UNIVERSITY *of the*
WESTERN CAPE

Influence of a selected endophyte consortium on salinity responses in *Medicago sativa.*

Supervisor: Prof. Ndomelele Ndiko Ludidi

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape.

Eden Keyster
3673616

General Plagiarism declaration

UNIVERSITY of the WESTERN CAPE

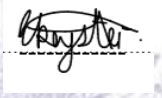
60 YEARS of hope, action & knowledge

FACULTY OF NATURAL SCIENCE

Name: Eden Keyster

Student number: 3673616

1. I, **Eden Keyster**, declare that this thesis entitled "**Influence of a selected endophyte consortium on salinity responses in *Medicago sativa*"** is my own work.
2. I know what plagiarism entails, namely, to use another's work and to present it as my own without attributing the sources in the correct way. (Refer to University Calendar part 1 for definition)
3. I know that plagiarism is a punishable offence because it constitutes theft.
4. I understand the plagiarism policy of the Faculty of Natural Science of the University of the Western Cape.



Signature

-----09/03/2022-----

Date

Acknowledgements

First and foremost, I would like to give all thanks to the Father Almighty. Only He knows and understands the extent of my efforts generated towards this paper. This year was accompanied with many obstacles but somehow with faith and great mercy, He has carried me through and therefore I will be forever grateful.

Also, a great thanks to my supervisor, Ndomelele Ndiko Ludidi, who continues to inspire and support me as his student. His calmness, compassion and ambition are used as a driving force to continue my research. I would also like to offer my gratitude towards my co-supervisor, Marshall Keyster. His great efforts to expose and support me throughout my life does not go unnoticed. He continues to set the bar as a great scientist and academic.

To my parents, Deon and Elizabeth Keyster. A lifetime would never be enough for me to express the amount of gratitude and thankfulness I owe to you. The continuous support, tough love and energy you invest into my life is something deeply recognized. I know my actions speak otherwise, but my mind and soul are forever grateful. The Almighty truly went out of His way to bless me with your union.

I would like to extend my thanks towards my siblings, Dyon and Amber Keyster, who are always willing to lend a hand, shoulder or time to help. There is something truly special between the bond of siblings and I am so blessed to be able to experience it with you two. You are a true representation of eternal friendship. I would also like to show appreciation towards my family and friends. All of you play your own role in my life and I am aware that this role is what I need to make a success. You all are part of the driving force behind my happiness.

I would like to extend my thanks to Gerhard Basson, Ali Ali and Yoneal Bless, members from my research group, who are always willing to help and guide. They show me that science is truly amazing in all forms. Their compassion and care for life is a perfect display of humanity. Also, thank you to the Environmental Biotechnology Laboratory (EBL) and the Plant Biotechnology Research Group (PBRG) for creating an environment in which I could complete my research as well as enjoy it. Lastly, I would like to offer gratitude toward the National Research Foundation (NRF), who, through the DSI-NRF Centre of Excellence in Food Security, is in support of the work put forward in this thesis.

List of Abbreviations

APX: Ascorbate peroxidase

CAT: Catalase

EDTA: Ethylenediaminetetraacetic acid

H_2O_2: Hydrogen peroxide

MDA: Malondialdehyde

NaCl: Sodium chloride

NBT: Nitro blue tetrazolium chloride O_2

PAGE: Polyacrylamide gel electrophoresis

PVPP: Polyvinylpolypyrrolidone

PGPB: Plant growth promoting bacteria

ROS: Reactive oxygen species

TBA: Thiobarbituric acid

TBRAS: Thiobarbituric acid reactive substances

TCA: Trichloroacetic acid

TEMED: N, N, N, N'- Tetramethylethylenediamin

List of Figures

CHAPTER 1

CHAPTER 3

List of Tables

Influence of a selected endophyte consortium on salinity responses in *Medicago sativa*

Eden Keyster

MSc. Thesis, Department of Biotechnology, University of the Western Cape

Abstract

Salinity is one of the major limiting factors to crop production, which consequently contributes to the risk of reduced food security. Among other factors, food security depends on availability of sufficient and nutritious food for humans. Livestock such as cattle and sheep are fed with various plant-based feeds; with *Medicago sativa* (commonly known as alfalfa or lucerne) being a very important forage/feed crop, so much that it is regarded as the queen of forage crops. However, alfalfa is severely affected by high soil salinity and thus its growth and yield are drastically reduced in soils with high NaCl content. Among the various alfalfa genotypes/varieties examined in this study, Agsalfa was identified as salt tolerant because it performed better under salt treatment compared to Magna601. The changes observed under salt stress in Agsalfa appear to be mediated by an underlying signalling mechanism. Bacterial endophytes aid in relieving salt stress toxicity in both Magna601 and Agsalfa, changing antioxidant activity or physiology of the plant itself. Among the bacterial endophytes identified, all of them are recognized soil-inhabiting microorganisms, but some have been identified as plant growth promoting bacteria (PGPB). From the preliminary results in this the study, it has been found that the bacterial endophytes are not halophytic but they possess the ability to alleviate salt stress in the plants. These bacterial endophytes are capable of maintaining the growth of the plant when the plant is subjected to salinity stress in its environment. The results shown in this study prove that the application of technological approaches and biofertilizing with bacterial endophytes can be applied in agricultural systems to sustain crops under salinity conditions. This study provides further insight on how plants regulate responses to salt stress, adding to can improve our understanding of this interaction.

Keywords: Salinity stress, bacterial endophytes, oxidative stress, Alfalfa, plant development, H_2O_2, ascorbate peroxidase, catalase

Table of contents:

Chapter 1: Literature Review

1. Introduction

Increases in the human population and the reduction of arable land for cultivation poses a major threat to sustainable agriculture (Shahbaz and Ashraf, 2013). Abiotic environments are critical for the optimum growth of plants, with continuous stress in these environments leading to limited plant growth (Li and Li, 2017). These abiotic stresses include drought, low and high pH of soil, and salinity (Nakashima *et al.,* 2021). The latter is regarded as a significant environmental constraint that limits the productivity of plants, especially when high levels of salt are present in soil (Acosta-motos *et al.,* 2016; Li and Li, 2017). Salinization applies a lot of pressure on the agricultural sector worldwide, specifically in arid and semi-arid regions (Acosta-motos *et al.,* 2016; Li and Li, 2017). Salt-affected lands have been aggravated by irrigation, making it a global concern as 20 % of land has already been damaged by salinization (Flowers and Yeo, 1995; Scasta *et al.,* 2012; Camlica and Yaldiz, 2017).

In Africa, salinization accounts for 50 % of irrigated land (Ceuppens and Woperies, 1999; Vengosh, 2003). According to the World Bank, 60 million hectares of land have been impacted by soil salinization worldwide (Russ *et al.,* 2020). Figure 1 bares as evidence to an area in Southern Africa effected with salinity. Salt in the form of KCl is regarded as an important component of soil, making it an essential nutrient element for plant growth (Kaiwen *et al*., 2020). When the salt content in the soil, especially NaCl, exceeds the threshold, it will cause higher water potential in the seed than that in the soil, thus inhibiting water absorption of the seed from the external environment (Zhang *et al.,* 2011). Without sufficient water content in the seed, its germination and growth will be inhibited. Saline soil contains a high concentration of metal ions, therefore the toxic effect of metal ions on seeds is another reason for inhibiting seed germination (Zhang *et al.,* 2011). Seed germination is a critical stage in plant growth and therefore directly affects the final yield of plants. To maintain growth and yield, plants adapt to abiotic stresses *via* specific tolerance mechanisms (Kaiwen *et al*., 2020). There are three main mechanisms of salt tolerance in plants, namely osmotic stress tolerance, maintenance of ion balance and reduction of the Na^+ concentration in the cytoplasm (Kaiwen *et al*., 2020; Figure 3). Kotula *et al*. (2015) reported that exposure of chickpea genotypes to 50

mM of NaCl decreased plant growth and yield. Areas with high salinity indicate how crucial it is to investigate the level of salt tolerance in different plants, as many plants do not possess this trait. It is also of utmost importance that different mechanisms be investigated to improve the tolerance of plants to salinity.

Figure 1. Salinity affected area in the Northern Cape, South Africa (Photo credit to Ali Ali).

Methods to increase the tolerance of crop plants to salt stress include genetic engineering and beneficial microorganisms (Radhakrishnan *et al.,* 2013). Many chemical fertilizers can be toxic to both human health and the environment (Bilkay *et al.,* 2010, Radhakrishnan *et al.,* 2013). Therefore, recent studies have focused on identifying alternative methods to increase soil productivity by using endophytic plant-bacteria as biofertilizers (Audipudi *et al.,* 2017). These microorganisms can enter the roots and establish their population in plants (Audipudi *et al.,* 2017). Endophytic fungi and bacteria promote plant growth; however, some plant-microbe relationships are yet to be identified (Audipudi *et al.,* 2017). These microorganisms can produce phytohormones, convert complex organic substances to simple forms and solubilize insoluble phosphate (Radhakrishnan *et al.,* 2013, Audipudi *et al.,* 2017). This thesis aims to isolate and determine if isolated endophytic bacteria can alleviate salt stress in the *Medicago sativa L.* (Alfalfa) cultivars.

1.1. Salt-tolerant legumes

Salinity continues to plaque soil fertility and has a drastic influence on the growth of important crop plants used in the agricultural sector (Manchanda and Garg, 2008). Dating back to the early 1900s, salt-tolerant plants have been studied to develop plants that are tolerant or even resistant to salt stress (Norlyn, 1980). Plants have developed ways to tolerate their harsh conditions; either through changing methods of distribution or adapting its root system (Chen *et al*., 2018). Halophytes are known as plants that are well adapted to saline environments with concentrations exceeding 200 mM or more (Chen *et al*., 2018). Halophytes can also be categorised further according to their salt requirements and their range of adaptation (Rogers *et al*., 2006; Chen *et al*., 2018). Cereal crops such as sorghum, wheat and barley are some of which are known to be tolerant to salt (Chen *et al*., 2018). Barley can tolerate up to 250 mM of NaCl, making it the most tolerant crop to salt conditions (Hanin *et al.,* 2016). Rhizophora, Suaede, Atriplex and Salicornia are among some plant species that can grow at 1000 mM NaCl (Khan *et al.*, 2001). Only 5-10 % of flowering plants are identified as halophytes (Khan *et al*., 2001). The protein content in legumes is high, making it an important crop for the human population (Khan *et al*., 2001). Some of these legumes are also drought and salt-sensitive, making them susceptible to poor growth and yield production in the field where these environmental constraints occur (Khan *et al*., 2001). Some farming systems depend on forage conservation, and some forage crops can provide a sustainable solution for protein supply and food security. The farming of cereal and forage crops together can improve yield, especially in consideration of the protein content provided by forage crops to livestock farming (Kulkarni *et al*., 2018).

1.1.1. Medicago sativa (Lucerne/Alfalfa)

Grain legumes belonging to the Fabaceae family are rich in proteins and have proven to be very important components of the human diet (Jukanti *et al.,* 2012). Alfalfa is a perennial legume that possesses characteristics such as high yield, good forage/feed quality for livestock and a soil fertilizer; which makes it a beneficial crop plant (Jukanti *et al.,* 2012, Kulkarni *et al*., 2018). Some cultivars of *M. sativa* have different levels of tolerance to salinity, and therefore, many cultivars of alfalfa are being studied for salt tolerance (Jukanti *et al.,* 2012, Kulkarni *et al*., 2018). Alfalfa is currently grown as a commercial crop in some countries and is described as the “Queen of forage” attributed to its importance as a high-quality

protein crop (Kulkarni *et al*., 2018). Some forage crops are already under the threat of changing climatic conditions and therefore the need for breeding different cultivars is increasing (Kulkarni *et al*., 2018). Alfalfa is a perennial legume is cultivated with subspecies/varieties of *sativa* cultivars (Kulkarni *et al*., 2018). This plant can also be grown for 3-4 years continuously; with the forage quality, yield, early spring vigour and resistance to lodging being some traits that make alfalfa important for improvement in agriculture (Kulkarni *et al*., 2018). These are also the traits that make alfalfa beneficial and grown throughout most temperate regions (Kulkarni *et al.,* 2018). *M. sativa L.* has great importance as it is not only used for livestock feed but is also used to improve soil fertility (Arshad *et al.*, 2017). Alfalfa sprouts a purple flower and can be grown in large fields (Figure 2).

Figure 2. *Medicago sativa* L. in the field, (Photo credit to Eden Keyster).

Little was once known of this forage crop under saline conditions, especially among various cultivars, but some studies have shown that Lucerne can grow at 200 mM of sodium chloride (NaCl), making it moderately tolerant to salt (El-Sharkawy *et al.,* 2017; Lei *et al*., 2018; Table 1). Some cultivars of Alfalfa have been confirmed to be salt-tolerant, like *Medicago sativa* L. cv. Gongnong No. 1, but when placed in high salt concentration its performance was poor

(Guo *et al.,* 2016). Alfalfa possesses traits that make it salt tolerant, but the response of the plant to high salt concentrations under short term conditions is still unclear (Guo *et al.,* 2016).

Table 1. Information of *M. sativa L.* and *Trifolium fragiferum* based on their capabilities to survive under salt conditions (McDonald, 2008).

Annual rainfall (mm)	Salinity	Waterlogging risk	Texture	pH ($CaCl_2$)	Suitable species - common name	Suitable species - cultivars	Common
>400	Moderate	Low	Sands - clay loams	5.6-9.0	Lucerne (*Medicago sativa*)	Many cultivars	winter active - and highly winter active cultivars perform best in WA
>550	Low-Moderate	High	Sandy loams - clay	5.6-9.0	Strawberry clover (*Trifolium frageriferum*)	O'Connors, Palestine	

Lucerne has a high protein content and displays high biomass production compared to other crops (El-Sharkawy *et al.,* 2017). Various cultivars have been studied (Lei *et al.*, 2018), where the response of mature-stage alfalfa plants to salt conditions was studied, in which it was found that one cultivar (ZM) has a salt-resistance strategy it utilizes but has slow growth whereas the other cultivar (XJ) has an inducible strategy but is not able to survive under salt stress. The study continued to describe how an underlying mechanism can provide salt tolerance to the ZM cultivar (Lei *et al*., 2018). Alfalfa provides a high yield because it has a deep-rooted system that helps it obtain water and has a symbiotic relationship with Rhizobia, which inhabit the root nodules, to help with nitrogen fixation (Castillo *et al*., 1999). This capability makes this forage legume even more attractive. In the leaves of alfalfa; the regulation of Cl^- and Na^+ is synchronized, also preventing these ions from increasing excessively (Munns, 2005). The perennial feature of alfalfa makes it capable of the acquisition of nutrients or water in deep soil and might also be beneficial for adaptation traits. The

urgency of providing food for growing populations is increasing and finding new strategies to increase food production will help in attaining food security.

1.2. Plant signalling under salt stress

Salinity poses a threat by limiting the growth of crops and impacting their development through water and ionic stress, leading to low yield (Isayenkov and Maathuis, 2019). Nonetheless, plants have many coping mechanisms which they make use of in defence against drought and salinity stress (Hasanuzzaman and Tanveer, 2020). Among these mechanisms, molecular signalling mechanisms are regarded as important because they help with defence gene expression and regulate cellular activities that help the plant respond to these conditions (Hasanuzzaman and Tanveer, 2020). Understanding these signalling mechanisms can help plant breeders to develop tolerant plant varieties (Hasanuzzaman and Tanveer, 2020). Much remains unknown about the molecular mechanisms which plants use to survive in saline conditions (Rozema and Schat, 2013). To make improvements in crop yields, the molecular responses of plants in saline conditions are of high importance and therefore requires more understanding. Using advancements made in fields like proteomics, genomics and transcriptomics will help to advance research on plant responses to stress.

1.2.1. Physiological adaptations

Salinity impacts many different aspects of the plant's physiology, thus adding complexity to studying plant physiological responses to salinity (Negrão *et al*., 2017). With the help of "omics-driven" research, many advances have been made in studying plant physiological responses to salinity (Negrão *et al*., 2017). There are two main phases in which plants' responses to salinity are divided (Negrão *et al.,* 2017). Within minutes of exposure to salt, leaf expansion, cell expansion, transpiration and photosynthesis are reduced, hence the decrease in plant growth and yield in crop plants (Tillbrook *et al*., 2017). These effects all take place before salt ions accumulate in the shoot and have been termed the shoot-ion independent response (Negrão et al., 2017; Tillbrook *et al*., 2017). This is the first response and might be related to the sensing and signalling of Na^+, which usually occurs within the first few days of salt exposure (Negrão et al., 2017; Tillbrook *et al*., 2017). During the first phase, inhibition of leaf expansion and stomatal closure occurs due to the accumulation of Na^+, resulting in toxicity within the plant organs

(Munns, 1993). Na^+ is transported from roots to shoots and therefore these ions build up in the shoot (Munns, 1993; Tester and Davenport, 2003). Prevention of accumulation of Na^+ in the shoots due to the ability of some plants to exclude Na^+ from the shoot can be used to improve salinity tolerance (Tester and Davenport, 2003). *Arabidopsis thaliana* was used as a model to understand the transport of Na^+ when the plant is placed in a saline environment, where it was found that the Salt Overlay Sensitive (SOS) pathway is involved in transporting Na^+ from roots to shoots (Møller *et al.*, 2009). Therefore, *Arabidopsis* can continuously be used as a model for understanding the tolerance of important crop plants to salt and might also bring more insight into the complexities that are faced within *M. sativa* under similar conditions (Møller *et al.*, 2009). Shoot-ion independence is linked to the maintenance of plant growth in the presence of high salinity in the soil, where the shoots continue to grow because of an ability to exclude salt from shoot tissue (Tillbrooke *et al*., 2020). This phenomenon has been linked to long-distance signalling mechanisms that confer salinity tolerance (Tillbrooke *et al*., 2020). The collection of Na^+ in the shoot is also the net result of processes in which Na^+ is transported. Each of these processes are also involved in the salinity tolerance of the plant (Tester and Davenport, 2003; Negrão *et al.,* 2017). The second response is called an ion-dependent response. This phase occurs at a later stage and involves ions that build up within the shoot, causing toxicity (Negrão *et al*., 2017). This causes chlorosis, leading to low yield and will eventually lead to plant death (Munns and Tester, 2008; Negrão *et al*., 2017). The abovementioned responses have led to the proposal of three mechanisms that suggest how plants can tolerate salinity (Figure 3).

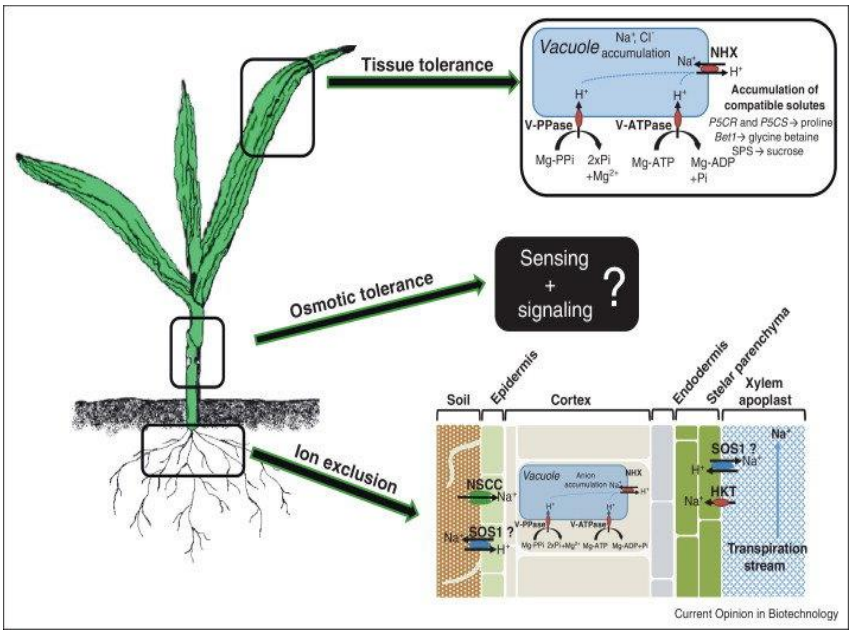


Figure 3. Three salinity tolerance mechanisms were proposed by Munns and Tester (2008). Ion-exclusion in which toxic ions are excluded from the shoot; osmotic tolerance in the shoot that maintains growth and water uptake regardless of the build-up of Na^+; and tissue tolerance, which entails the compartmentalization of toxic ions in specific tissues (Munns and Tester, 2008; Roy *et al.*, 2014; Negrão et al., 2017).

Many other physiological components can also contribute to the tolerance of the plant to salinity. However, because very little is known about these components, it remains important to focus on the above three mechanisms (Figure 3) to understand salinity tolerance (Negrão *et al*., 2017). These mechanisms are mutually exclusive and might also be genotype-specific (Bhattarai *et al.*, 2020; Roy *et al*., 2014). Alfalfa has underlying mechanisms that help it tolerate salt, some of these processes involve photosynthesis, secondary metabolism, antioxidants, detoxification and ion transport (Bhattarai *et al.*, 2020). Finding ways in which the genetic control of alfalfa can be improved is difficult as alfalfa has complex physiological and genetic processes (Bhattarai *et al.*, 2020; Guo *et al*., 2016). By understanding basic salt tolerance in crop plants, the discovery of how different alfalfa cultivars can adapt to saline conditions can be useful in improving alfalfa tolerance to salinity.

1.2.2. Molecular and cellular signalling

The regulation of the plant gene expression during salinity stress occurs at post-translational and transcriptional levels (Long *et al.,* 2016). Different plant models have been used to investigate molecular and cellular signalling mechanisms involved in plant responses to salinity, among them are *Arabidopsis thaliana*, rice, wheat, soybean and *Medicago truncatula* (Long *et al*., 2016). The root is among the first tissues that sustain damage when the plant is subjected to stress, hence the productivity of the plant is compromised due to the sensitivity of the root to the stress (Long *et al*., 2016; Stepphun *et al*., 2010). This is among the reasons why many researchers focus on the molecular mechanisms within the root (Stepphun *et al*., 2010). Drought and salinity are the common stresses in which metabolic capabilities are affected (Khan and Hakeem, 2013). Signal transduction pathways are key in identifying adaptive pathways during such stressful conditions (Khan and Hakeem, 2013). Ion toxicity, hyperosmotic stress and oxidative damage occur under high salt levels (Lei *et al*., 2018). Cytosolic Ca^{2+} is increased when Na^{+} is in abundance in the plant and causes Ca^{2+} binding proteins to activate downstream pathways (Lei *et al*., 2018). SOS3 is a novel unit from the calcium-binding proteins, and besides its role in salt tolerance, it interacts with SOS2, leading to the mediation of osmotic stress induction of ABA biosynthesis (Zhu, 2002). Simultaneously, other molecules are also linked to the Ca^{2+} signalling such as reactive oxygen species (ROS), which are activated during the process (Lei *et al.*, 2018). There is limited information on the genetic factors that enable alfalfa to adapt to saline soils, but there have been reports of transcriptional responses in this forage legume (Postnikova *et al*., 2013). Some *M. sativa* cultivars have a higher tolerance to salinity, such as *Medicago sativa* cv. Zhongmu-1; compared to most *M. truncatula* cultivars which are more salt-sensitive than *M. sativa*, yet the latter is used as a model to study this family of legumes (Long *et al*., 2016).

1.2.3. Biochemical response of plants

The ability of some plants to tolerate salt stress can be determined by various biochemical pathways that help in retaining or acquiring water, protecting chloroplast functioning and maintenance of ion homeostasis (Parvaiz and Satyawati, 2008). Investigating more ways in which plants respond biochemically to salinity may help with the identification of specific pathways by which tolerance is achieved.

1.2.4. Enzymatic and non-enzymatic antioxidants

ROS scavenging molecules are released when the levels of ROS increase to a level that causes injury to the plant. The defence system consists of enzymatic and non-enzymatic antioxidants which neutralize the ROS (Huang *et al*., 2019; Khan and Hakeem, 2014). The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and ascorbate peroxidase (APX) (Huang *et al*., 2019; Khan and Hakeem, 2014). The properties of antioxidant enzymes have been intensely studied, but controversy persists on whether the enzymes have any significance in salt tolerance (Huang *et al*, 2019). The reason is that high levels of antioxidant enzymes activity are associated with both salt tolerance and salt sensitivity (Huang *et al.*, 2019). Some studies have shown the correlation between overexpression of antioxidant enzymatic activity and improved salt tolerance, but in contrast, salt tolerance did not depend on high antioxidant activity (Huang *et al*., 2019). Ascorbate, carotenoids and tocopherol are among the non-enzymatic antioxidants (Huang *et al*., 2019). Ascorbate limits ROS production under salt stress, but a study conducted by Athar *et al.* (2008) used ascorbic acid exogenously to alleviate oxidative stress and showed that ascorbate may not be enough to act as a defence system against salt stress and a *de novo* synthesis is required. For example, ascorbate peroxidase was over expressed in the chloroplasts of tobacco and enhanced the salt tolerance in turn (Badawi *et al*., 2004). The manipulation of these antioxidants can provide possible ways in which tolerance to salt can be improved. Exogenous application of melatonin enhanced antioxidant activity in response to salt stress but further research on this type of application is still required. The results also showed that melatonin can indeed increase plant growth and alleviate salt damage (Cen *et al*., 2020). The salt tolerance was improved by reducing oxidative stress but also enhancing the activity of both enzymatic and non-enzymatic antioxidants (Cen *et al*., 2020). This provides insight as to how a bioactive molecule can enhance salinity tolerance by improving the performance of the plant antioxidant system, among others.

1.3. Strategies for improved salt-tolerance

We are currently facing a period in which freshwater is a limited resource for sustainable agriculture. Desertification is mainly caused by salinity and therefore soil degradation is increased (Ladeiro, 2012). Semi-arid and arid regions are extreme environments; therefore, adjustments should be made to find alternative ways in which sustainable agriculture can be provided (Flowers, 2004; Ladeiro, 2012). Salinization remains a huge threat to crop productivity, especially in regions in which irrigation practices are essential for their agriculture (Flowers, 2004). Attempts were made in plant breeding to improve salt tolerance in plants, but these have largely failed due to the complexities that are involved in the genetics and physiology associated with the salt tolerance trait (Flowers, 2004).

1.3.1. Application of plant growth-promoting bacteria (PGPB) as biofertilizer in agriculture

Plants have developed symbiotic associations with the members of the ecosystem to thrive in their natural environment (Santoyo *et al*., 2016). Some microorganisms can establish beneficial associations with plants (Santoyo *et al*., 2016). Plant-beneficial bacteria provide numerous benefits to their host plants, helping them tolerate different biotic and abiotic stresses that challenge plant growth. Some bacteria can live internally or externally in their host plant (Afzal *et al.,* 2019). Bacteria living outside their host plant are epiphytes, whereas bacteria living inside the plant are called endophytes (Afzal *et al.,* 2019). Endophytic bacteria are considered a subclass of rhizospheric bacteria that is commonly called plant growth-promoting bacteria (PGPB) (Afzal *et al.,* 2019). These are a specialized group of rhizobacteria that have acquired the ability to invade their host plant (Afzal *et al.,* 2019). Flowers and Yeo (1995) suggested ways in which salt-tolerant crops could be produced, for example developing halophytes as alternative crops and breeding for yield rather than salinity tolerance. These suggestions were appropriate to their time, yet the relevance to current times persists, nonetheless. The majority of the suggestions made included different methods of breeding crops, but none had referred to the possibility of utilizing plant growth-promoting bacteria (PGPB) to enhance salt tolerance in plants at the time. PGPB are a diverse group of microorganisms that can improve growth and yield (Kumar *et al.*, 2020). At the present time, the focus is placed on finding alternative methods of improving crop production, and

conventional agricultural techniques will not be enough to meet the demand (Kumar *et al*., 2020). Salinity alters soil physicochemical properties while simultaneously reducing the microbial diversity in soil, which in turn reduces the health of the soil (Ansari *et al*., 2019). Salinity can also change the functioning and community structure of soil microorganisms, wherein some microbes interact with one another and exchange roles when placed under stressful conditions (Ansari *et al*., 2019). Bacteria that are tolerant to salt can survive in a wide range of salt concentrations by making use of different mechanisms, such as the production of extracellular proteases (Ansari *et al*., 2019). Some microorganisms can truly change or enhance the response of the plant to salinity, with some increasing antioxidant levels and upregulating the production of hormones that mediate signalling for stress tolerance. Salinity decreases stomatal conductance and photosynthesis, but PGPB inoculated into the plant have the potential to enhance these physiological processes (Kumar *et al*., 2020). *Rhizobium phaseoli* in bean and *Pseudomonas putida* in canola are among the salt-tolerant-PGPB that alleviated salinity stress. The production of ACC deaminase and a decrease in ethylene levels are responsible for PGPB-mediated plant growth promotion under salinity (Ansari *et al*., 2019). In a study performed by Ansari *et al*. (2019), alfalfa was inoculated with PGPB under salt conditions. In this study, several features including dry weight, plant height and photosynthesis were improved after treatment with PGPB (Ansari e*t al*., 2019). This proves that a relationship between plants and microbes can indeed be beneficial. Certain traits of the microorganism must be studied and thereafter investigate the effect they may have on plants that are not their host. This way of sustainable agriculture is laborious and tedious but is less expensive (Kumar *et al.*, 2020). Knowledge of the various signalling events between the plant and the microbe is limited, and the mechanisms used in growth promotion during the presence of PGPB under salinity stress needs to be investigated further to characterise the genetic components that regulate plant-microbe interactions under these conditions (Ansari *et al*., 2019). Salt-tolerant PGPB has the potential to increase plant growth, crop yield and salt tolerance in plants affected by salinization (Ansari *et al.*, 2019).

1.4. Conclusion

Irrigation practices and impact on the environment will continue to increase salinity in the soil, leading to poor plant growth and reduced crop yield. This review highlights the issues we face and the future strategies we can mobilise to reach a point in research where we can understand the physiology and genetics behind the tolerance of plants to salinity. Until then, continuous research should be done on crop varieties that show great promise of tolerance to saline soils. Alfalfa holds great promise for future research in salt tolerance because several cultivars of alfalfa have some level of salt tolerance. The application of endophytic bacteria also holds great possibilities for sustainable agriculture, especially if they possess traits for stress tolerance. A renewed interest in the internal colonization of healthy plants by non-rhizobium bacteria and exploitation of their potential in agriculture becomes apparent.

1.5. Justification

The world is approaching an era in which demands are being made for food to be provided at a faster rate for a growing population. This places great pressure as the world is also facing climate change or other environmental factors that impact food production negatively.. Salinity is one such factor that limits crop productivity and yield. Finding alternative methods of breeding, especially using cultivars or species that are known to be salt tolerant, can provide great insight as to how sustainable agriculture can be maintained in saline conditions. The goal of this study is to compare salinity responses in two genotypes/accessions of *Medicago sativa* and to determine if a consortium of bacterial endophytes can improve salinity tolerance in *Medicago sativa*. It is of utmost importance to understand different mechanisms of salt tolerance and to identify traits for improved salt tolerance in the breeding of alfalfa. This will also help with identifying cellular machinery in which the plant activates its adaptive responses in order to survive in environmental stresses. Breeding more of these forage legumes can provide larger yield and improve feed for livestock, especially taking into consideration that demand for meat products is increasing.

1.6. Objectives of study

To address the limiting effect of soil salinity on *Medicago sativa* (alfalfa), this study will compare the growth of alfalfa accessions, from diverse genetic backgrounds, cultivated in saline soil in the presence or absence of a consortium of eight endophytic bacteria. Growth parameters will be used to determine which of the accessions is salinity sensitive and which one is salinity tolerant. The study will also assess the effect of the endophytes on the growth and salinity tolerance of the alfalfa accessions. Given that one of the biochemical processes that occur during salinity stress in plants is an increase in reactive oxygen species that cause oxidative stress as a result of the failure of plant antioxidant enzymes to sufficiently maintain physiologically appropriate levels of reactive oxygen species in plant tissue, the study will determine if the endophytes contribute to the regulation of the activity of the antioxidant enzymes. *Mentha spicata* (spearmint) is a traditional medicinal plant in the mint family. *M. spicata* is used worldwide as an infusion to treat respiratory ailments such as colds, nasal congestion, sore throat, and both upper and lower respiratory infections, diabetes, and intestinal infections. It also has been described to have antiseptic, antibacterial, and antifungal properties. Spearmint also has been identified as a salt tolerant plant and it is therefore important to understand how the plant achieves this salinity tolerance. However, to date spearmint has not been investigated with respect to microbial communities and hence this study, in which the consortium of 8 bacterial endophytes was isolated from spearmint roots, can provide some insight into the role of these endophytes in conferring salinity tolerance in spearmint.

Chapter 2: Methods

Table 2.1. List of chemicals and suppliers

2- Thiobarbituric acid (TBA)	Sigma-Aldrich
3,3'-Diindolylmethane (DIM)	Sigma-Aldrich
4-Nitro blue tetrazolium chloride (NBT)	Sigma-Aldrich
5-sulfosalicyclic acid dihydrate	Sigma-Aldrich
Acetone	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Bradford reagent	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ferric chloride	Sigma-Aldrich
Glycine 99 %	Sigma-Aldrich
Hydrogen peroxide	Sigma-Aldrich
L-Ascorbic acid	Sigma-Aldrich
N, N, N', N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Polyacrylamide 40%	Sigma-Aldrich
Polyvinylpolypyrrolidone (PVPP)	Sigma-Aldrich
Potassium ferricyanide	Sigma-Aldrich
Potassium phosphate dibasic	Merck
Potassium phosphate monobasic	Merck
Promix	Windell Hydroponics
Trichloroacetic acid 99%	Sigma-Aldrich
Tris (hydroxymethyl)	Sigma-Aldrich

Table 2.2. List of equipment

Instruments	Model	Company
Centrifuge	5415D	Eppendorf
Gel electrophoresis tank	Mini-Protean Tetra Cell	BioRad
Freeze drier	Freezone Plus 2.5 litre	Labconco
Heating block	Block heater	Stuart Scientific
Mass balance	AS 220R2	Radwag
pH meter	pH + DHS-S/N	ACCSEN
Power supply	MP 250V	Cleaver Scientific
Shaker	Mini orbital shaker (SSM1)	Stuart Scientific
Vfb Spectrophotometer (Microtitre plate reader)	POLARstar	Omega
Phenotyping robot	Sunbear 2.0	

2.1. Sterilization and isolation of endophytes

To identify candidate endophytes that may confer salinity stress tolerance, *Mentha spicata* (spearmint) was sampled because of its salinity tolerance. The whole plant was subjected to sterilization by first washing with 70 % ethanol for 1 minute, followed by a wash with 0.32 % of bleach for 10 minutes. The third wash included 100 % ethanol and, finally, the sterilization ended with a wash in autoclaved distilled water (Istain, 2019). An aliquot of the final distilled water wash was inoculated onto a Luria Broth (LB) agar plate to detect any possible microbial contamination on the plant surface. The LB agar plate was incubated at room temperature overnight (o/n). Endophyte extraction was performed after the roots, stem and leaves were separated and placed in 50 ml tubes. A salt solution (1.0034 g of sodium chloride in 100 ml of dH_2O) was prepared in which roots, stems and leaves were separately ground in 5-10 ml using a mortar and pestle. The liquid parts were poured into a separate 50 ml tube and incubated for 4-24 hours at room temperature with shaking at 150 rpm. In order to isolate and find pure colonies the solutions were prepared after a 1/10 dilution was performed. The samples were inoculated onto petri-dishes containing R2A growth media. The plates were then incubated at room temperature before isolated colonies were picked and streaked.

2.2. Identification of endophytes

2.2.1. Growth curve for halophyte identification

To determine whether each individual bacterial endophyte is capable of growing in saline conditions. A growth curve was constructed using LB broth media inoculated with each endophyte in 5 ml and grown until it reached an optical density (OD) of 0.3 at 530 nm. Thereafter each culture medium was aliquoted into LB media containing no salt or salt (200 mM NaCl) respectively. The readings were taken hourly at a wavelength of 530 nm. The readings were then used to identify the bacterial endophyte(s) capable of growing in salinity.

2.2.2 Sequencing of bacterial endophytes

A colony PCR was conducted, in which a single colony was chosen and heated in 10 µl in nuclease-free water @ 95°C for 5 minutes. For 16S rRNA gene sequencing, total genomic DNA was extracted using a DNA Purification Kit (Promega) as described by the manufacturer. With

genomic DNA as the template, a portion of the bacterial 16S rRNA gene was amplified with the bacterial universal primers (E9F:5'-GAG TTT GAT CCT GGC TCAG'3 and 1492R: 5'-AGA GTT TGA TCC TGG CTC AG'3). The 25 µl of PCR mixture contained Mastermix (Taq DNA Polymerase 2x Mastermix RED), 1 µl of forward and reverse primer, 2 µl of DNA sample and nuclease-free water. A negative control (PCR mixture without DNA template) was included for each PCR reaction. Amplifications were carried out in an Eppendorf Mastercycler using the following conditions: 95 °C for 5 min, 30 denaturation cycles (at 95 °C for 30 s, annealing temperature of 55 °C for 30 sec, primer extension at 72 °C for 1 min and 30 sec), followed by a final extension at 72 °C for 10 min. In each case, the PCR product was run on a 0.8 % agarose gel run for 40 min at 90 volts to confirm the integrity of the DNA. The DNA sequences obtained were analyzed with basic sequence alignment BLAST program run against the database from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

2.3. Surface sterilization of seeds

Medicago sativa seeds were surface sterilized with bleach solution (0,35% commercial bleach) for 10 minutes, washed with sterile water for another 30 seconds before it was washed in 70 % ethanol for 20 seconds. The final wash was done 3 times before it was plated overnight on R2A- media at room temperature for each cultivar of alfalfa seeds to assure no contamination is still present.

2.3.1. High-throughput phenotyping of Agsalfa and Magna 601 seeds

All seeds of *M. sativa* L. (genotypes/ accessions Agsalfa and Magna 601) were imbibed in a consortium of the bacterial endophytes for 2 hours before they were filtered on Miracloth. Seeds for the control was also prepared where no endophytes were inoculated on the seeds. The seeds were placed on ¼ MS plates (Murashige & Skool Basal, MES and phytagar, pH 5.7) and ¼ MS plates containing 200 mM NaCl in petri dishes. The seeds were allowed to germinate and grow for 9 days in the Sunbear 2.0 robotic imager for image-based phenotyping, followed by measurement of root growth.

2.3.2. Preparation of bacterial endophyte treatment

The liquid solution of bacterial endophyte was prepared after a single colony of each bacterial culture (from different 8 isolates) was inoculated into 10 ml of Luria Broth (BioRad). Each liquid culture solution was grown at room temperature for five days before it was mixed into a consortium of all 8 cultures. Water was added to the liquid endophyte solution and made up to five litres to treat plants.

2.4. Plant growth and treatments under field conditions

The study was conducted on a 240 m^2 field (at Lukholweni village, Matatiele, Alfred Nzo District, Eastern Cape province in South Africa, GPS coordinates 30°37'54.8" S 28°51'29.3" E), consisting of a 120 m^2 block for the salt stress treatment and 120 m^2 for the non-saline (control) experiment, as illustrated in Figure 4. The cultivars of *Medicago sativa L.* were commercially obtained from Agricol (Brackenfell, South Africa). The seeds were of the cultivars Magna 601 and Agsalfa. The experiment was initiated in June 2021 and terminated at maturity on the 14th of October 2021. For the 120 m^2 salt block, the field was dug up to a depth of 30 cm and the dug-up soil was set aside such that a plastic waterproof membrane was laid at the 30 cm deep base of the dug-up field. Following the plastic membrane laying, the dug-up soil was returned on top of the plastic membrane to make an even layer of soil that is 15 cm thick. This was followed by applying salt (NaCl) on this soil layer at a rate of 0.4 kg/m^2, ensuring that the salt was evenly spread on the soil layer. Another 15 cm layer of soil was then evenly returned on top of the salt. A 'no salt' control block was set up in a similar way as the salt block at a space of 5 meters away from the salt block, except that no salt was applied to the control block. The other blocks contained plants that was treated with the consortium of bacterial endophytes whereas the last block contained both salt- and endophyte treated plants. Seeds of the 2 *Medicago sativa L.* accessions were sowed 6 cm deep with a spacing of 10 cm between seeds and 40 cm between rows such that each accession occupied 40 m^2. The soil was irrigated daily with water at a rate of 2 L/m^2. After 123 days from sowing, the plants were harvested and yield parameters such as the shoot length, stem diameter (at the cotyledonary node), number of branches, shoot- and root weights were measured. The level of salinity in both the salt-treated and non-saline field was measured in soil sampled at a depth of 10 cm, using the Extech EC410 ExStik Conductivity/TDS/Salinity Kit

(Extech instruments, New Hampshire, USA). The resulting salinity in the salt treatment was 8 ± 1.2 $dS.m^{-1}$ and was 0.7 ± 0.08 $dS.m^{-1}$ for the 'no salt' soil.

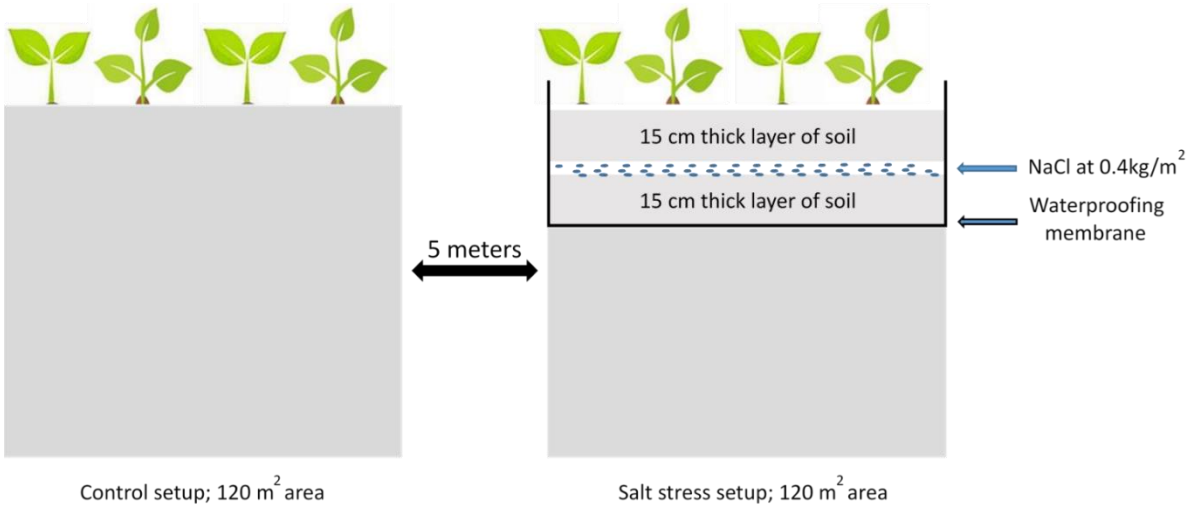


Figure 4. The salt stress field setup (Created by Mr Musa Akanbi). A field measuring 120 m^2 was used for each of the salt and non-salt stress study. The distance between the control setup and the salt-stressed setup was 5 meters. The salt-stress setup was 30 cm deep and was covered with a waterproofing membrane to restrict salt penetration to other parts of the field. The membrane was covered with soil layer 15 cm thick, following which NaCl (0.4 kg/m^2, thus 48 kg for the 120 m^2 area) was evenly distributed on the soil. The salt-treated soil was then layered with a soil layer 15 cm thick. The seeds were sowed. The control filed was also 120 m^2 and set up similarly to the salt block but without any addition of salt.

2.5. Protein-Free Metabolite Extractions

To obtain the protein-free extracts to be used in the determination of hydrogen peroxide (H_2O_2), a tricloroacetic acid (TCA)-based extraction was performed using a modified version of the method described by Velikova *et al.* (2000). The frozen plant material (200 mg) was added to 1.5ml centrifuge tubes. A volume (5 times the plant material) of 6 % (w/v) TCA was added to the tubes and the samples were homogenised using a vortexer. Following this step was centrifugation at 12 000 x *g* for 10 minutes to pellet the plant material. The supernatant was transferred to sterile tubes to be used for subsequent experiments.

2.5.1. Determination of hydrogen peroxide (H_2O_2) in M. sativa L. leaves and roots

Hydrogen peroxide (H_2O_2) content was determined by homogenising 50 µl of the TCA extract with 100 µl of reaction buffer, which contained 5 mM dipotassium hydrogen phosphate (K_2HPO_4, pH 5.0), and 0.5 M potassium iodide (KI). This was followed by samples being incubated at 25 °C for 20 min. Standards for H_2O_2 (0 nM, 500 nM, 1000 nM, 1500 nM, 2000 nM and 2500 nM) were prepared by diluting H_2O_2 deionised water. Following the incubation, 200 µl of both the sample and standard curve were loaded in triplicate and the absorbances were measured at 390 nm on a spectrophotometer. The hydrogen peroxide content was then calculated using the standard curve constructed with the absorbance of H_2O_2 standards read at 390 nm.

2.6. Protein extraction

Total soluble protein was extracted from both the leaves and roots by first grinding 200 mg of plant tissue in liquid nitrogen, followed by addition of 0.6 ml of protein extraction buffer [1 mM ethylenediaminetetraacetic acid (EDTA) and 5 % (w/v) Polyvinylpolypyrrolidone (PVPP) dissolved in 40 mM potassium phosphate buffer (KPO_4, pH 7.4). The sample was mixed by vortexing and the homogenate was then pelleted by centrifugation at 12 000 x *g* for 20 min at 4 °C. Thereafter, the supernatant was carefully transferred to sterile tubes for use in subsequent experiments, whereas the pellet was discarded. The protein concentration was then quantified using the Bradford assay. Bradford reagent was purchased from Sigma-Aldrich and assay was performed according to the manufacturer's instruction using bovine serum albumin as a standard. Protein samples were stored at -20 °C for future experiments.

2.7. Ascorbate peroxidase (APX) activity in M. sativa L. leaves and roots

Ascorbate peroxidase (APX) activity was determined using native in-gel activity-based staining. The protein extract (200mg, in section 2.6) was loaded on a non-denaturing 10 % polyacrylamide gel and then subjected to native polyacrylamide gel electrophoresis (PAGE) at 4 ^{o}C. The gels were run in an APX running buffer consisting of 192 mM glycine, 25 mM Tris (hydroxymethyl) aminomethane (Trizma Base) and 2 mM L-Ascorbic acid]. Following electrophoresis, gels were equilibrated in a 50 mM potassium phosphate buffer (KPO4, pH 7) containing 2 mM L-Ascorbic acid for 20 minutes. Thereafter a second incubation was carried

out in 50 mM KH_2PO_4 (pH 7.8) buffer containing 4 mM L-Ascorbic acid and 2 mM H_2O_2 for 20 minutes, followed by an incubation in 50 mM KH_2PO4 (pH 7.8) for 1 minute. The gel was then stained with a solution containing of 50 mM KH_2PO4 (pH 7.8), 28 mM (N, N, N', N'-Tetramethylethylenediamine) TEMED and 0.5 mM 4- Nitro blue tetrazolium chloride (NBT) for 20 min in the absence of light. After the incubation, gels were exposed to light and APX activity was detected by the formation of achromatic bands against the darker purple background. At this point, the reaction was stopped by the removal of the staining solution and addition of deionized water. Additionally, pixel intensity ratios were also determined using the AlphaEaseFC Software.

2.8. Catalase (CAT) activity in M. sativa L. leaves and roots

Catalase (CAT) activity was determined using native in-gel activity staining. The method entailed using a non-denaturing 7.5 % polyacrylamide gel, subjected to native polyacrylamide gel electrophoresis (PAGE) at 4 ^{o}C. Protein extracts (400 mg, from section 2.6) were loaded on the gel and run in a running buffer containing 192 mM glycine and 25 mM Tris (hydroxymethyl) aminomethane (Trizma Base. Following electrophoresis, gels were incubated in 0.003 % H_2O_2 for 20 minutes in the absence of light. Thereafter, gels were stained with 2 % ferric chloride and 2 % potassium ferricyanide simultaneously and exposed to light. CAT activity was detected by the formation of achromatic bands against a blue/green background. The reaction was then stopped by the removal of the staining solution and addition of deionized water. Pixel intensity ratios were determined using the AlphaEaseFC Software.

2.9. Statistical analysis

All data were statistically validated using Oneway analysis of variance (ANOVA) on GraphPad Prism 6.01 software. The Tukey-Kramer test at 5% level of significance was done to compare the mean

Chapter 3: Results

3.1. Halophytic display of bacterial endophytes

Over the course of 8 hours under controlled conditions, all bacterial cultures grew well, with C7 thriving better the rest. Under salt conditions, C7 was outperformed by C5.1 at the 6th hour. None of the bacterial endophytes thrived to the 8th hour under salt stress, although the growth of C6 was sustained until the 7th hour.

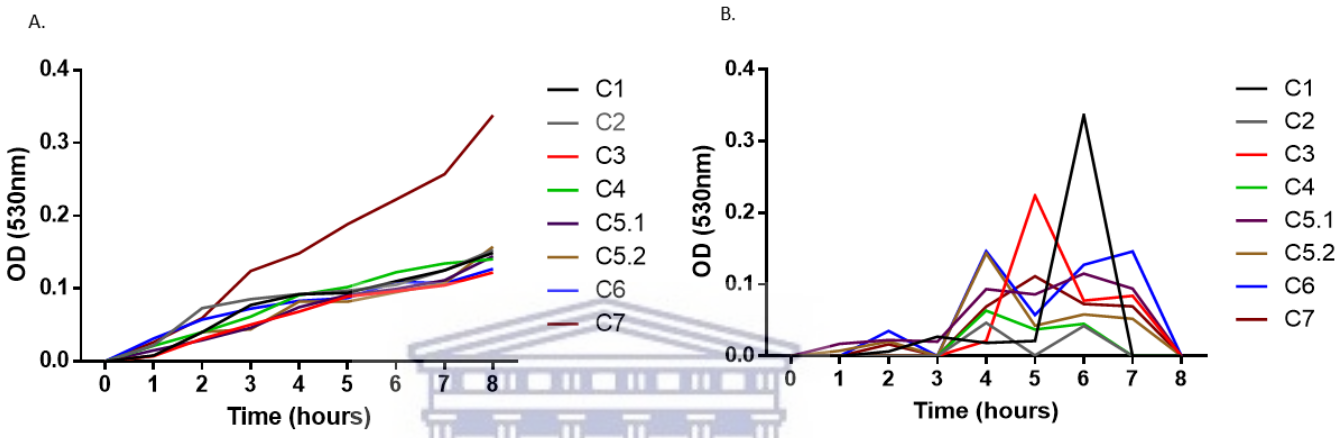


Figure 3.1. Growth of bacterial endophytes in saline media. Graph used to identify whether bacterial endophytes can grow under salt stress. A represents control and B represents the curve with salt. Data presented are means (±SE) of three independent experiments (n=3).

3.2. Sequence-based identification of bacterial endophytes

The extracted bacterial endophytes belonged to a range of soil microorganisms, which have been identified as *Rhizobium sp.*, *Rhodococcus erythropolis*, *Stentrophomonas sp.*, *Rhodococcus sp.*, *Stenotrophomonas melophilia*, *Brucella rhizosphraerae* and *two Ochhrobacterum sp.* (Table 2).

Table 2. The DNA sequence data obtained after BLAST analysis of bacterial endophytes obtained from *Mentha spicata* leaves (See Appendix 6.3).

Isolated endophytes	Genus-species names	Genbank Accession number:
C1	*Rhodococcus erythropolis*	MT423706.1
C2	*Rhizobium sp.*	MN227294.1
C3	*Stenotrophomonas sp.*	AB921262
C4	*Rhodococcus sp.*	MF526418.1
C5.1	*Stenotrophomonas maltophilia*	MT472052
C5.2	*Brucella rhizosphaerae*	MT505117
C6	*Ochrobactrum sp.*	MT271895
C7	*Ochrobactrum sp.*	MK034246

3.3. Application of endophytes improves Agsalfa and Magna 601 tolerance to salinity on MS media.

In the pre-screening of Magna 601 and Agsalfa cultivars, Magna 601 did not germinate in the saline media (Figure 3.3.1 B and Figure 3.3.2). The root length of Agsalfa was also reduced under the saline conditions (Figure 3.3.2 B). Figure 3.3.1. C shows that Magna 601 grew better than Agsalfa when it was inoculated with bacterial endophytes compared to when no endophytes were applied (Figure 3.3.1. A). However, a slight reduction in root length occurred with the inoculation of bacterial endophytes (Figure 3.3. A and C). The addition of bacterial endophytes into the saline media increased root length in Magna 601 and Agsalfa. Agsalfa was outperformed by Magna 601 after day 3 (Figure 3.3.2 B). The consortium of bacterial endophytes improved the performance of both Agsalfa and Magna 601 under salinity (Figure 3.3.2). The failure of Magna 601 to germinate under salinity when no endophytes were applied led to the selection of Agsalfa for further study.

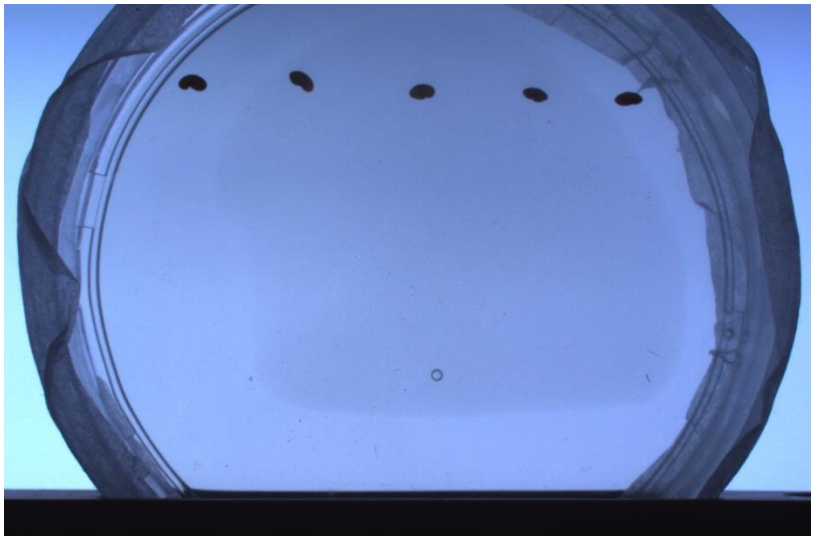


Figure 3.3.1. Seedling growth of Magna 601 under salinity at day 3.

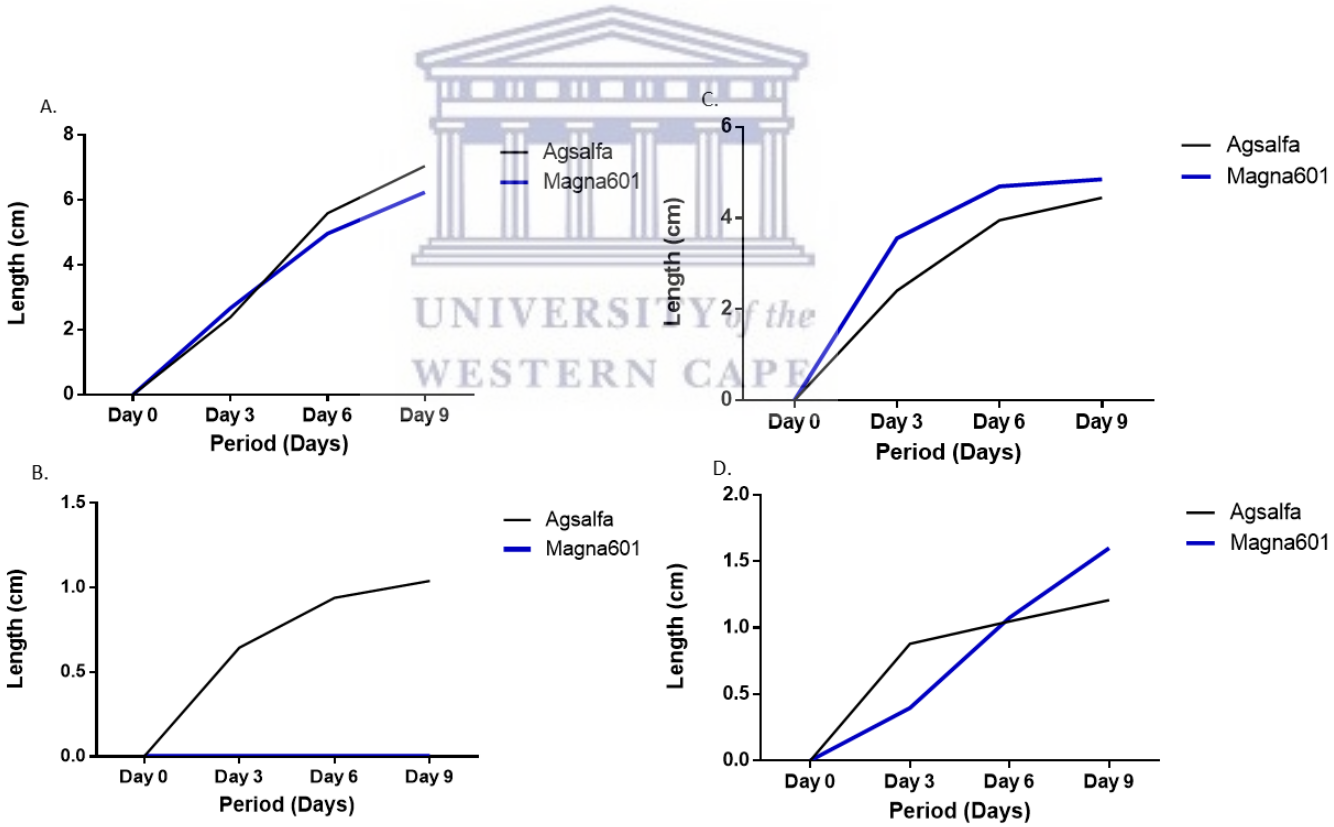


Figure 3.3.2. The effect of salt stress on root growth in Magna 601 and Agsalfa in the presence and absence of bacterial endophytes. Control (No salt, no Endophyte) (A), Salt only (B), Endophytes only (C) and Salt & endophyte (D) of *M.sativa L.* grown under normal and salt conditions in the presence and absence of bacterial endophytes.

Agsalfa was identified as the tolerant cultivar, because it grew better under salt and the combination treatment (salt and endophyte) (Figure 3.3.2). The shoot fresh weight, shoot length and stem diameter were reduced under salt stress by ~70 %, ~ 65 % and ~68 %, respectively (Figure 3.3.3 A-C). The endophytes treatment resulted in a reduction to ~40 % and ~42% of the control shoot weight (Figure 3.3.3 B) and stem diameter (Figure 3.3.3 (C), respectively. The combination treatment (Salt and Endophyte, SE) improved the salt-induced weight, length and stem diameter loss in the Agsalfa cultivar (Figure 3.3.3. A-C). The number of stems/branches was not affected by salt but was increased by the endophyte treatment (Figure 3.3.3 D). The combination treatment with salt and endophytes resulted in branch numbers higher than in the salt treatment and similar to the endophyte treatment (Figure 3.3.3).

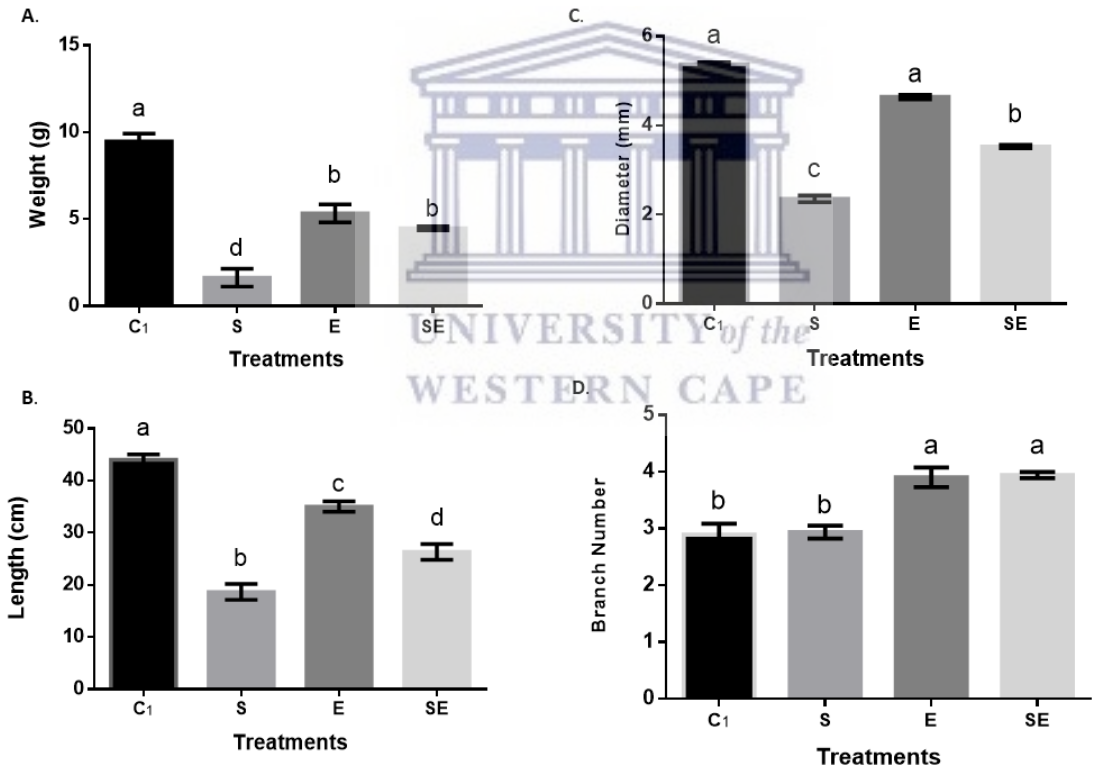


Figure 3.3.3. The effect of salt stress on the weight, length, branch number and shoot diameter of Agsalfa in the presence and absence of bacterial endophytes. Fresh shoot weight (A), shoot length (B), Shoot diameter (C) and Branch number (D) of *Agsalfa* grown under control or salt conditions in the presence or absence of bacterial endophytes. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, with indicated statistically significant differences at $P \leq 0.05$. Abbreviations in the figure are as follows: C_1 (Control); S (Salt); E (Endophyte-treated); SE (Salt and Endophytes).

3.4. The effects of salt and bacterial endophytes on H_2O_2

Hydrogen peroxide (H_2O_2) levels decreased under salt stress in the leaves by ~14 % and increased by ~7 % in the roots in comparison to the results obtained in the controls (Figure 3.4. A and B). The salt plus endophyte treatment resulted in levels of hydrogen peroxide returning to similar levels as the endophyte treated plants in the leaves of Agsalfa compared to the lower levels of H_2O_2 in the salt-treated plants. (Figure 3.4. A). Agsalfa treated with salt-only had higher levels of H_2O_2 in comparison to the lowered levels found in the leaves (Figure 3.4. A and B). In the roots of Agsalfa, the endophytes lowered the H_2O_2 levels compared to its control (Figure 3.4. B). The combination of salt and endophyte also resulted in reduced levels, lower than both control and endophyte treatment (Figure 3.4. B).

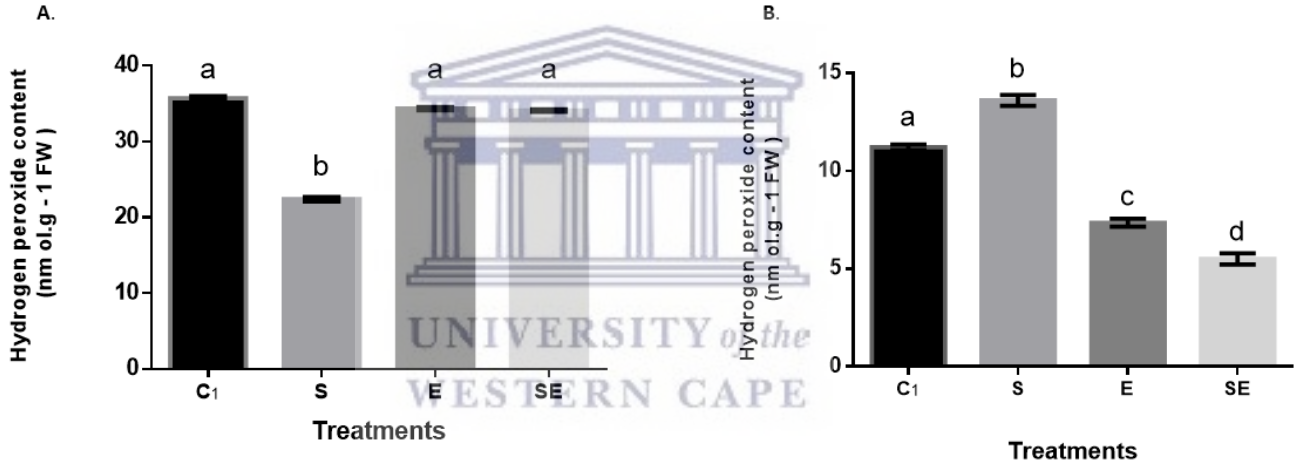


Figure 3.4: The effect of salt stress on the hydrogen peroxide accumulation in *M. sativa* in presence or absence of bacterial endophytes. Hydrogen peroxide in leaves (A) and roots (B). These contents were observed *Agsalfa* cultivars grown under control and salt conditions in the presence or absence bacterial endophytes. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, similar indicated statistical differences where $P \leq 0.05$. Abbreviations in the figure are as follows: C1 (Control); S (Salt); E (Endophyte-treated); SE (Salt and Endophytes)

3.5. Ascorbate peroxidase (APX) activity is altered in Agsalfa under salt and endophyte application

In this study, six isoforms of APX were identified in the leaves (Figure 3.5.1 A). The activity of APX was statistically similar for all levels across the various treatments (Figure 3.5.1. B and C).

In APX5 there is a spike in APX activity when treated with both salt and endophyte compared to the resulting treatments with a difference of ~36.

In the roots, seven isoforms for APX were identified (Figure 3.5.2). The levels of APX were higher in the control compared to the results found in the other treatments which was statistically similar from APX1-APX3 across the three treatments with decreases by ~9, ~24 and ~16% respectively (Figure 3.5.2 B). No statistical differences were observed in APX6 and APX7 across all treatments but differences were observed in the activity of APX under salt treatment which was higher than the levels found in the other treatments (Figure 3.5.2 C). The difference of APX activity in the salt treated plants had a difference of ~12% in APX4 and APX5, increasing and decreasing respectively (Figure 3.4.2 C). Higher activity of APX was observed in roots than leaves as more isoforms were observed in the roots.

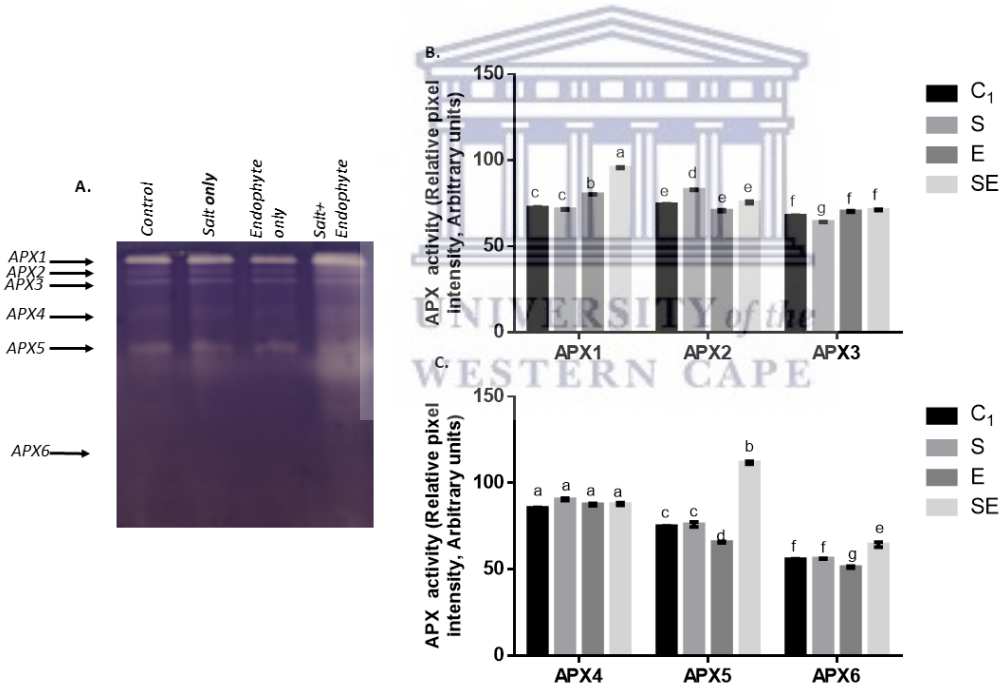


Figure 3.5.1: In-gel activity assays for APX activity in *Agsalfa* leaves in response to salt stress and bacterial endophyte treatment. The in-gel activity assay of APX isoforms in response to the various treatments is represented in (A), APX1-APX3 (B) and APX4-APX6 (C), from which pixel intensities of from 6 APX isoforms Agsalfa were determined. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, bars with the same letter indicate means with no statistically significant differences whereas bars with different letters indicate means with statistically significant differences; where $P \leq 0.05$. Abbreviations in the figure are as follows: C_1 (Control); S (Salt); E (Endophyte -treated); SE (Salt and Endophytes).

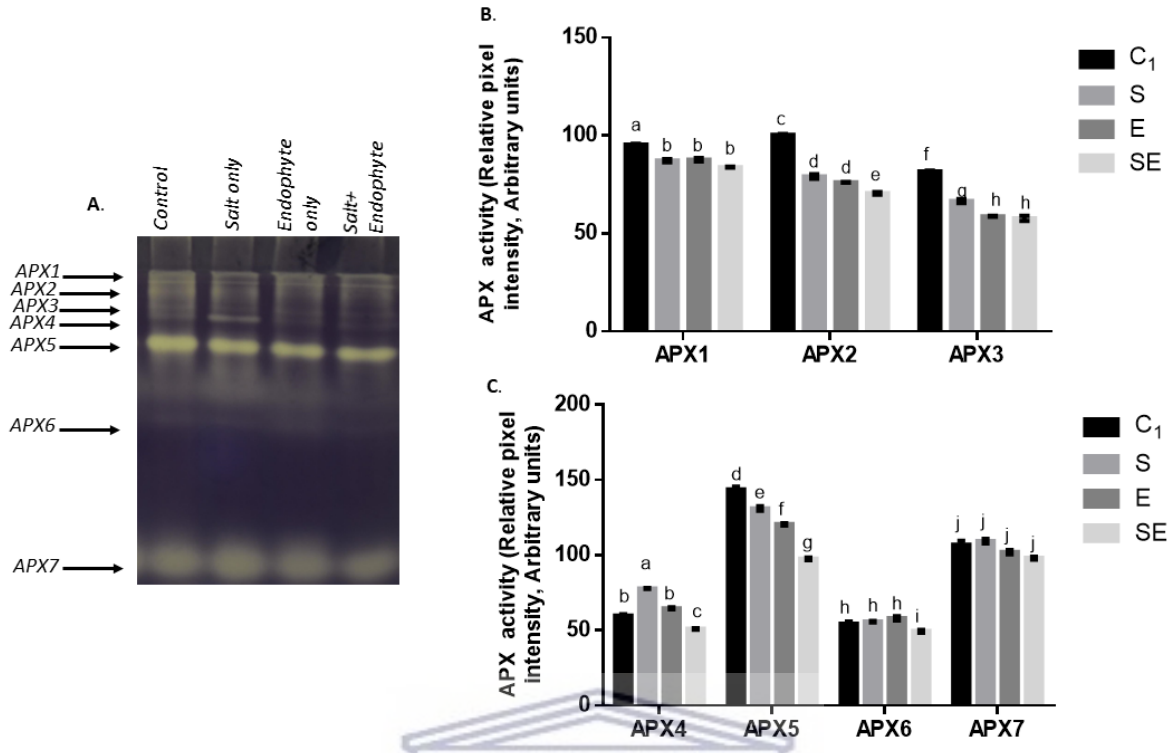


Figure 3.5.2: In-gel activity assays for APX activity in *Agsalfa* roots in response to salt stress and bacterial endophyte treatment. The in-gel activity assay of APX isoforms in response to the various treatments is represented in (A), APX1-APX3 (B) and APX4-APX7 (C), from which pixel intensities of from 7 APX isoforms Agsalfa were determined. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, statistically different means are indicated with different letters, where $P \leq 0.05$. Abbreviations in the figure are as follows: C_1 (Control); S (Salt); E (Endophyte-treated); SE (Salt and Endophytes).

3.6. Catalase (CAT) activity is regulated in Agsalfa under combination of salt and endophyte application

This study resulted in the identification of two CAT isoforms and no catalase activity observed in the leaves in response to any of the treatments (Figure 3.6.2). The salt treatment lowered CAT activity by ~10 % in both isoforms CAT1 and CAT2 (Figure 3.6.1.B). The activity of CAT decreased when Agsalfa was subjected to endophyte treatment alone and elevated (by ~18 %) in response to the combination treatment (SE) (Figure 3.6.1. B). The CAT2 isoform were close in statistical significance in all four treatments (Figure 3.6.1. B).

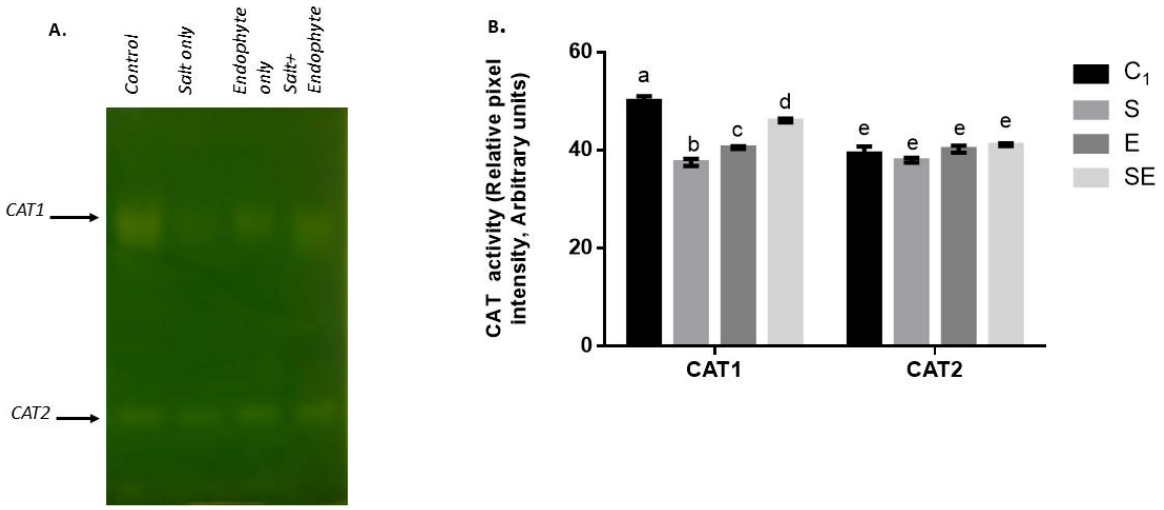


Figure 3.6.1: In-gel activity assays for CAT activity in *Agsalfa* roots in response to salt stress and bacterial endophyte treatment. The in-gel activity assay of APX isoforms in response to the various treatments is represented in (A), CAT1-CAT2 (B), from which pixel intensities of from 2 CAT isoforms Agsalfa were determined. Data presented are means (±SE) of three independent experiments (n=3). Error bars denote standard deviation, similar indicated statistical differences where $P < 0.05$. Abbreviations in the figure are as follows: C1 (Control); S (Salt); E (Endophyte-treated); SE (Salt and Endophytes).

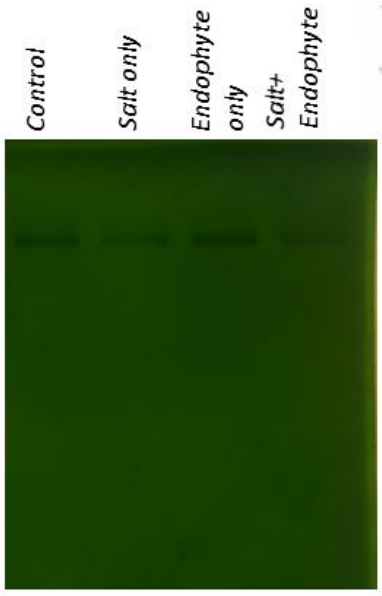


Figure 3.6.2: In-gel activity assays for CAT activity in *Agsalfa* leaves in response to salt stress and bacterial endophyte treatment. The in-gel activity assay of CAT isoforms in response to the various treatments is represented in (A).

Chapter 4: Discussion and Conclusion

4.1. Identification of bacterial endophytes

The bacterial endophyte extraction resulted in a range of soil microorganisms, one which has been identified as a *Rhizobium sp.* (Table 2). This *Rhizobium sp.* is a nitrogen-fixing microorganism already identified as a PGPB (Grover *et al.,* 2011). The *Rhizobium sp*. was previously identified as a plant growth-promoting bacteria and is not commonly found in *Mentha spicata.* Bacteria belonging to the genera *Rhizobium, Bacillus* and *Pseudomonas* are some of the diverse soil microbes that have been reported to provide tolerance to host plants under different abiotic stresses (Grover *et al.,* 2011). The use of these microorganisms can aid in stress alleviation in agriculture (Shrivastava and Kumar, 2015).

Microorganisms like the *Rhodococcus erthropolis, Stenotrophomonas sp.* and *Ochrobacterum sp.* are some of the soil microorganisms identified that have the potential to be pathogenic to people but are common soil bacteria. Microorganisms previously identified to have been extracted from *Mentha spicata* were *Bacillus* and *Pseudomonas*, among others (Grover *et al.,* 2011). The area in which *Mentha spicata* was sampled was a conservation area with a diversity of plant species. This could also shed light on the diversification of bacterial endophytes that were extracted from this plant, none of which were truly halophytic (Table 2). Isolate C1, C3 and C6; identified *as Rhodococcus erythropolis, Stenotrophomonas* sp. and *Orthrobactrum sp*., respectively, were among the isolated bacterial endophytes that had reached a high OD in the presence of salt before a declined (Figure 3.1). This proves that some microorganisms do not need to be halophytic to provide alleviation of stress.

Some microorganisms such as *Rhodococcus* sp. metabolize abscisic acid (ABA) *in vitro* (Qin *et al.*, 2016). The reduction of ABA levels in plants acts as another variable to cope under salinity stress (Qin e*t al*., 2016). The fluctuations on the curve can represent the possibility of a decline in nutrient availability, space or a change in environment (Qin *et al.,* 2016). All bacteria isolated showed the ability to produce IAA and also have siderophore activity (Table 6.1), which can be an endophyte-mediated mechanism to maintain plant growth under salt stress. A study conducted by Khan *et al.* (2019), bacterial endophytes were isolated from *Artemisia princeps* Pamp. To investigate salinity stress, the isolated bacterial endophyte SAK1, was applied to *Glycine max cv.* Pungsannamule (Khan *et al*., 2019). The results showed that SAK1

relieved salinity stress by producing different phytohormones such as ABA, IAA and GAs (Khan *et al*., 2019). Another study by Bianco and Defez (2010) reported that IAA-producing bacterial endophytes increased root and shoot length of *Medicago* plants in a saline environment, compared to uninoculated plants.

4.2. Bacterial endophytes regulate physiological responses in Agsalfa.

Seed germination is a crucial stage in the life cycle of plants and salt tolerance during germination is critical for stand establishment in plants and growth in saline soils (Khan et al., 2000). Several investigations of seed germination under salinity stress have indicated that seeds of most species attain their maximum germination in salt-free water and are very sensitive to elevated salinity at the germination and seedling phases of development (Ghoulam and Fares, 2001; Gulzar *et al*., 2003). Seed germination is defined as the emergence of the radicle through the seed coat (Copeland and Mcdonald, 1995). The study presented here made use of a phenotyping robot that captured images of the germination and growth of both Agsalfa and Magna 601 seedling varieties of *M. sativa* L. under different treatments. The growth of the seedlings from each cultivar was affected by salt stress, as there was the reduction in root length in Agsalfa and no growth of Magna 601. A study by Wang *et al.* (2020) showed that the rate of germination and growth of seedlings was decreased under salt stress, confirming the detrimental effect salt stress has on seedling growth in these cultivars. The lack of growth of Magna 601 indicates the sensitivity of this cultivar to salt stress. Gulzar et al. (2003) reported that seed germination can be initiated by water imbibition and any shortage in water supply will subject the seed to stress. Shokohifard et al. (1989) reported that salt stress negatively affected seed germination; either osmotically through reduced water absorption or ironically through the accumulation of Na^+ and Cl^-, which causes an imbalance in nutrient uptake and an ion toxicity effect.

Endophytes provide support in acclimatizing crop plants under abiotic stress conditions, growth promotion and management of phytopathogens, and they help in activating plant stress-responsive genes that are not usually activated under stress conditions (Saika *et al*., 2018). During initial colonization to the host surface, endophytic microbes are confronted with the immune response of the host (Saika *et al*., 2018). This may be overcome depending

on the endophytic microbial strains and the particular host colonized. This can explain why there were no significant changes in root length in Agsalfa and Magna 601 when compared.

Seed germination is a complex process and is sensitive to many hormones' environmental factors (Wang *et al*., 2020). The action of the endophytes in alleviating salinity stress in the plants was achieved in the study presented here, as there was still root length increases in both cultivars. Alternate management strategies include the use of beneficial microbes (endophytes and PGPB), capable of producing IAA for plant growth and ACC-deaminase for chelating Na^+. (Glick e*t al.*, 2007; Saikia *et al*., 2018). These microbes are also capable of reducing salt stress in plants through the production of ROS-scavenging enzymes, modulating osmotic adjustment, enhancing uptake of K^+ to counteract Na^+, and modulation of signalling pathways (Glick e*t al.*, 2007; Saikia *et al*., 2018).

The cultivar Magna 601 did not germinate under salt stress but as soon as the bacterial endophytes were applied, the root length had surpassed the length of the Agsalfa cultivar. A study by Pal *et al*. (2021) showed that isolated endophytes were capable of increasing root length during germination when the plants were subjected to salt stress. Bacterial endophytes are also capable of enhancing root growth (Pal *et al.* 2021). Bacterial endophytes have co-evolved with plants and help them to acclimatize to the terrestrial ecosystem while transiting from aquatic life (Han and Lee, 2005).

An important bottleneck faced by crop plants has been their inability to maintain growth and biomass production in abiotic stress conditions, resulting in stunted growth and reduced yield (Glick e*t al.,* 2007; Saikia *et al*., 2018). Salt stress has the potential to reduce plant growth due to its influence on turgor pressure, photosynthesis and the activity of specific enzymes within the plant (Bhattarai *et al*., 2020). Such imbalances affect different physiological and biochemical mechanisms related to the growth and development of the plant (Zhang *et al.,* 2013). Different plant species have developed different mechanisms to help them cope under changing conditions (Neto *et al.,* 2004). The results of the present study show how growth parameters of Agsalfa decline under saline conditions. A decrease in fresh shoot weigh and shoot length is among the many changes the plants undergo when subjected to salt stress (Kumar *et al.,* 2021). Salinity increases the osmotic stress that inhibits absorption and transportation of water (Kumar *et al.,* 2021). This leads to a hormone-induced sequential reactions, which reduce stomatal opening and the photosynthetic rate (Kumar *et al.* 2021).

The increase of shoot fresh weight in Agsalfa under salinity conditions, when supplemented with endophytes, may be caused by the ability of the plant to increase its sap vacuoles with the help of the bacterial endophytes (Acosta-Motos *et al.,* 2017). This allows for the collection of water to dissolve salt ions that have accumulated, leading to an increase of fresh weight (Acosta-Motos e*t al.,* 2017). The increase in the number of branches, seen in the work reported here, can act as compensation for the decline of other growth parameters under saline conditions. In a study by Bernstein *et al.* (2001), avocado had grown a new flush of branches when salt stress was induced and resulting in a lowered biomass.

The results obtained from the shoot length, shoot fresh weight and stem diameter shows the effect of salt on the plant. The inhibition of shoot development is a primary response to stresses (Nirit, 2013). According to Torabi and Halim (2010), shoot growth in alfalfa is affected more by salinity than root growth. The present study supports this statement as the shoot lengths had decreased when subjected to salt stress.

Based on all phenotypic results of the current study, it is suggested that the decrease in growth and biomass could be due to the adverse effects of salinity on cell division and elongation (Ali *et al*., 2017). Moreover, salinity also causes nutrient imbalance, overproduction of ROS, and inhibition of enzymatic activities, which significantly affect the cellular components and biological membranes, and cause a decrease in biomass production (Ali *et al*., 2017). The combination of salt stress and bacterial endophytes led to an increase in the fresh weight, shoot length and diameter of Agsalfa compared to salt alone. In a study conducted by Han and Lee (2005), plant growth-promoting endophytic bacteria from *Rhizobium* and *Serratia* were able to increase the availability of nutrients to roots.

It is possible that plant growth promoting endophytic bacteria are able to promote plant growth better under stress conditions than normal conditions (Fan *et al.,* 2020). This supports the results obtained from the present study as the isolates were applied as a consortium, it is possible that not all microorganisms were always active at the same time. Some aid the plant in a different aspect, protecting the plant against pathogenic soil microbes. Some PGPR were reported to promote plant growth under both normal and stressed conditions, while others were effective only under stressed conditions, exerting no growth promotion effect under optimal conditions, suggesting that the PGP activity of some endophytic bacteria depend on stress (Fan *et al.,* 2020). The increase in root to shoot ratio is a common response to salt

stress, related to factors associated with water stress (osmotic effect) rather than a salt-specific (ionic) effect (Fan *et al.,* 2020).

4.3. Bacterial endophytes help regulate ROS (H_2O_2) in M. sativa L.

The generation of ROS in response to stresses is the primary response in plants (Gill and Tuteja, 2010). The accumulation of ROS induces oxidative damage to membrane lipids, proteins and nucleic acids (Mitler, 2002). For this reason, tight control of the steady-state concentration of ROS is necessary to avoid oxidative damage, while simultaneously allowing ROS to perform their useful functions as signal molecules (Gomez *et al.,* 2004; Rubio *et al.,* 2009). Hydrogen peroxide (H_2O_2) is a molecule involved in several cell processes under both normal and stress conditions (Quan *et al*., 2008). Under stressful conditions, H_2O_2 is produced and its excessive accumulation leads to oxidative stress in plants (Quan *et al*., 2008).

This can be a possible explanation for the increase in H_2O_2 levels in the roots of Agsalfa in response to salinity. The opposite occurred in the leaves, with levels of H_2O_2 decreasing in Agsalfa during the salt stress. The lowering of H_2O_2 levels for the salt treatment in the cultivar could be the result of less oxidative damage in the leaves and with more oxidative damage occurring the roots. A reduction of oxidative stress is achieved in three levels; lowering exposure to environmental constituents with oxidizing properties, the increase of exogenous and endogenous antioxidant levels or mitochondrial stabilization by lowering its oxidative stress (Poljsak, 2011). Kim *et al*. (2005) observed a decrease in H_2O_2 content in response to salinity stress, which was a result of an increase in antioxidant enzyme activities in barley. A study by Wang *et al.* (2009) investigated two alfalfa cultivars under salt stress and reported lower levels of H_2O_2, suggesting an enhanced capacity to protect the tissues from oxidative damage imposed by salt stress.

The sensitivity of the roots to changes in its environment can be a result of the roots being the first sensors of stress in the soil (Wang *et al.,* 2009). In this study, the results obtained by Wang *et al.* (2009) agree with this statement, where the majority of the damage occurred in the roots than the leaves of Agsalfa. When the plants were inoculated with the endophytes, the H_2O_2 levels changed to levels close to normal. In a study conducted by El-Awady (2015), endophytic and rhizospheric bacteria were isolated and it was found that the endophytes

have growth promoting capabilities, aiding in the alleviation of salt stress in plants. Some studies have shown that PGPB enhance root and shoot growth by inducing antioxidant defence system in crops such as pea (*Pisum sativum L.),* rice (*Oryza sativa*) and soybean (*Glycine max*) (Egamberdieva *et al.*, 2016). The results from the present study show that the bacterial endophytes alleviated stress in the roots of Agsalfa when it was subjected to a combination treatment (salt and endophyte). A study conducted by Wang *et al.* (2020) found that, under Cd stress, inoculation with a bacterial endophyte ($SAMR_{12}$) could activate the antioxidant response and decrease levels of H_2O_2 in *B. jancea*.

4.4 Bacterial endophytes increase plant adaptive responses to salt stress through the activation of antioxidant enzymes.

Ascorbate peroxidase (APX) and catalase (CAT) are among the key enzymes that scavenge H_2O_2. APX is a fundamental component of the Ascorbate-Glutathione (ASCGSH) cycle and reduces H_2O_2 to H_2O and dehydroascorbate (DHA). In this study, the upregulation of APX activity was observed in the plants, in both the leaves and roots compared to the control. This increase in APX activity is most-likely an effort to prevent oxidative damage from H_2O_2 accumulation. Many studies have shown that APX activity increases under stress conditions such as drought and salinity, leading to higher salt tolerance (Yabuta *et al.,* 2002).

Salt-tolerant lucerne (alfalfa) genotypes are equipped with a diverse array of enzymatic and non-enzymatic antioxidants that help them to scavenge ROS under salt stress. For example, Ferreira *et al.* (2015) associated improvements in nutrient composition and forage parameters with improved total antioxidant capacity of four lucerne genotypes under salt stress. Redman *et al.* (2017) observed that endophyte inoculations significantly decreased the accumulated ROS in the plant cell by activating antioxidant enzymes. Endophytes induce the synthesis of antioxidants to balance an array of free radicals that are produced in the cell. In addition, the production of osmolytes maintains the sodium-potassium ratio, which overcomes the osmotic effect caused by stress factors (Redman *et al., 2017)*.

Additionally, the results show that the majority of APX activity occurred in the roots of the Agsalfa cultivar. This is most likely the result of roots being the first sensors of stress in the environment. APX is regarded as one of the most widely distributed antioxidant enzymes in

plant cells and isoforms of APX have much higher affinity for H_2O_2 than CAT, making APXs efficient scavengers of H_2O_2 under stressful conditions (Ferreira *et al.,* 2015). This is evident when plants were placed under salt stress, levels for CAT were lower than that of APX.

No CAT activity was detected in the leaves of Agsalfa. During salinity stress treatment, a decrease in CAT activity was observed in the roots. Baltruschat *et al.* (2008) reported reduced levels of CAT, APX, GR DHAR in root tissues of barley under saline conditions. However, root colonization by *Piriformospora indica* elevated the antioxidant enzyme activity (Baltruschat *et al*., 2008). The bacterial endophytes are capable of increasing CAT activity and can aid as proof to the mediation of antioxidants by bacterial endophytes. A study by Ferreira *et al.* (2015) showed that, after inoculating plants with bacterial endophytes, enhanced levels of antioxidant enzymes were attained, mainly SOD, POD, CAT in roots.

4.5. Conclusion and future work

This study aimed at investigating if bacterial endophytes are capable of alleviating any form of salt stress. This study makes use of Mentha spicata as plant material to isolate bacterial endophytes from. This medicinal plant has shown potential to have moderate salt tolerance. The study shows that Agsalfa is more tolerant to salt and Magna 601 is salt-sensitive. The bacterial endophytes alleviate salt stress in both cultivars, especially the sensitive cultivar Magna601. The bacterial endophytes, as a consortium, can also regulate antioxidant activity (in this case CAT).

Under salt stress, these bacterial endophytes change the physiology of the plant to promote survival under salinity stress. Some of the identified endophytes were found to be plant growth-promoting bacteria (PGPB). This is quite important, as this can pave a way for sustainable agriculture in relation to abiotic stress resilience and also making use of microorganisms as biofertilizers. Although none of the bacterial endophytes proved to survive at high salt concentrations, they were still able to regulate some form of responses to help maintain growth and overall survival in the plant.

For future work, the bacterial endophytes with more favourable traits can be isolated and tested on their own in a saline environment. The use of the phenotyping image robot was useful in capturing time-lapse data, in this case germination of the two alfalfa cultivars.

Overall, the best performing cultivar was Agsalfa. Agsalfa showed growth adaptations that allowed it perform better under salt stress without bacterial endophyte inoculation. The ability to improve plant adaptive responses to stress is important in the development of more tolerant crops and, based on the results above, Agsalfa could be a potential candidate in that process. It will be important to assess alteration in gene expression for the antioxidant's enzymes investigated, as well as investigating genes involved in both the plant and endophyte in conferring salinity stress tolerance.

Chapter 5: References

Acosta-Motos, J.R., Ortuño, M.F., Álvarez, S., López-Climent, M.F., Gómez-Cadenas, A. and Sánchez-Blanco, M.J. (2016). Changes in growth, physiological parameters and the hormonal status of *Myrtus communis* L. plants irrigated with water with different chemical compositions. *Journal of plant physiology*, 191. 12-21.

Acosta-Motos, J., Maria F. Ortuño, A., Pedro Diaz-Vivancos, M. and Jose, A. (2017). Plant responses to salt stress: Adaptive mechanisms. *Agronomy* 7: 18. https://doi.org/10.3390/agronomy7010018

Afzal, I., Shinwari, Z.K., Sikandar, S. and Shahzad, S. (2019). Plant beneficial endophytic bacteria: Mechanisms, diversity, host range and genetic determinants. *Microbiological Research* 221, 36-49.

Ali, S., Charles, T.C. and Glick, B.R. (2017). Endophytic phytohormones and their role in plant growth promotion. In Functional Importance of the Plant Microbiome, *Springer, Cham.* 89-105.

Ansari, M., Shekari, F., Mohammadi, M.H., Juhos, K., Vegvari, G. and Biro, B. (2019). Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (*Medicago sativa* L.) cultivars at high salinity. *Acta Physiologiae Plantarum* 41, 195.

Audipudi, A., Chakicherla, B. and Bhore, S. (2017). Bacterial endophytes as biofertilizers and biocontrol agents for sustainable agriculture. *Biotechnology for Sustainability*, 223-247.

Athar, H., Khan, A. and Ashraf, M. (2008). Exogenously applied ascorbic acid alleviates salt induced oxidative stress in wheat. *Environmental and Experimental Botany* 63, 224–231.

Arshad, M., Khan, A.H.A., Hussain, I., Anees, M., Iqbal, M., Soja, G., Linde, C. and Yousaf, S. (2017). The reduction of chromium (VI) phytotoxicity and phytoavailability to wheat (*Triticum aestivum L.)* using biochar and bacteria. *Applied Soil Ecology* 114, 90-98.

Badawi, G. H., Kawano, N., and Yamauchi, Y. (2004). Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. *Physiologiae Plantarum* 121, 231–238.

Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., Schäfer, P., Schwarczinger, I. and Zuccaro, A. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytologist* 180, 501-510.

Bernstein, N., Ioffe, M. and Zilberstaine, M. (2001). Salt-stress effects on avocado rootstock growth. I. Establishing criteria for determination of shoot growth sensitivity to the stress. *Plant and Soil* 233, 1-11.

Bhattarai, S., Biswas, D., Fu, Y.B. and Biligetu, B. (2020). Morphological, physiological, and genetic responses to salt stress in Alfalfa: *A Review. Agronomy* 10, 577.

Bianco, C. and Defez, R. (2010). Improvement of phosphate solubilization and Medicago plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. *Applied and Environmental Microbiology* 76, 4626-4632.

Bilkay, I.S., Karakoç, Ş. and Aksöz, N. (2010). Indole-3-acetic acid and gibberellic acid production in *Aspergillus niger*. *Turkish Journal of Biology* 34, .313-318.

Çamlica, M. and Yaldiz, G. (2017). Effect of salt stress on seed germination, shoot and root length in basil (*Ocimum basilicum*). *International Journal of Secondary Metabolite* 4, 69-76.

Castillo, M., Flores, M., Mavingui, P., Martínez-Romero, E., Palacios, R. and Hernández, G. (1999). Increase in alfalfa nodulation, nitrogen fixation, and plant growth by specific DNA amplification in *Sinorhizobium meliloti*. *Applied and Environmental Microbiology* 65, 2716-2722.

Cen, H., Wang, T., Liu, H., Tian, D. and Zhang, Y. (2020). Melatonin application Improves Salt Tolerance of Alfalfa (*Medicago sativa L.)* by enhancing antioxidant capacity. *Plants* 9, 220.

Ceuppens, J. and Wopereis, M.C.S. (1999). Impact of non-drained irrigated rice cropping on soil salinization in the Senegal River Delta. *Geoderma* 92, 125-140.

Chen, M., Yang, Z., Liu, J., Zhu, T., Wei, X., Fan, H. and Wang, B. (2018). Adaptation mechanism of salt excluders under saline conditions and its applications. *International Journal of Molecular Sciences* 19, 3668.

Copeland, L.O. and McDonald, M.B. (1995). Principles of seed science and technology, Chapman & Hall, New York. Bioclimatic map of Natal. Extension Offices of the Department of Agricultural Development, Pietermaritzburg.

Downie, L., Proiddle, J., Hawes, C. and Evans, D.E. A calcium pump at the higher plant nuclear envelope? *FEBS Letters* 429, 44-48.

Egamberdieva, D., Wirth, S., Behrendt, U., Abd_Allah, E.F. and Berg, G. (2016). Biochar treatment resulted in a combined effect on soybean growth promotion and a shift in plant growth promoting rhizobacteria. *Frontiers in Microbiology* 7, 209.

El-Awady, M.A., Hassan, M.M. and Al-Sodany, Y.M. (2015). Isolation and characterization of salt tolerant endophytic and rhizospheric plant growth-promoting bacteria (PGPB) associated with the halophyte plant (*Sesuvium verrucosum*) grown in KSA. *International Journal of Applied Sciences and Biotechnology* 3, 552-560.

EL-Sharkawy, M.S., EL-Beshsbeshy, T.R., Hassan, S.M., Mahmoud, E.K., Abdelkader, N.I., AlShal, R.M. and Missaoui, A.M. (2017). Alleviating salt stress in barley by use of plant growth stimulants and potassium sulfate. *Journal of Agricultural Science* 9, 136.

Fan, D., Subramanian, S. and Smith, D.L. (2020). Plant endophytes promote growth and alleviate salt stress in Arabidopsis thaliana. *Scientific Reports*, 10.

Ferreira, J.F., Cornacchione, M.V., Liu, X. and Suarez, D.L. (2015). Nutrient composition, forage parameters, and antioxidant capacity of alfalfa (*Medicago sativa, L.)* in response to saline irrigation water. *Agriculture* 5, 577-597.

Flowers, T.J. and Yeo, A.R. (1995). Breeding for salinity resistance in crop plants: where next. *Functional Plant Biology* 22, 875-884.

Flowers, T.J. (2004). Improving crop salt tolerance, *Journal of Experimental Botany* 55, 307-319,

Ghoulam, C., Foursy, A. and Fares, K. (2001). Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environmental and experimental Botany* 47, 39-50.

Gill, S.S. and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48, 909-930.

Gomez L.D., Noctor G., Knight M. & Foyer C.H. (2004). Regulation of calcium signaling and gene expression by glutathione. *Journal of Experimental Botany* 55, 1851-1859.

Glick, B.R., Cheng, Z., Czarny, J. and Duan, J. (2007). Promotion of plant growth by ACC deaminase-producing soil bacteria. In: Bakker P.A.H.M., Raaijmakers J.M., Bloemberg G., Höfte M., Lemanceau P., Cooke B.M. (eds) New Perspectives and Approaches in Plant Growth-Promoting Rhizobacteria Research. Springer, Dordrecht, 329-339.

Grover, M., Ali, S.Z., Sandhya, V., Rasul, A. and Venkateswarlu, B. (2011). Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World Journal of Microbiology and Biotechnology* 27, 1231-1240.

Gulzar, S., Khan, M.A. and Ungar, I.A. (2003). Effects of salinity on growth, ionic content, and plant–water status of *Aeluropus lagopoides*. *Communications in Soil Science and Plant Analysis* 34, 1657-1668.

Guo, P., Wei, H., Zhang, W. and Bao, Y. (2016). Physiological Responses of Alfalfa to high-level salt stress: root ion flux and stomatal characteristics. *International Journal of Agriculture and Biology* 18, 125–133.

Han, H.S. and Lee, K. (2005). Plant growth promoting Rhizobacteria effects on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity. *Research Journal of Agriculture and Biological Sciences* 1, 210-215.

Hanin, M., Ebel, C., Ngom, M., Laplaze, L. and Masmoudi, K. (2016). New insights on plant salt tolerance mechanisms and their potential use for breeding. *Frontiers in Plant Science* 7, 1787.

Hasanuzzaman, M. and Tanveer, M. (2020). Salt and Drought Stress Tolerance in Plants: Signaling Networks and Adaptive Mechanisms. 1st ed. Cham, Switzerland: Springer.

Huang, H., Ullah, F., Zhou, D.X., Yi, M. and Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. *Frontiers in Plant Science* 10, 800.

Isayenkov, S.V. and Maathuis, F.J. (2019). Plant salinity stress: many unanswered questions remain. *Frontiers in Plant Science* 10, 80.

Istain, B. (2019). Isolation and characterization of plant growth promoting endophytic bacteria from *Medicago lupulina* for enhanced drought tolerance in maize. http://hdl.handle.net/11394/7870

Jukanti, A.K., Gaur, P.M., Gowda, C.L.L. and Chibbar, R.N. (2012). Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. *British Journal of Nutrition* 108, S11-S26.

Kaiwen, G., Zisong, X., Yuze, H., Qi, S., Yue, W., Yanhui, C., Jiechen, W., Wei, L. and Huihui, Z. (2020). Effects of salt concentration, pH, and their interaction on plant growth, nutrient uptake, and photochemistry of alfalfa (*Medicago sativa*) leaves. *Plant Signaling & Behavior* 15, 1832373.

Kim, Y.J. and Park, M. (2005). Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under saline stress. *Journal of Biochemistry and Molecular Biology* 38, 218-224.

Kulkarni, K.P., Tayade, R., Asekova, S., Song, J.T., Shannon, J.G. and Lee, J.D. (2018).

Kumar, A., Singh, S., Mukherjee, A., Rastogi, R.P. and Verma, J.P. (2021). Salt-tolerant plant growth-promoting *Bacillus pumilus* strain JPVS11 to enhance plant growth attributes of rice and improve soil health under salinity stress. *Microbiological Research* 242, 126616.

Harnessing the potential of forage legumes, alfalfa, soybean, and cowpea for sustainable agriculture and global food security. *Frontiers in Plant Science* 9, 1314.

Khan, F., Bhatti, A.U. and Khattak, R.A. (2001). Soil and nutrient losses through sediment and surface runoff under maize mono-cropping and maize-legume, intercropping from up-land sloping field. *Pakistan Journal of Soil Science* 19, 32-40.

Khan, F., Hakeem, K.R., Siddiqi, T.O. and Ahmad, A. (2013). RAPD markers associated with salt tolerance in soybean genotypes under salt stress. *Applied Biochemistry and Biotechnology* 170, 257-272.

Khan, F. and Hakeem, K.R. (2014). Cell signaling during drought and salt stress. In *Plant signaling: Understanding the molecular crosstalk* (pp. 227-239). Springer, New Delhi.

Khan, A. and Ashraf, M. (2008). Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. *Environmental and Experimental Botany* 63, 224-231.

Khan, M.A., Asaf, S., Khan, A.L., Ullah, I., Kang, S. and Lee, I. (2019). Alleviation of salt stress response in soybean plants with the endophytic bacterial isolate *Curtobacterium* sp. SAK1. *Annals of Microbiology* 69, 797-808.

Kotula, L., Khan, H.A., Quealy, J., Turner, N.C., Vadez, V., Siddique, K.H., Clode, P.L. and Colmer, T.D. (2015). Salt sensitivity in chickpea (*Cicer arietinum* L.): ions in reproductive tissues and yield components in contrasting genotypes. *Plant, Cell & Environment* 38, 1565-1577.

Ladeiro, B. (2012). Saline agriculture in the 21st century: using salt contaminated resources to cope food requirements. *Journal of Botany* 2012, 310705.

Lei, Y., Xu, Y., Hettenhausen, C., Lu, C., Shen, G., Zhang, C., Li, J., Song, J., Lin, H. and Wu, J. (2018). Comparative analysis of alfalfa (*Medicago sativa L.)* leaf transcriptomes reveals genotype-specific salt tolerance mechanisms. *BMC Plant Biology* 18, 35.

Li, L. and Li, L. (2017). Plant growth-promoting endophyte *Piriformospora indica* alleviates salinity stress in *Medicago truncatula. Plant Physiology and Biochemistry* 119, 211-223.

Long, R., Li, M., Zhang, T., Kang, J., Sun, Y., Cong, L., Gao, Y., Liu, F., & Yang, Q. (2016). Comparative proteomic analysis reveals differential root proteins in *Medicago sativa* and *Medicago truncatula* in response to salt stress. *Frontiers in Plant Science* 7, 424..

Manchanda, G. and Garg, N. (2008). Salinity and its effects on the functional biology of legumes. *Acta Physiologiae Plantarum* 30, 595-618.

Mitller, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7, 405-410.

Munns, R. (2005). Genes and salt tolerance: bringing them together. *New Phytologist* 167, 645-663.

Munns, R. (1993). Physiological processes limiting plant growth in saline soils: Some dogmas and hypotheses. *Plant Cell & Environment* 16, 15-24.

Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology* 59, 651-681.

Møller, I. S., Gilliham, M., Jha, D., Mayo, G. M., Roy, S. J., Coates, J. C., Haseloff, J., and Tester, M. (2009). Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type specific alteration of Na^+ transport in Arabidopsis. *Plant Cell* 21, 2163-2178.

Nakashima, K., Takasaki, M., Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012). NAC transcription factors in plant abiotic stress responses. *Biochimica et Biophysica Acta,* 1819, 97-103.

Negrão, S., Schmöckel, S. M., and Tester, M. (2017). Evaluating physiological responses of plants to salinity stress. *Annals of Botany* 119, 1–11.

Neto, A., Prisco, J., Eneas-Filho, J., Lacerda, C., Silva, J., Costa, P. and Gomes-Filho, E.

(2004). Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes. *Brazilian Journal of Plant Physiology* 16, 31-38.

Nirit, B. (2013). Effects of salinity on root growth. In *Plant Roots: The Hidden Half*, 4th Ed, (pp. 1-18). CRC Press.

Norlyn, J.D. (1980). Breeding salt-tolerant crop plants. In *Genetic Engineering of Osmoregulation* (pp. 293-309). Springer, Boston, MA.

Pal, K.K., Dey, R., Sherathia, D.N., Mangalassery, S., Kumar, A., Rupapara, R.B., Mandaliya, M., Rawal, P., Bhadania, R.A., Thomas, M. and Patel, M.B. (2021). Alleviation of salinity stress in peanut by application of endophytic bacteria. *Frontiers in Microbiology* 12, 791.

Parvaiz, A. and Satyawati, S. (2008). Salt stress and phyto-biochemical responses of plants-a review. *Plant, Soil and Environment* 54, 89-99.

Poljsak, B. (2011). Strategies for reducing or preventing the generation of oxidative stress. *Oxidative Medicine and Cellular Longevity* 2011, 194586.

Postnikova, O., Shao, J. and Nemchinov, L.G. (2013). Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant and Cell Physiology* 54, 1041-1055.

Qin, Y., Druzhinina, I.S., Pan, X. and Yuan, Z. (2016). Microbially mediated plant salt tolerance and microbiome-based solutions for saline agriculture. *Biotechnology Advances* 34, 1245-1259.

Quan, L.J., Zhang, B., Shi, W.W. and Li, H.Y. (2008). Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *Journal of Integrative Plant Biology* 50, 2-18.

Radhakrishnan, E.K., Jasim, B., John-Jimtha, C., and Jyothis, M. (2013). Plant growth promoting potential of endophytic bacteria isolated from Piper nigrum. *Plant Growth Regulation* 71, 1-11.

Redman, R.S., Kim, Y.O., Woodward, C.J.D.A., Greer, C., Espino, L., Doty, S.L. and Rodriguez, R.J. (2017). Increased fitness and adaptation of rice plants to cold, drought and salt stress via habitat adapted symbiosis: A strategy for mitigating impacts of climate change. *PLoS One*, 6, 14823.

Rogers, M.E., Craig, A.D., Munns, R.E., Colmer, T.D., Nichols, P.G.H., Malcolm, C.V., Barrett Lennard, E.G., Brown, A.J., Semple, W.S., Evans, P.M. and Cowley, K. (2006). Corrigendum to: The potential for developing fodder plants for the salt-affected areas of southern and eastern Australia: an overview. *Australian Journal of Experimental Agriculture* 46*,* 1665-1665.

Roy, S., Negrao, S. and Tester, M. (2014). Salt resistant crop plants. Current Opinion in Biotechnology. 26, 115–124. Rozema, J. and Schat, H. (2013) Salt tolerance of halophytes, research questions reviewed in the perspective of saline agriculture. *Environmental and Experimental Botany* 92, 83-90.

Rozema, J. and Schat, H. (2013). Salt tolerance of halophytes, research questions reviewed in the perspective of saline agriculture. *Environmental and Experimental Botany* 92, 83-95.

Rubio, V., Bustos, R., Irigoyen, M.L., Cardona-López, X., Rojas-Triana, M. and Paz-Ares, J. (2009). Plant hormones and nutrient signalling. Plant Molecular Biology 69, 361-373.

Russ, J.D., Zaveri, E.D., Damania, R., Desbureaux, S.G., Escurra, J.J. and Rodella, A.S. (2020). Salt of the earth: quantifying the impact of water salinity on global agricultural productivity. *World Bank Policy Research Working Paper*, (9144).

Santoyo, G., Moreno-Hagelsieb, G., del Carmen Orozco-Mosqueda, M. and Glick, B.R. (2016). Plant growth-promoting bacterial endophytes. *Microbiological Research* 183, 92-99.

Saika, A., Nagatake, T. and Kunisawa, J. (2018). Host-and microbe-dependent dietary lipid metabolism in the control of allergy, inflammation, and immunity. *Frontiers in Nutrition* 6, 36.

Scasta, J.D., Trostle, C.L. and Foster, M.A. (2012). Evaluating alfalfa (*Medicago sativa L.)* cultivars for salt tolerance using laboratory, greenhouse and field methods. *Journal of Agricultural Science* 4, 90.

Shahbaz, M. and Ashraf, M. (2013). Improving salinity tolerance in cereals. *Critical Reviews in Plant Science* 32, 237-249.

Shokohifard, G., Sakagami, K., Hamada, R. and Matsumoto, S. (1989). Effect of amending materials on growth of radish plant in salinized soil. *Journal of Plant Nutrition* 12, 1195-1214.

Shrivastava, P. and Kumar, R. (2015). Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi journal of Biological Sciences* 22, 123-131.

Steppuhn H., Falk K. C. and Zhou, R. (2010) Emergence, height, grain yield and oil content of camelina and canola grown in saline media. Can. J. Soil Sci. 90, 151–164. Tester, M., and Davenport, R.J. (2003) Na+ tolerance and Na+ transport in higher plants. Annals of Botany (Lond.) 91, 503-527.

Tester, M. and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. *Annals of Botany* 91, 503-527.

Tilbrook, J., Schilling, R.K., Berger, B., Garcia, A.F., Trittermann, C., Coventry, S., Rabie, H., Brien, C., Nguyen, M., Tester, M. and Roy, S.J. (2017). Variation in shoot tolerance mechanisms not related to ion toxicity in barley. *Functional Plant Biology* 44, 1194-1206.

Tilbrook, J., Asif, M.A., Garcia, M., Brien, C., Dowling, K., Berger, B., Schilling, R.K., Short, L., Trittermann, C., Gilliham, M. and Fleury, D. (2020). Identification of salt tolerance QTL in a wheat RIL mapping population using destructive and non-destructive phenotyping. *Functional Plant Biology* 48, 131-140.

Torabi, M. and Halim, M. (2010). Variation of root and shoot growth and free proline accumulation in Iranian alfalfa ecotypes under salt stress. *Journal of Food Agriculture and Environment* 8, 323-327.

Velikova, V., Edreva, A. and Loreto, F. (2000). Endogenous isoprene protects Phragmites australis leaves against singlet oxygen. *Physiologia Plantarum* 122, 219-225.

Vengosh, A. 2003. Salinization and saline environments. Treatise on geochemistry, 9, 612.

Wang, W.B., Kim, Y.H., Lee, H.S., Kim, K.Y., Deng, X.P. and Kwak, S.S. (2009). Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. *Plant Physiology and Biochemistry* 47, 570-577.

Wang, Z., Hong, Y., Li, Y., Shi, H., Yao, J., Liu, X., Wang, F., Huang, S., Zhu, G. and Zhu, J.K. (2021). Natural variations in SlSOS1 contribute to the loss of salt tolerance during tomato domestication. *Plant Biotechnology Journal* 19, 20.

Yabuta, Y., Motoki, T., Yoshimura, K., Takada, T., Ishikawa, T. and Shigeoka, S. (2002). Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. *Plant Journal* 32. 912–925.

Zhang, J. and Mu, C.S. (2011). Effects of saline and alkaline stresses on the germination, growth, photosynthesis, ionic balance and anti-oxidant system in an alkali-tolerant

Zhang, J.L., Ismail, A. and Shi, H. (2013). Physiological and molecular mechanisms of plant salt tolerance. *Photosynthesis Research* 115, 1-22.

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* 53, 247-273.

Chapter 6: Appendix

Table 6.1. Comparative study of the potential of endophytes for their biochemical characteristics.

Characteristics features	Endophytes							
	C1	C2	C3	C4	C5.1	C5.2	C6	C7
IAA production	+	+	+	+	+	+	+	+
Siderophore production	+	+	+	+	+	+	-	-
Zinc solubilisation	+	-	-	-	-	-	-	-
Phosphorus solubilization	-	-	-	-	-	-	-	-
Catalase activity	+	+	+*	+	-	+	+*	+*

+*: a weak positive

6.2. Videos from robot

Agsalfa (endophyte only)

https://drive.google.com/file/d/1NWRaplWZc5XhLDR2g0yHHwiEPbBD4OWz/view?usp=sharing

Agsalfa (Salt and endophyte)

https://drive.google.com/file/d/1DNZH9-WFOx8h-2r61TpDO73rWWeDkn9f/view?usp=sharing

Control (No salt, no endophyte)

https://drive.google.com/file/d/1iJonCL-ST923S0cxlsIP7WmeL-ONbPCT/view?usp=sharing

Control (Salt, No endophyte)

https://drive.google.com/file/d/1yKzR9tfjJchyk84Dn1tnGYSFo7JsIGRO/view?usp=sharing

Magna601 (Endophyte only)

https://drive.google.com/file/d/1Z9KObFeV6W6L6jc8EWKnW-aLplqFeQPA/view?usp=sharing

Magna601 (Salt and endophyte)

https://drive.google.com/file/d/1vzt9VsiNS0HSLo9xz5Ki18AonMDQSVWE/view?usp=sharing

6.3. Unedited sequences obtained from BLAST queried with sequences from the PCR reactions of isolated endophyte DNA

Cultures	Sequences
1	GGGGCGGGAGGGCGCAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGT TGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTA CCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAA GCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAA AGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGGACTAGAGTGTGGTAGAGGGT AGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAA GGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATT AGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACT
2	TGCAAGTCGAACGCCCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGCGTGGGAACATA CCCTTTCCTGCGGAATAGCTCCGGGAAACTGGAATTAATACCGCATATCGCCCTACGGGGG AAAGATTTATCGGGGAAGGATTGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGGCC TCACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAGA CACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCAGGGA AGAAGCCATAAGTGACGGTATCCGGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCC GCGGTAATACGAAGGGGGCTAGCGTTGTCCGGAATTACTGGGCG
3	GGGGCGGGAGGGCGCAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGT TGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTA CCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAA GCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAA AGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGGACTAGAGTGTGGTAGAGGGT AGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAA GGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATT AGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGT
4	TGACCTAGACTGTTCTCCGACGCCGCCGTGCAGTTGAGGGCCCTGGGTTGTAAACCTCCTTC ATCAGGGACGAAGCGGGGTGACGGTACCTGTAAAAGAAGCACCGGGTAACTACGTGCTAG CAGCAGCGGTAATTCGTAGGGTGCAAGCGTATCGGAATTACTGGGGTAAAGAGCTCGTAG GCGGTTGTTGCGGGGTTGTAAAAACCAGCAGCTCCACTGATGGGTTGCAGACGAGACGGG CGACTTGAGTCTGAGGGGAAACTGGAATTCCTGGTGTAGCGGTGAAATGCGAGTTAACGG AGGAACATCGGTGAAAAAGGAATACTCGCCTTACTAAGCTGAGCAATGAAAGCGTGACCG CTAACAGGATTAAATAGCTGGTCACCTGATGAGAAGTGGGCACCCGGGTGTGTTCCTTCCA CGGAATCTGTCTGATTCGCATAATGGCCCCACCCGGTAACGTTATGTCACTGTAAAACT
5.1	GGGGCGGGAGGCGCAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTT GTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTAC CCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAG CGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAA GCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGAGACTAGAGTGTGGTAGAGGGTA GCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAG GCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTA GATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACG CAGTATCGAAGCTAACGCGTTAAGTTCGCC
5.2	GGTAAAGGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGT AAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCG TGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAG CGCACGTAGGCGGATTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCT TTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAA

	TTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCT GAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC GATGAATGTTAGCCGTCGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTC CGCCTGGGGAGTACGGTCGCA
6	GATGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAG CTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCC AGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCA CGTAGGCGGATTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGA TACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCG TAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTGACGCTGAG GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GAATGTTAGCCGTCGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTCCGC CTGGGGAGTACGGTCGCAAGA
7	GGGGGGGATGGGGCGCAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGG TTGTAAAGCTCTTTCACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAAC TTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGT AAAGCGCACGTAGGCGGATTTTTAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAAC TGCCTTTGATACTGGAAGTCTTGAGTATGGTAGAGGTGAGTGGAATTCCGAGTGTAGAGGT GAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGACCATTACTG ACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGAATGTTAGCCGTCGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAA CATTCCGCCTGGGGAGTACGGTCGC