted into singular pots. Plants were inoculated with *S. fredii* and grew in limited-K⁺ (LK, 0.05 mM) or sufficient-K⁺ (SK, 3.75mM) and exposed to five levels of NaCl (0, 25, 50, 100, and 200 mM). Biomass, nodule count, and shoot K⁺, Na⁺, and Ca²⁺ concentrations were determined.

Materials and Methods

Plant and bacterial materials

Soybean seeds from a commonly planted maturity group six variety were prepared the same way as explained in **Chapter 2**; the only difference is the use of a different substrate that is more suited for nodulation experiments. Seeds were surface sterilized in 0.6% bleach for four minutes, rinsed five times with milli-Q water, and germinated in double-rinsed potting substrate (Safe T Sorb®; Ep Minerals®, Nevada, USA) for around 10 days in a growth chamber at 23 °C/14 h and 18 °C/10 h day/night cycle.

The bacterial strain *S. fredii* USDA 208 was cultured from glycerol stock in three 50 ml tubes containing Ty medium and placed on a shaker at 28 °C. After two days, each culture was transferred into autoclaved 250 ml flasks and placed back at 28 °C to reach their stationary growth phase that took 2 additional days. A spectrophotometer was used to obtain an optical density (O.D.) of 2.25. The culture was separated into four 50 ml falcon tubes to be centrifuged at 4,000 rpm for 5 mins. Supernatant was poured out and replaced by 10 ml of tap water. After vortexing and re-centrifuging, the final O.D. was recorded at 4.0. Through calculations, plants were inoculated with the bacterial cultures with an O.D. of 0.1.

Potassium and sodium conditions

Plants were supplied with different N-free modified Fahraeus media (Catoira et al., 2000) in which K⁺ and Na⁺ concentrations were changed as follow: two treatments containing either limited (LK, 0.05 mM K⁺) or sufficient (SK, 3.75 mM K⁺) K⁺ levels were used, as well as five Na⁺ conditions with the addition of 0, 25, 50, 100, and 200 mM NaCl (Table 3.1). Both K⁺ and Na⁺ treatments

were mixed to make a total of ten different treatment media, abbreviated below as LK+0/25/50/100/200Na or SK+0/25/50/100/200Na. The pH of the solutions were then adjusted with 13.5 M Ca(OH)₂ to reach a pH of 6.5.

Table 3.1: Recipe of N-free modified Fahraeus media used in this study to prepare the limited (LK) and sufficient (SK) potassium and sodium (NaCl) conditions. All components were dissolved in milli-Q water. The media solution's pH was adjusted with 13.5 M Ca(OH)₂ to 6.5. All components were stored at room temperature after autoclaving.

Components	Concentration in LK and SK solutions
MgSO ₄	8.3 mM
NH ₄ H ₂ PO ₄	43.5 mM
Fe-EDTA	7.3 mM
MnSO ₄	0.7 mM
CuSO ₄	0.6 mM
ZnSO ₄	0.6 mM
H ₃ BO ₃	1.6 mM
Na ₂ MoO ₄	2.4 mM
CaCl ₂	9.0 mM
KCl	0.05 mM (LK), 3.75 mM (SK)
NaCl	0, 50, 100, & 200 mM

Rhizobial symbiosis

Germinated soybean seedlings were transplanted into pots and inoculated with *S. fredii* USDA 208 (Nod+) or kept non-inoculated (Nod-) two days after transplant. Six pots were placed in a plastic tray, corresponding to six replicates per treatment. Seven-to-ten-day old plants were watered at the base of the tray with 200 ml of LK+0/25/50/100/200Na or SK+0/25/50/100/200Na every three days for a duration of twenty-one days post-inoculation.

Biomass, nutrient content, and nodule number determination

After harvest, fresh shoot and root biomass were obtained, and the shoots were dried in an oven for five days at 70 °C, and dry shoot biomass was obtained. Root nodules were immediately counted after obtaining root wet weight by using a microscope before they were placed in the oven for five days at 70 °C followed to obtain the root dry weight. All non-inoculated plants were checked for nodules. Dried shoot samples were grinded, and digested in nitric acid to determine K⁺, Na⁺, and Ca²⁺ concentrations by ICP-OES at the Environmental and Agricultural Testing Service (EATS) at North Carolina State University.

Statistical analyses

All data analyses were based on 6 replicates for each treatment (5 for non-inoculated plants at SK+100Na). All the figures were made in R v.4.0.3 (R Core Team, 2020), using the ggplot2 package. Depending on the experiment, two-way or three-way analysis of variance (ANOVA) was conducted to determine the differences among means followed by Fisher's post-hoc LSD test using the agricolae package (Mendiburu 2010).

Results

Increasing salinity negatively affects root nodule formation and soybean biomass

To assess the impact of salinity and K⁺ supply on rhizobial symbiosis and biomass in soybean, plants were inoculated with *S. fredii* USDA 208 and compared to non-treated plants through placement in single pots under various NaCl and K⁺ regimes (Table 3.1). Root nodule count and plant dry weight were determined after twenty-one days post inoculation (dpi). In both LK and SK, root nodule count was significantly different at +0Na and +25Na, and decreased to about 0-

17 nodules on average at +50Na, +100Na, and +200Na (Fig. 3.1). This shows an impact of salinity on root nodule count. Plants at SK+0Na significantly had more nodules than plants at LK+0Na, and plants in LK+25Na had significantly higher root nodule count than plants in SK+25Na (Fig. 3.1).



Figure 3.1: Root nodulation of soybean seedlings inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Root nodules were determined on soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to a three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

At SK, shoot biomass of non-inoculated (Nod-) plants and those treated with *S. fredii* (Nod+) remained similar as NaCl levels increased except at +100Na with Nod- plants having a higher shoot biomass than Nod+ plants (Fig. 3.2). Overall, Nod- and Nod+ plants in SK had a decreasing trend from the control to +200Na, showing that shoot biomass is negatively impacted by salinity (Fig. 3.2). At LK, shoot biomass of Nod- and Nod+ had significant differences only at

+0Na and +50Na with Nod- plants having a higher shoot biomass than Nod+ plants (Fig. 3.2). Nod+ plants show a significant increase in LK at +0Na to +25Na followed by a significant decrease at +50Na (Fig. 3.2).

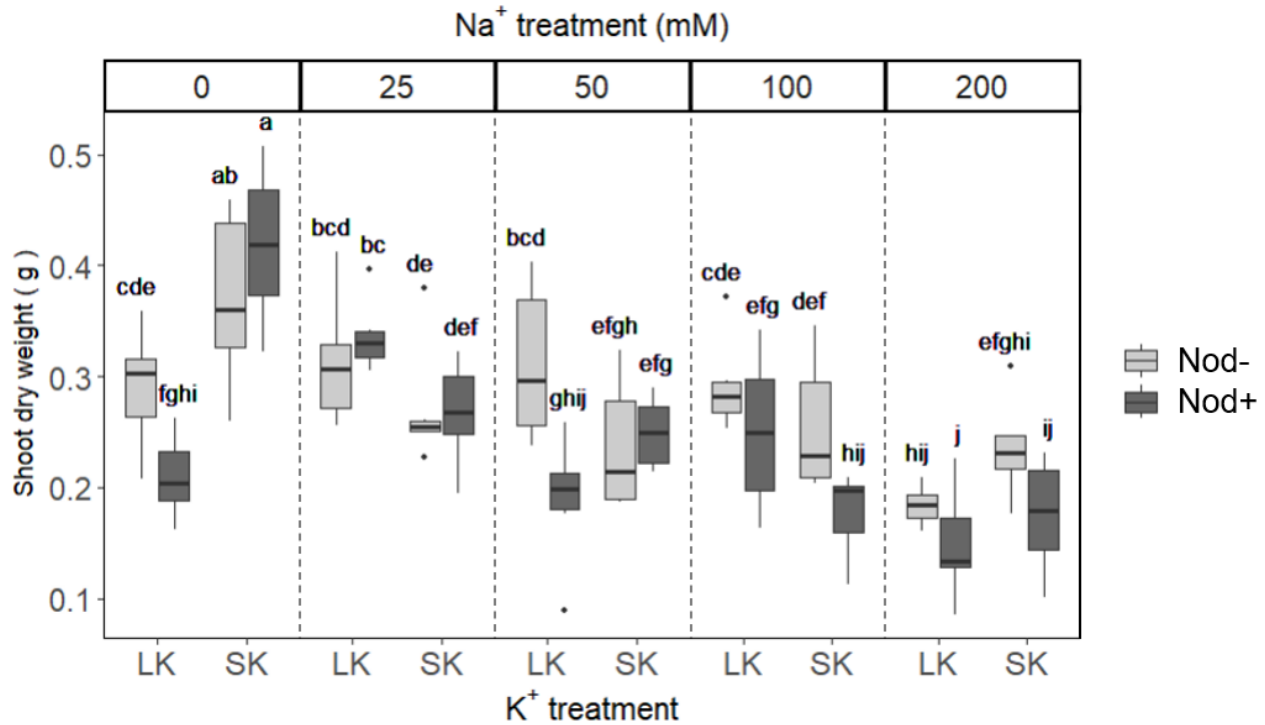


Figure 3.2: Shoot biomass of soybean seedlings inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Shoot biomass was determined on inoculated (Nod+) or non-inoculated (Nod-) soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

In SK, root biomass of Nod- and Nod+ only significantly differed at +100Na and +200Na with Nod- plants having a higher root biomass. Overall, Nod- and Nod+ plants of SK showed a decreasing trend from +0Na to +200Na (Fig 3.3). Nod- plants significantly decreased from +0Na to 200Na (Fig. 3.3). In LK, root biomass of Nod- and Nod+ significantly differed at +0Na, +25Na, and +50Na (Fig. 3.3). Nod- plants in LK remained similar at +0/25/50Na and then significantly

decreased at +100Na and +200Na (Fig. 3.3). Nod+ plants in LK significantly increased from +0Na to +25Na and significantly decreased to +100Na. Then it remains similar at +100Na and then significantly decreases at +200Na (Fig. 3.3).

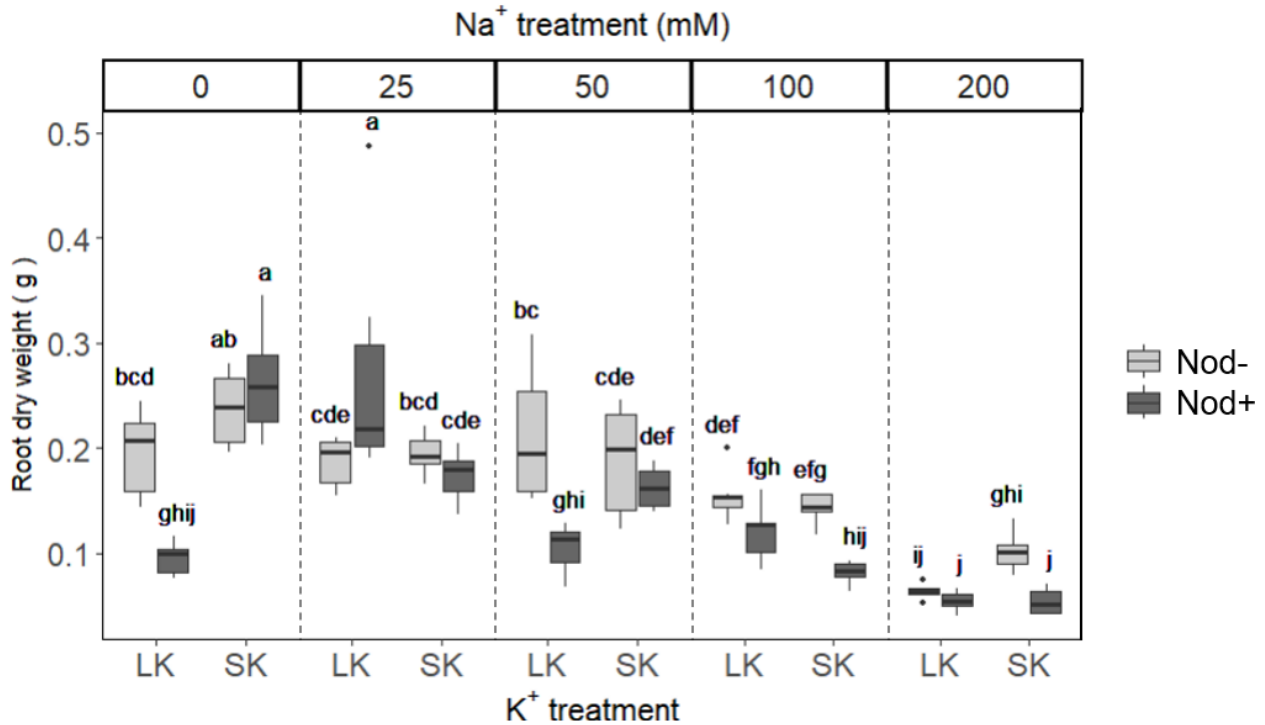


Figure 3.3: Root biomass of soybean seedlings inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Root biomass was determined on inoculated (Nod+) or non-inoculated (Nod-) soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K⁺ conditions and 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

Analysis of shoot potassium, sodium and calcium concentrations in soybean colonized by *S. fredii* USDA 208

Shoots of soybean plants inoculated with *S. fredii* USDA 208 or not were digested to determine K⁺, Na⁺, and Ca²⁺ concentrations by ICP-OES. In both LK and SK conditions at +0Na, shoot K⁺ concentrations were significantly lower compared to the other NaCl treatments (Fig. 3.4).

Inoculated plants in LK+0Na had a significantly higher shoot K^+ concentration than non-inoculated plants, while no difference was shown between Nod- and Nod+ plants at LK+0Na (Fig. 3.4). In SK conditions, shoot K^+ concentrations of Nod- and Nod+ plants remained similar with an exception at +200Na with Nod- plants accumulating significantly more K^+ than Nod+ plants (Fig. 3.4). Nod+ plants in SK show a significant difference by a bell shape at +25Na to +100Na, Nod- plants remained similar at +25/50/100/200Na (Fig. 3.4) In LK, Nod- and Nod+ plants showed a significant difference at +50Na with Nod- plants having a higher shoot K^+ concentration (Fig. 3.4). Nod- and Nod+ plants in LK showed a very slow increase from +25Na to +100Na followed by a significant decrease at +200Na (Fig. 3.4). Nod- plants in LK increased from +0Na to +50/100Na and then decreased at +200Na. Nod+ plants in LK increased from +0Na to +25/50/100Na and then decreased at +200Na (Fig. 3.4).

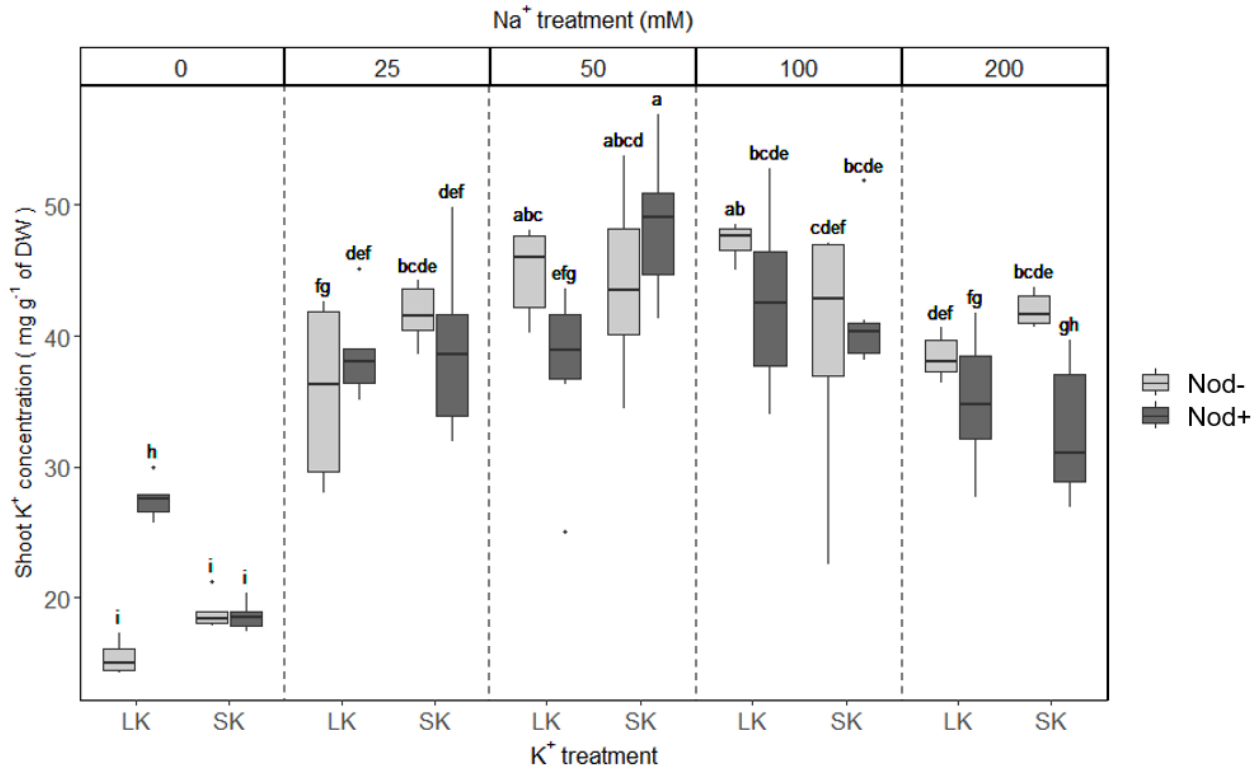


Figure 3.4: Shoot potassium concentrations in soybean plants inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Potassium (K^+) concentrations were determined by ICP-OES in the shoots of inoculated (Nod+) or non-inoculated (Nod-) soybean plants grown limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

Shoot Na^+ concentrations in the control and at +25Na did not have any differences between any plant, and concentrations were significantly lower than most of the NaCl treatments (Fig. 3.5). From +50Na to +200Na, a significant increase of shoot Na^+ concentration was observed in LK and SK of Nod- and Nod+ plants (Fig. 3.5). In SK conditions, Nod+ plants had higher shoot Na^+ concentrations than Nod- ones at +100Na, and Nod+ plants had lower shoot Na^+ concentrations than Nod- plants at +200Na (Fig. 3.5). In LK conditions, Nod+ plants had significantly higher shoot Na^+ concentrations than Nod- plants at +50Na, and then remained similar at +100Na and +200Na (Fig. 3.5).

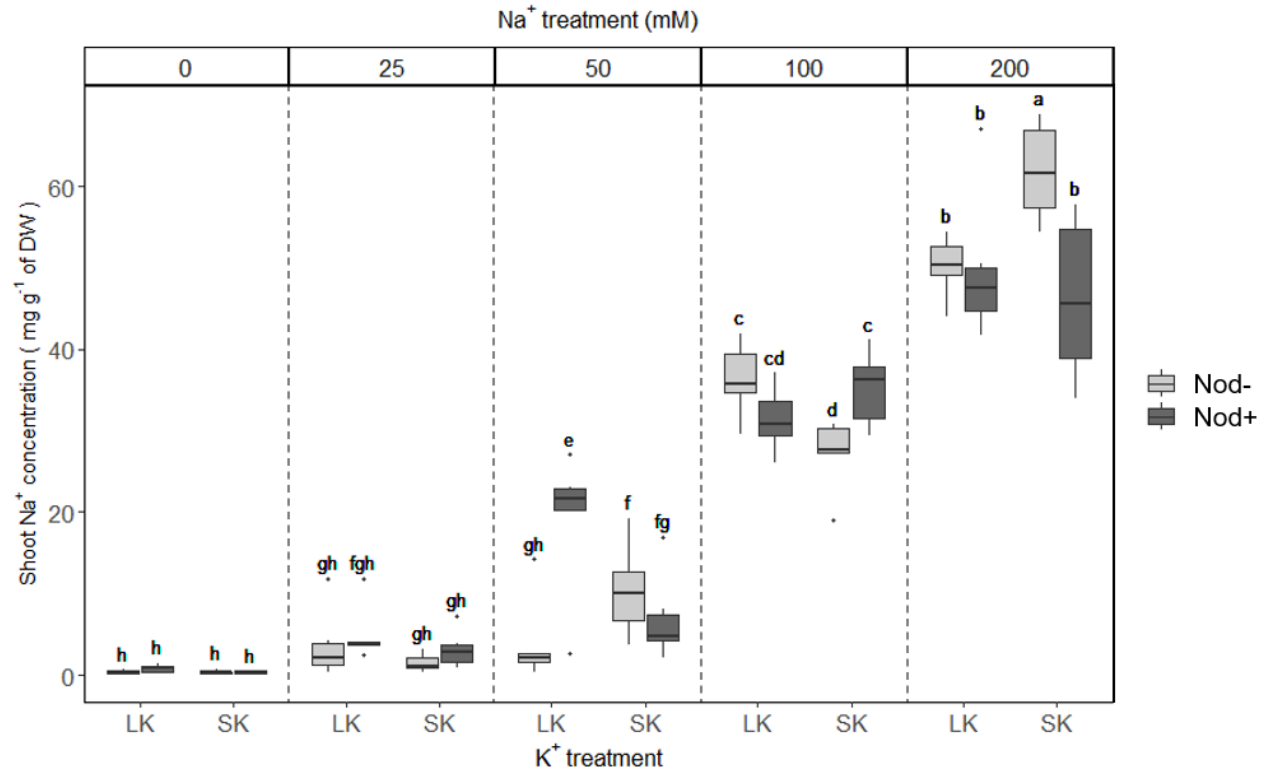


Figure 3.5: Shoot sodium concentrations in soybean plants inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Sodium (Na^+) concentrations were determined by ICP-OES in the shoots of inoculated (Nod+) or not inoculated (Nod-) soybean plants grown limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

Shoot Ca^{2+} concentrations are good indicators of a working symbiosis and overall stress response. Overall, all plants in +0Na did not differ from one another and had significantly lower shoot Ca^{2+} concentrations compared to the remaining NaCl levels (Fig. 3.6). There was an increase at +25/50Na followed by a drop at +100Na and +200Na (Fig. 3.6). In SK, there was only a significant difference between Nod- and Nod+ plants at +50Na with Nod+ plants having higher shoot Ca^{2+} concentrations than Nod- plants (Fig. 3.6). In LK, there were significant differences between Nod- and Nod+ plants at +25Na with Nod+ being higher than Nod-. This could be due to

an outlier. Nod- plants at LK+50Na had higher shoot Ca^{2+} concentrations than Nod+ plants (Fig. 3.6).

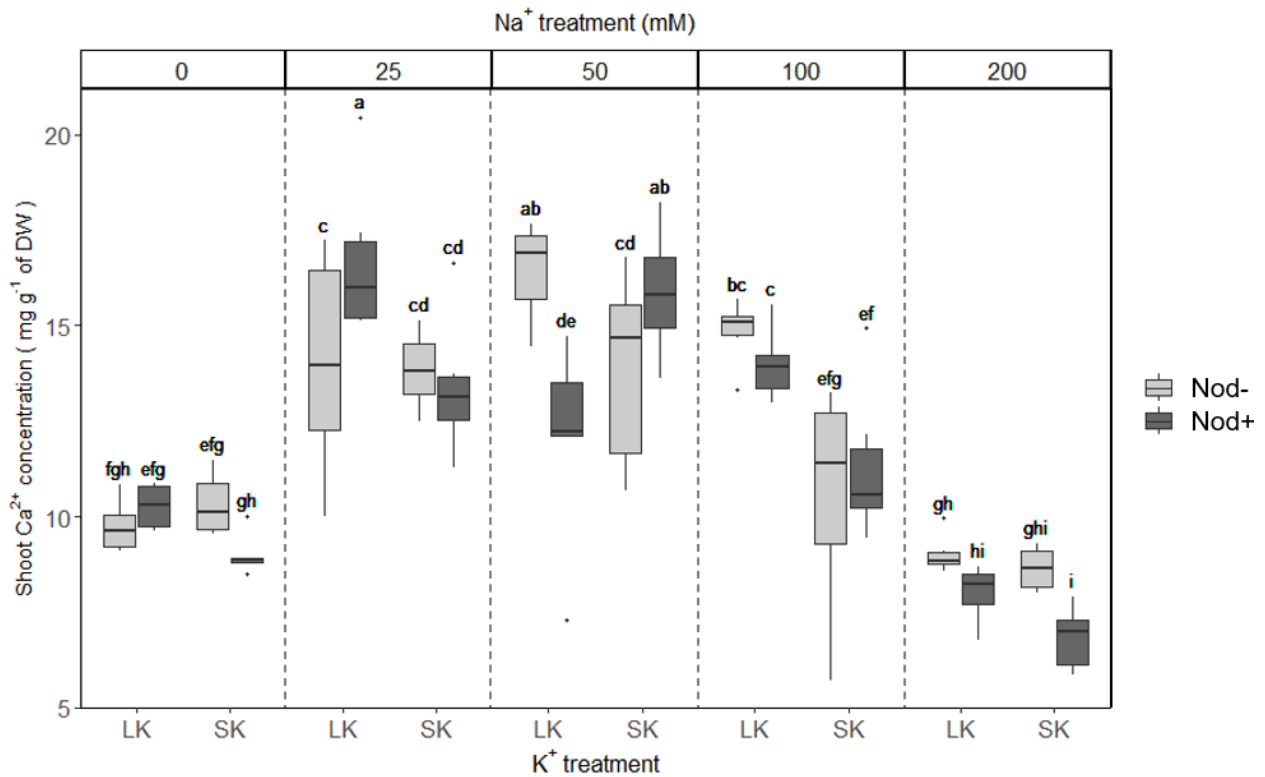


Figure 3.6: Shoot calcium concentrations in soybean plants inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Calcium (Ca^{2+}) concentrations were determined by ICP-OES in the shoots of inoculated (SF) or not inoculated (NI) soybean plants grown limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and exposed for four weeks to either 0, 25, 50, 100, or 200 mM of NaCl, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 5-6$.

Discussion

Crops planted in low lying coastal fields are threatened by increasing salinity caused by rising sea levels and salt intrusion (Mondal et al., 2023). When Na^+ ions accumulate in the cytosol, a disturbance in ion homeostasis occurs. This causes an efflux of K^+ out of the cytosol, and Na^+ can take over K^+ binding sites which results in chlorophyll degradation and the alteration of optimal

protein functions. Maintaining K^+ homeostasis can enhance plant tolerance to salinity stress (Kumari et al, 2021). Recruiting symbiotic microbes such as salt-tolerant rhizobia can alleviate salinity stress too. There is limited research on whether and how K^+ nutrition impacts rhizobial symbiosis in plants, and particularly soybeans, under salinity (Liu et al., 2022). Here, we observed that root nodule formation was higher in the plants placed in SK conditions and not exposed to NaCl. At moderate salinity (25 mM), it was the opposite and plants in LK conditions had more root nodules than plants at SK. This indicates that the external K^+ availability had a direct effect on the development of soybean root nodules with this particular bacterial species, *S. fredii*, but this effect was influenced by salinity. At higher salinity levels (50-200 mM NaCl), a decrease in root nodule count was observed, but without any impact of external K^+ concentration.

A general decrease in shoot and root biomasses was observed with increasing exposure to NaCl. Also, under non-saline conditions, both shoot and root biomass were higher in plants in SK compared to those in LK, which shows better nutrition. In SK+0Na conditions, Nod+ plants appeared to have had higher shoot and root biomass than Nod- plants but there were no significant differences between them. In LK+0Na, Nod- plants had significantly higher biomass than Nod+ plants. In moderate to higher salinity, we observed multiple differences in biomass with Nod- plants being significantly higher than Nod+ plants. With no salt, nodule count was higher in plants at SK, plant shoot and root biomass were higher in Nod+ plants at SK compared to Nod+ plants at LK. This suggests a positive impact of K^+ availability on nodulation and plant biomass in NaCl-free conditions. There was variation in the plant's growth to K^+ levels applied but salinity negatively impacted these parameters. Shoot K^+ concentrations were also recorded. In no salinity, plants in LK and SK were lower than plants in salinity. As NaCl was applied, shoot K^+ concentrations increased, indicating a dual impact of K^+ availability and salinity. A peak was

observed followed by a decrease. There was an odd effect of K^+ and NaCl on K^+ acquisition that could be due to the increase in K^+ availability from the substrate when NaCl was added. Indeed, although we used a slightly different substrate, we have shown in **Chapter 2** that adding NaCl to it results in the release of K^+ and Rb^+ ions. A similar phenomenon may have occurred here, making more K^+ ions available for plant root uptake. The compatibility of this symbiosis between this soybean cultivar and *S. fredii* USDA 208 could also have a potential impact on the morphological responses observed here.

In Liu et al. (2022), two of their K^+ conditions (0.02 and 2.0 mM) were comparable to ours (0.05 and 3.75mM) and they did not observe any difference in root nodule numbers. However, they did observe increased nodule fresh weight being the highest at 2 mM K^+ . Another observation was the increase of nitrogenase activity in plants under sufficient K^+ . The shoot and root biomass had a general increase as more K^+ was supplied, at 2 mM K^+ inoculated plants had higher biomass compared to non-inoculated plants. Lastly, shoot K^+ content was higher in 2 mM K^+ than 0.02 mM K^+ . At sufficient- K^+ , they observed inoculation with rhizobia enhanced K^+ uptake. This study showed that more K^+ available enhanced nodule growth, plant biomass, and nitrogenase activity. However, this study used a different soybean cultivar (Williams-82) and rhizobial species (*Bradyrhizobium japonicum* USDA 110). As mentioned earlier, the compatibility of the plant and rhizobia chosen can highly impact the results. Also, in our study, nodule fresh weight, K^+ content in nodules, and nitrogenase activity were not obtained, making it difficult to compare the work done by Liu et al. (2022) and ours. It would be wise to perform similar analysis in a future study. That being said, our study and Liu et al. (2022) concluded in a positive impact on nodulation and shoot and root biomass when plants were in sufficient- K^+ conditions.

Shoot Na^+ and Ca^{2+} concentrations were also obtained in Nod- and Nod+ plants. Shoot Na^+ concentrations generally increased as more NaCl was added to the plants. We also observed variation in Na^+ uptake between Nod- and Nod+ plants in both LK and SK conditions at moderate to high salinity. Altogether, we conclude in an impact of salinity but a lesser impact of K^+ availability. Shoot Ca^{2+} concentrations showed low levels at no salinity followed by an increase, peak, and decrease as NaCl levels increase. There was a mixed response of shoot Ca^{2+} concentrations to K^+ availability. A direct impact of NaCl was evident. These mixed results could partially be due to ion balance defects in infected tissue by the bacteria (Fedorova et al., 2020). In *Medicago truncatula* (Fedorova et al., 2020), the K^+ content in the root nodules gradually decreased within 12-15 days since the rhizobia was released in the host cell. The host plant and the bacteria shared the K^+ supply so that could have led to the ion imbalance. In another previous study, two alfalfa cultivars (salt sensitive and tolerant) were inoculated with a *Sinorhizobium meliloti* A2 and salt-tolerant Rm1521 and placed under different levels of NaCl (Bertrand et al., 2015). They observed that both cultivars resulted in decreased shoot and root biomass when exposed to salt. When inoculated with the salt-tolerant-Rm1521, nodule sink strength and metabolism were maintained in both cultivars under NaCl stress. This suggests that combining both salt-tolerant plants and rhizobia can further help in mitigating salt tolerance. Our study suggests that the salt sensitive soybean MG 6 and salt tolerant *S. fredii* USDA 208 are mainly compatible under no salt to moderate salt due to how nodulation was impacted by salt.

Conclusion

In this study, we investigated the dual impact of external K^+ availability and salinity on the formation of nodules by *S. fredii* USDA 208 and some nutrient accumulation on soybean. Overall, our results show that soybean biomass, root nodule count, and shoot K^+ and Ca^{2+} concentrations

were negatively impacted by increasing salinity, and limited effects of K^+ availability were observed. Suggested studies to complete are to extend symbiosis time from 21 days to 28 days or even longer time, and to obtain more data such as nodule weight and nitrogenous activity. There is limited research in the interrelationship between rhizobial symbiosis, K^+ availability, and NaCl stress. More research is therefore needed to help determine what are the effects of K^+ and NaCl on nodulation in soybean.

Additional work: Dual impact of nodulation and external potassium availability on salt tolerance in another soybean cultivar (MG 5)

The same experiment as described above was done for the other soybean cultivar from MG 5 that we also used in **Chapter 2** (submitted manuscript). All K^+ , $NaCl$, and growing conditions were the exact same. The goal of the experiment was to compare two soybean cultivars: the excluder is MG 5 which excludes Cl^- , and the includer is MG 6 which is more sensitive to Cl^- . This experiment with cultivar MG 5 failed due to nodules being present in non-inoculated plants (Fig. 3.7). Non-inoculated plants at LK+0/25Na and SK+50Na resulted in high numbers of nodules, indicating that contaminations occurred. Therefore, the experiment was not analyzed further and needs to be re-conducted in the future.

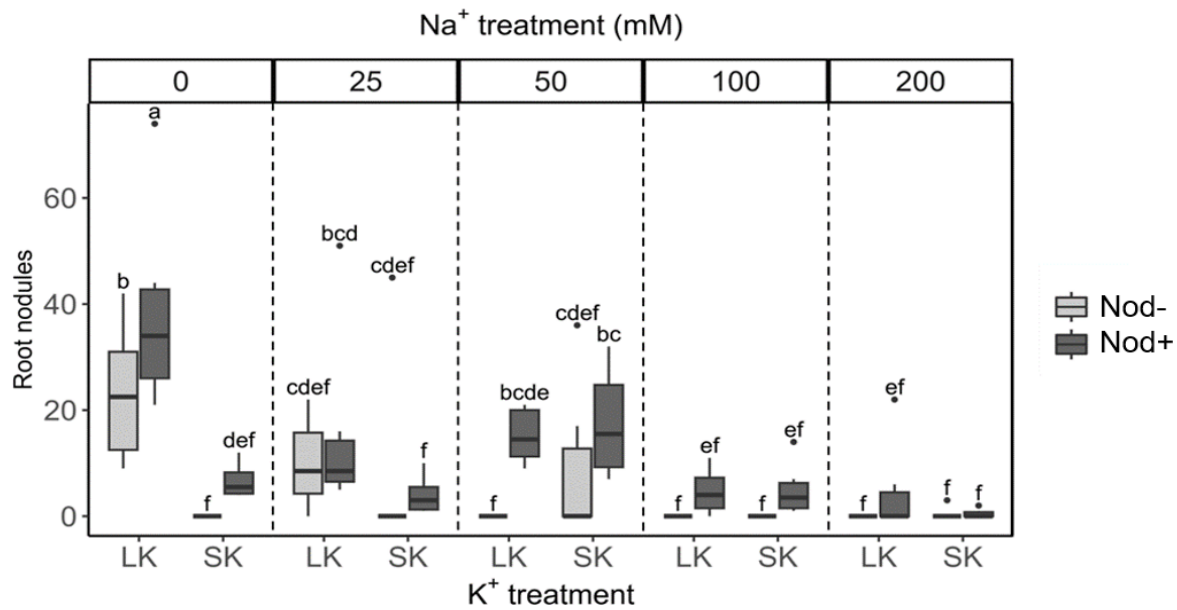


Figure 3.7: Root nodulation of soybean (MG 5) seedlings inoculated with *Sinorhizobium fredii* USDA 208 and grown in limited or sufficient potassium conditions at varying levels of sodium chloride exposure. Root nodules were determined on soybean plants grown in limited (LK, 0.05 mM) or sufficient (SK, 3.75 mM) K^+ conditions and 0, 25, 50, 100, or 200 mM of $NaCl$, at 21 dpi. Median values are indicated by horizontal lines within boxplots, and whiskers on top and bottom extend from first quartile to minimum and from third quartile to maximum values, respectively. Significant differences between treatments are indicated by different letters according to a three-way ANOVA followed by a Fisher's post-hoc LSD test ($P < 0.05$). $n = 6$.

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