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Isolation and characterization of bacterial endophytes for growth promotion of *Phaseolus vulgaris* under salinity stress

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ABSTRACT

Isolation and Characterization of Bacterial Endophytes taken from South African Halophytic Plants for the Growth Promotion of *Phaseolus vulgaris* under Salinity Stress

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As the global human population grows, so does the demand for faster food production rates. Owing to this, agricultural practices have had to expand and move into semi-arid and arid regions, too, where frequent irrigation is essential. However, irrigated ground water contains many salt ions (mainly Na^+ and Cl^-) which contribute to soil salinization on croplands. Soil salinity negatively impacts crop growth and yield and thus, strategies for the alleviation of salt stress on crop plants have had to be developed. This study assessed the use of plant growth promoting bacteria (PGPB).

The aim of this study was to isolate, identify and characterize bacterial endophytes isolated from the halophyte, *Arctotheca calendula*. Endophytes were identified using 16S rDNA and were screened for plant growth promoting properties including nitrogen fixation, phosphate and zinc solubilization, siderophore, ammonia and indole-3-acetic acid (IAA) when exposed to 0 mM, 300 mM and 600 mM NaCl. The endophytes had been identified as *Erwinia persicina* NBRC 102418^T, *Bacillus marisflavi* JCM 11544^T, *Ochrobactrum rhizosphaerae* PR17^T, *Microbacterium gubbeenense* DSM 15944^T and *Bacillus zhangzhouensis* DW5-4^T and all of which had demonstrated some plant growth promoting characteristics. Thereafter, we aimed to demonstrate plant growth promotion of *P. vulgaris* cv. Star 2000 inoculated with PGPB under salinity stress. *P. vulgaris* cv. Star 2000 seeds were inoculated with the PGPB and exposed to 0 mM and 100 mM NaCl. Post-harvest, plants were assessed for their dry mass, cell death, superoxide concentration and nutrient content. It was discovered that salinity negatively impacted *P. vulgaris* cv. Star 2000's dry mass, NaCl-induced cell death, and differentially influenced superoxide concentration, nutrient uptake and content of the leaf and root material in the inoculated and control treatments. However, the isolated PGPB had been able to mitigate the negative effects of soil salinity on *P. vulgaris* cv. Star 2000.

Keywords: Salinity stress, 16S rDNA, PGPB, IAA, *Phaseolus vulgaris, Arctotheca calendula, Erwinia, Bacillus, Ochrobactrum, Microbacterium*

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LIST OF ABBREVIATIONS

ROS	Reactive Oxygen Species
PGPG	Plant Growth Promoting Bacteria
R2A	Reasoner's 2A agar
IAA	Indole Acetic Acid
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
SA	Solubilization Activity

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Plants are immobile organisms, lacking the ability to move from one locality to another. As such, it is essential that they have a broad range of responses to stimuli received from the environment. Plant growth is significantly influenced by abiotic factors (Singh et al., 2018). These include abiotic stresses, such as salinity, extreme heat, extreme cold, drought and many more which are responsible for large reductions in agricultural yields (Kawasaki et al., 2001; Singh et al., 2018). Of these stresses, one faced globally is that of increasing salinity in agricultural systems (Kawasaki et al., 2001; Isayenkov, 2012; Shrivastava et al., 2015; Orhan, 2016; Torche et al., 2018). Numan et al. (2018) estimated that of the 5,2 billion hectares of fertile agricultural land, a total of 50% are immensely impacted by saline stress. Numan et al. (2018) described saline stress as the osmotic forces exerted on plants, with sodium chloride (NaCl) as the main constituent (Yahya, 1998; Isayenkov, 2012; Torche et al., 2018). As the demand for increasing crop yield grows globally, agriculturalists have had to expand their croplands into semi-arid and arid regions (Chaves et al., 2009; Orhan, 2016). As such, the need for intensive irrigation in these regions have increased (Shrivastava et al., 2015; Orhan, 2016), resulting in the secondary salinization of the soils (Chaves et al., 2009).

In semi-arid to arid regions where high evaporative rates and low precipitation rates are commonplace, water used for irrigation often contain a considerably higher concentration of soluble salts (Torche et al., 2018). Soil salinity is affected by soil type and soil composition (Numan et al., 2018). As such, irrigation water can be composed of a number of salt ions including bicarbonates, calcium, carbonates, chlorides, magnesium, sodium, sulphates and potassium (Hu et al., 2005; Numan et al., 2018). Poor water management techniques, such as intensive irrigation and insufficient drainage, increases the concentration of these salt-associated ions in the soil all the more often (Numan et al., 2018; Torche et al., 2018). Salt stress limits plant productivity, metabolism and growth by reducing plant yield, restricting CO_2 diffusion, altering the water potential in the roots (osmotic stress), impairing ionic balance and inducing oxidative damage through the production of reactive oxygen species (ROS) (Kawasaki et al., 2001; Chaves et al., 2009; Orhan, 2016; Torche et al., 2018). As such, highly saline soils pose dire problems for farmers depending on crops for economic benefit and for human populations as food security decreases (Ali et al., 2014; Shrivastava et al., 2015; Numan et al., 2018).

The common bean (*P. vulgaris* L.) is a significant crop species belonging to the legume family, Fabaceae (Kumar et al., 2012; Torche et al., 2018; Padilla-Chacón et al., 2019). It is a staple crop species in low-income countries in Africa, Asia and Latin America (Blair et al., 2003; Kumar et al., 2012; Chekanai et al., 2018; Diana et al., 2018; Padilla-Chacón et al., 2019). It is a glycophytic, herbaceous annual species, which is primarily self-pollinating (Graham et al., 1997). It plays an essential role in agriculture, owing to the symbiotic associations it forms with nitrogen-fixing bacteria in root nodules (Chekanai et al., 2018; Torche et al., 2018). It contains significant amounts of vitamins, minerals, fibre, protein, carbohydrates and phytochemicals (including polyphenolic compounds) which help prevent bodily disorders such as obesity, cardiovascular disease and elevated blood glucose levels (Chekanai et al., 2018; Torche et al., 2018; Chigwedere et al., 2019; Mendoza-Sánchez et al., 2019). Owing to its importance as a global agricultural crop and its sensitivity to saline conditions (Torche et al., 2018), it is essential that strategies for alleviating salinity stress are found for *P. vulgaris L*.

Strategies for the alleviation of salinity stress include growing transgenic plants (Zhu, 2001), breeding of salt-tolerant lines (Yokoi et al., 2002; Chaves et al., 2009; Torche et al., 2018) and the use of plant growth promoting bacteria (PGPB) (Orhan, 2016; Numan et al., 2018; Singh et al., 2018). In this paper, the focus is placed on using PGPB for the mitigation of salt stress in *P. vulgaris L*. PGPB are microorganisms which colonise the rhizosphere or roots of plants which facilitate plant growth either directly or indirectly (Orhan, 2016, Numan et al., 2018). These mechanisms for stimulating plant growth occur through the production of phytohormones, solubilization of minerals and decomposition of organic matter (Orhan, 2016; Numan et al., 2018). PGPB have shown to facilitate plant growth in several important agricultural crops, including cereal and legume crops (Orhan, 2016; Numan et al., 2018). Singh et al. (2018) suggests that the use of PGPB poses a safe, cost efficient and eco-friendly choice for management of agricultural systems. Additionally, Singh et al. (2018) has shown that using PGPB, *Achromobacter piechaudii ARV8*, seedling survival and the fresh and dry weight of tomato (*Solanum lycosperscum L.*) seedlings was enhanced under salt stress up to salinity concentrations of 172 mM. Additionally, Orhan (2016) has shown that under salt stress, *Triticum aestivum*, a wheat species, inoculated with halotolerant and halophilic PGPB displayed increased root and shoot length.

Therefore, this study will aim to provide evidence for increased crop productivity and growth in *P. vulgaris* cv Star 2000. under salt stress, when inoculated with plant growth promoting endophytic bacteria taken from South African halophytic plant species.

1.2 Soil salinity

Global food security is being threatened by the increase in cultivated soils affected by salinity (Etesami et al., 2018). Saline soils play a large role in the decline in global crop production (Ali et al., 2014), with major agricultural crops such as rice, wheat, barley and maize displaying significant declines in crop yield (Shrivastava et al., 2015; Etesami et al., 2018). Agricultural lands, particularly irrigated croplands, are affected by salinity stress (Flowers et al., 1986; Chaves et al., 2009; Ali et al., 2014; Shrivastava et al., 2015). Saline soils are caused by a number of factors, including higher evaporative rates, low precipitation, weathering of sodium-containing rocks and poor irrigation management (Jha et al., 2012). Saline soils are described as soils which have a higher concentration of dissolved salt ions (Jha et al., 2012) and a higher electrical conductivity (EC), where the saturation extract (EC_e) in the rhizosphere surpasses 4 dS m^{-1} at 25°C (Shrivastava et al., 2015). In these soils, the total exchangeable sodium concentrations exceed 15% or 40 mM NaCl (Shrivastava et al., 2015). However, productivity of many glycophytic or salt-intolerant species are reduced at salinity levels a little more than 2 dS m^{-1} (Shrivastava et al., 2015; Orhan, 2016; Torche et al., 2018). Na^+ concentrations above 100 mM severely affect enzymatic activity, particularly those involved in photosynthesis (Chaves et al., 2009). Enzymes dependent on K^+ as cofactor are particularly sensitive to high sodium ion concentrations (Chaves et al., 2009). This poses dire problems for agriculturalists, as most crop species are glycophytic with a low soil salinity threshold (Glenn et al., 1999; Yokoi et al., 2002; Torche et al., 2018).

Salinity on croplands are often the result of secondary salinization, whereby the use of frequent irrigation and poor irrigation management lead to the excess of soluble salt ions in soils (Flowers et al., 1986; Chaves et al., 2009; Shrivastava et al., 2015; Torche et al., 2018). As human populations grow, agricultural practices have had to expand into semi-arid and arid regions to account for the increase in the global demand for food (Flowers et al., 1986; Etesami et al., 2018). Low annual rainfall and high evaporative rates in semi-arid and arid regions drives the need for frequent irrigation in these areas (Shrivastava et al., 2015). The consequence of such frequent irrigation are soils with large deposits of soluble salt ions accumulating in the top layer of the soil (Flowers et al., 1986; Torche et al., 2018). Excessive amounts of salt ions in the soil reduces the plant germination rate, growth rates and crop production by reducing the osmotic potential of the rhizosphere resulting in a water deficit to the plant, ion toxicity in the

plant tissues owing to the excess of Na^+ and Cl^- ions, inducing oxidative stress owing to the production of reactive oxygen species (Singh et al., 2018) and nutrient imbalances as a result of the reduction in nutrient uptake from the roots (Torche et al., 2018). Thus, the need for mechanisms that will improve the salt-tolerance of crop species are necessary.

1.3 Impacts of salinity on nutrient uptake in plants

As most of the world's modern crops are derived from glycophytic plant species (Glenn et al., 1999), understanding the impacts of increasing soil salinity on crop yield and plant mineral nutrition is essential. Not only does highly saline soils decrease the water potential in the rhizosphere (resulting in drought-like, water deficit conditions for the plant), it incurs a nutrient imbalance and Na^+ or Cl^- ion toxicity in the shoots and roots, thus adversely affecting the plant growth (Yahya, 1998; Gorham, 1999; Shannon et al., 1999; Hu et al., 2005; Shrivastava et al., 2015; Numan et al., 2018; Torche et al., 2018). Plant shoots are significantly more sensitive to excess cation ions, such as Na^+, than root material (Yahya, 1998) which is highly problematic as damage to the photosynthetic material decreases plant productivity and crop yield. When soils contain Na+ in excess, it disrupts potassium (K^+) and calcium (Ca^{2+}) ion uptake, thus resulting in potassium and calcium deficiencies as well as sodium toxicity (Yahya, 1998).

1.3.1 Nitrogen

Nitrogen is the main mineral element required by plants (Hu et al., 2005) and makes up 80% of the total minerals taken up by the plant roots (Grattan et al., 1998). It forms the building blocks of many cellular components such as amino acids and nucleic acids, and as such plants deficient in nitrogen often display growth retardation (Hu et al., 2005). Plants take up nitrogen in the form of nitrate, NO_3^-, and ammonium, NH_4^+ (Grattan et al., 1998; Gorham, 1999). Studies looking at crop yield and plant growth on saline soils deficient in nitrogen determined that the addition of nitrogen fertilizer improved plant growth as well as crop yield (Hu et al., 1997; Grattan et al., 1998). However, the efficiency of nitrogen fertilizer on plant growth and yield was the greatest only on soils on the lower salinity spectrum (Ravikovitch et al., 1967; Hu et al., 2005). This has been shown in many crop species including wheat (*T. aestivum* L.) (Soliman et al., 1994), cotton (*Gossypium hirsutum* L.) (Chen et al., 2010), cow peas (*Vigna unguiculate* L.), clover (*Trifolium alexandrinum*), common bean (*P. vulgaris* L.), corn (*Zea*

mays L.), African millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), tomato (*Lycopersicon esculentum*), carrots (*Daucus carota* L.) (Ravikovitch et al., 1967), apple (*Malus pumila*), grape (*Vitis vinifera* L.) and spinach (*Spinacia oleracea* L.) (Grattan et al., 1998).

The interaction between salinity and nitrogen remains highly complex in plant and soils, as the nitrogen ions most abundant in soils, NO_3^- and NH_4^+, respond differently when in the presence of salt ions such as Na^+, Cl^-, Ca^{2+} and K^+ (Grattan et al., 1998; Hu et al., 2005). An increase in soil Cl^- concentration has been shown to decrease shoot NO_3^- concentration and is known as the chloride-nitrate antagonism (Grattan et al., 1998, Hu et al., 2005). This has been shown in tomato (*L. esculentum* L.) (Kafkaki et al., 1982); melon *(Cucumis melo* L.) (Feigin et al., 1987); eggplant (*Solanum melongena* L.) (Savvas et al., 1996) and cucumber (*C. sativus* L.) (Martinez et al., 1989). However, the counter-cation greatly affects the influence that Cl^- has on NO_3^- uptake (Grattan et al., 1998). Kafkaki et al. (1982) demonstrated this in tomato plants where the plants were grown in 100 L containers in a factorial experiment of 4 chlorine: 3 nitrogen: 3 phosphorus. It was found that $CaCl_2$ inhibited NO_3^- uptake in plants at a lower concentration (60 mol/m3), when compared to KCl, which only inhibited NO_3^- uptake between 100 – 200 mol/m3 (Kafkaki et al., 1982). It was inferred that NaCl would respond similarly to KCl, as both counter cations are monovalent ions (Grattan et al., 1998). Additionally, studies such as the one performed by Hu et al. (1998) displayed that salinity may not always impact the total nitrogen concentration within the plant individual, however, the spatial distribution of that nitrogen may be greatly affected. In the study, spring wheat (*T. aestivum* L.) was grown under two salt concentrations, namely 0 mM and 120 mM NaCl, and the spatial distribution of a number of different mineral ions was assessed, including NO_3^- and total nitrogen (N) (Hu et al., 1998). The study determined that total N remained consistently higher along the leaf axis, from the leaf base to tip, in the 120 mM NaCl treatment when compared to the 0 mM NaCl treatment (Hu et al., 1998). Conversely, NO_3^- displayed significantly lower concentrations in the leaf elongation zone (Hu et al., 1998). As such, the inhibition of NO_3^- deposition to the growing leaf tissue resulted in the inhibition of plant growth under 120 mM NaCl treatment (Hu et al., 1998).

The form of nitrogen given to plants determines their sensitivity to salinity too (Grattan et al., 1998). In a study conducted by Flores et al. (2001), investigating the interaction between salinity and ammonium-nitrate fertilizer ratios on the development and nutrition of tomato plants (*L. esculentum* Mill.) in a hydroponic system. Tomato plants were grown at 0 mM, 30 mM and 60 mM NaCl and supplemented with 3 millimolar of ammonium and nitrate at the

ratios 14:0, 12:2 and 10:4 (Flores et al., 2001). Unsurprisingly, the saline treatments decreased plant growth, however, the deleterious effects of the saline treatments were mediated partially by NH_4^+, particularly the ammonium-nitrate fertilizer with higher NH_4^+ to NO_3^- ratios (12:2 and 10:4) (Flores et al., 2001). Shoot fresh weight declined with saline treatments under the 14:0 ammonium-nitrate ratio but increased with the 12:2 and 10:4 fertilizer ratios (Flores et al., 2001). Furthermore, it was revealed that with increasing salinity, NO_3^- in the shoots decreased, whereas NH_4^+ remained fairly constant (Flores et al., 2001), thus supporting the notion that the two nitrogen ions not only respond differently in the presence of NaCl, but that the increasing NH_4^+ increases plant sensitivity to salinity (Grattan et al., 1998; Hu et al., 2005).

However, this study was conducted within a hydroponic system and evidence suggests that plants respond to the supplied nitrogen differently when in different growth mediums (Grattan et al., 1998). An example of this can be seen in a study performed on wheat (*T. aestivum* L.) (Leidi et al., 1991; Silberbush et al., 1991). When grown in a hydroponic system and sand solution, wheat displayed a greater sensitivity to an increased NH_4^+:NO_3^- ratio. However, when grown on salinized soil, wheat appeared more salt-tolerant when assessing its crop yield under NH_4^+:NO_3^- and NO_3^- -only treatment (Shaviv et al., 1991). Shaviv et al. (1991) compared the growth of wheat on sandy loam and clay soils under salt stress (0 g, 3 g, and 8 g NaCl per 3L pot) with different ratios of NH_4^+:NO_3^- (0:100, 25:75 and 50:50). They found that salinity decreased plant dry matter yield, but that the effects were mitigated by the addition of 50:50 mixed NH_4^+ and NO_3^- and that the dry matter yield of the 50:50 mixed treatment was significantly greater than that of the NO_3^- treatments on both non-salinized and salinized growth media (Shaviv et al., 1991). This is further supported by a study performed by Arshad et al. (1999). A pot experiment was conducted on wheat grown in loam soil under saline conditions (0, 6 and 12 dSm^{-1}) supplemented with NH_4^+ and NO_3^- in the ratios, 0:0, 0:100, 25:75, 50:50, 75:25 and 100:0 (Arshad et al., 1999). After the wheat plants reached maturity, the grain and straw yield was measured at the time of harvesting and the plant nitrogen concentration was recorded (Arshad et al., 1999). Additionally, nitrogen uptake and recovery were measured, too (Arshad et al., 1999). The findings of the study showed increased plant tillers, straw and grain yield under a 50:50 NH_4^+ and NO_3^- ratio in comparison to the NH_4^+ only and NO_3^- only treatments under saline stress (Arshad et al., 1999). Furthermore, increasing soil salinity decreased efficient nitrogen utilization; however, nitrogen uptake and nitrogen recovery in wheat was significantly higher in the 50:50 NH_4^+ and NO_3^- treatment, when compared to the NH_4^+ and NO_3^- only treatments (Arshad et al., 1999). Thus, supporting the

notion that in soil, NH_4^+ concentrations equivalent to NO_3^- concentrations improves crop yield and plant growth.

1.3.2 Phosphorus

Phosphorus is a major constituent of nucleic acids, phospholipids, phosphoproteins, dinucleotides, and adenosine triphosphate (Hu et al., 2005). It is necessary for a number of different processes including photosynthesis, enzyme regulation, carbohydrate transportation and both energy storage and energy transfer (Hu et al., 2005). The interplay between salinity and phosphorus (P) is highly complex, as P does not impact all crop plants in a similar manner (Grattan et al., 1998; Hu et al., 2005). The interaction between salinity and P is dependent on the plant species, crop cultivar, plant age, soil salinity, soil P concentrations and other soil composition attributes (Grattan et al., 1998; Hu et al., 2005). In some instances, increasing soil salinity has been linked to plant P deficiency (Shrivastava et al., 2015). However, this is highly species specific (Grattan et al., 1998). Saline soils have been known to reduce P availability owing to ionic strength effects which reduce P activity in the soil (Grattan et al., 1998; Hu et al., 2005). Additionally, P concentrations in soil are highly regulated by sorption activities and by Ca-P mineral interaction (Grattan et al., 1998; Hu et al., 2005). A study by Navarro et al. (2001), melon seedlings were grown in a hydroponic system at 80 mmol/L of NaCl at two concentrations of P namely, 25 µmol/L and 1 mmol/L, in order to assess the effects of salinity on P uptake and translocation. It was found that salinity reduced P uptake in the low P treatment, even though no P-ion Cl^- ion competitiveness was observed (Navarro et al., 2001). In contrast, in the higher P treatment, salinity increased P uptake, even though salinity was found to reduce P flux through the xylem (Navarro et al., 2001). It was hypothesized that at greater saline concentrations, the mobility of P stored in plant vacuoles decreased, thus inhibiting the export of P to the rest of the plant (Navarro et al., 2001). This was found in the study where root and shoot P concentrations were significantly different owing to reduced translocation of P between the two plant parts (Navarro et al., 2001). Additionally, it was concluded that net P uptake rates under saline conditions were dependent on the concentration of P in the growth medium (Navarro et al., 2001). This, too, was found where salinity decreased P uptake when P concentration in the growth solution was low, thus limiting the supply of P to young tissues (Navarro et al., 2001). In contrast, when P concentration in the growth medium

was not limiting, salinity increased P uptake so much so that plants experienced P toxicity (Navarro et al., 2001)

In a study by Champagnol (1979), a total of 17 publications were reviewed and it was concluded that of the 37 crops assessed, P supplementation improved crop growth and yield in 34 of the plant species. However, the same could not be said about the plants' tolerance to salinity, as P supplementation showed either no effect or a reduced tolerance to salt stress in horticultural crops such as carrot, maize, tomato and sugar beet (*Beta vulgaris* L.) (Champagnol, 1979). Yet, a study by Kaya et al. (2001) and Awad et al. (1990) on spinach (*S. oleracea*) cv. "Matador" and tomato plants demonstrated an increased salt tolerance when plants were supplemented with P. Kaya et al. (2001) conducted an outdoor pot experiment using sand cultures. They exposed spinach to 0 mM and 60 mM of salt and supplemented some of the treatments with a foliar spray consisting of 5 mM KH_2PO_4 (Kaya et al., 2001). They found that spinach vegetative growth, seedling growth and chlorophyll concentration was greatly reduced when exposed to the saline solution (Kaya et al., 2001). However, when added the 5 mM KH_2PO_4 foliar spray was used, it mediated the effects of the saline treatment almost entirely, as the plants in the treatments that received it produced similar fresh weights and chlorophyll values as the control – nutrient solution only treatment (Kaya et al., 2001). Additionally, in a study by Awad et al. (1990) performed on tomato plants using a flow-through solution culturing system (NaCl: 10 – 100 mM), the addition of P at concentrations 0.1 to 10 µM to the plants increased the salt tolerance of the plants.

1.3.3 Potassium

Potassium (K) is one of the principal inorganic solutes crucial to plants as they are necessary for protein synthesis, photosynthesis, maintenance of cell membrane integrity and xylem turgor pressure processes for solute transport (Grattan et al., 1999; Hu et al., 2005). Under saline conditions, K^+ uptake is reduced, and K deficiencies increase (Grattan et al., 1999). It has been shown that greater K^+/Na^+ ratios are able to increase plants' tolerance to salinity (Hu et al., 2005). However, under low K^+/N Na^+ ratios, the excess Na^+ disrupts K^+ uptake (Bhivare et al., 1984) through the damage to root membranes, thus altering their ability to select for particular mineral acquisition (Grattan et al., 1999) and reducing plant growth (Loupassaki et al., 2002). Unlike P, higher concentrations of Na^+ ions are almost always associated with reductions in K^+ concentrations in plant tissues (Hu et al., 2005). Loupassaki et al. (2002) conducted a study

assessing the effects of saline stress on the concentration of a number of macro- and micronutrients in the roots, shoots and leaves of six olive cultivars, namely Amphissis, Koroneiki, Megaritiki, Kalamon, Kothreiki and Mastoidis. They assessed the growth of self-rooted year-old plants grown under salt concentrations of 0, 25, 50, 100 and 200 mM NaCl for a period of five months (Loupassaki et al., 2002). In the control treatments, it was found that the mineral elements were significantly more concentrated in the shoots (Ca, Mg, P and Na), with nitrogen and potassium as the only exceptions (Loupassaki et al., 2002). However, in the salt treatments, Na concentrations increased, and K concentrations decreased significantly in all tissue types, so much so that at a mere 25 mM NaCl, in all the tissue types of all the cultivars the K concentration had already reduced to 66.93% of controls (Loupassaki et al., 2002). A similar study by Bhivare et al. (1984) was performed on *P. vulgaris* (L) cv. Vaghya where twelve-day old plants were exposed to salt conditions of 0, 2.5, 5, 7.5 and 10 dS/m NaCl: $CaCl_2$ (1:1) and Na_2SO_4. At the 60^{th}-day mark, the plants mineral elements were analysed, and they found that K concentrations decreased significantly in all plant tissue types in all the salt treatments (Bhivare et al., 1984). Thus, in many plant species grown under saline conditions, a decrease in K uptake can be found which can often lead to K deficiency.

However, other studies have found that the preferential uptake and translocation of K^+ may occur even in the presence of high concentrations of Na^+ (Grattan et al., 1999). This was found in a study conducted by Ruiz et al. (1997) on four different citrus rootstocks namely Cleopatra mandarin, *Citrus macrophylla*, Cleopatra mandarin and sour orange. The rootstocks were grown at five different salt concentrations: 0, 10, 20, 40 and 80 mM NaCl for a period of 20, 40 and 60 days (Ruiz et al., 1997). The leaf and root mineral content were measured, and the findings show that K^+ levels decreased in the leaves and roots of all four rootstocks, except *C. macrophylla*, which displayed a significant increase in its K^+ concentration in the leaves of the plant (Ruiz et al., 1997). Ruiz et al. (1997) suggested that an increase in *C. macrophylla* leaf K^+ may owe to exchanges between Na^+ and K^+ within the basal stem and proximal root area, where the release of K^+ from the root to the xylem facilitated the transport of the mineral to the leaf. However, it is important to note that the whole-plant biomass of all rootstocks were significantly decreased by exposure to NaCl over the experimental time period (Ruiz et al., 1997). Therefore, the effect of salinity on plant K uptake and translocation is highly dependent on plant species.

K^+ uptake is highly reduced under greater soil salinities owing to the antagonism between K^+ and Na^+ at the uptake sites on the root tissue (Grattan et al., 1999; Hu et al., 2005). Yet, plant

potassium requirements increase significantly under saline conditions (Grattan et al., 1999). A study by Chow et al. (1990) provided evidence for increasing K^+ requirements in spinach leaves under 250 mM NaCl conditions, when compared to the lower saline conditions (50 mM NaCl) and the non-saline groups. However, the results for K^+ supplementation on salt stressed plants are contradictory (Grattan et al., 1999). A few studies suggest that the addition of K^+ to the growth medium may increase plant tolerance under salt stress (Grattan et al., 1999). This was seen in a study conducted by Delgadoa et al. (1999) on sunflower plants (*Helianthus annutis* L. cv. Dwarf) grown under saline conditions (0 mM, 50 mM and 100 mM NaCl), either supplemented with additional potassium or not. Their findings show that the addition of K increased plant growth and germination rates of plants grown under 100 mM NaCl conditions (Delgadoa et al., 1999). However, other studies have contradicted these results. Yurtseven et al. (2005) performed a study on a Central Anatolian tomato species (*L. esculentum*) grown under four salinities (0.25, 2.5, 5 and 10 $dS.m^{-1}$) and with three concentrations of supplemented K^+ (0, 5 and 10 $mmol.l^{-1}$). They found that increasing salinity reduced plant yield and fruit size and that the addition of K had no beneficial effect on neither plant yield nor fruit size (Yurtseven et al., 2005). As such, the need for K supplementation to plants under salt stress is variable and highly dependent on plant species.

1.3.4 Calcium

Calcium is an essential nutrient for plants as it controls many physiological processes when plants are growing under stressful environmental conditions (Hu et al., 2005). It regulates a number of different processes involving membrane integrity, solute transport, water movement, cell division, cell-wall synthesis, plant defence and metabolism (Hu et al., 1997; Hu et al., 2005). However, a number of different factors have the ability to limit plant uptake of Ca^{2+} such as the availability of Ca^{2+} in soil, soil pH, counter ions and the ratio of Ca^{2+} to antagonistic cations in the soil (Hu et al., 1997; Grattan et al., 1999). Saline soils in excess of Na^+ ions have a negative effect on plant Ca^{2+} uptake (Gorham, 1999; Grattan et al., 1999) owing to the antagonistic interaction between Na^+ and Ca^{2+} at root uptake sites and transport in the xylem (Hu et al., 1997; Hu et al., 2005; Maas et al., 1999). Under high soil Na^+: Ca^{2+} ratios, Ca^{2+} uptake is significantly reduced owing to increased precipitation, Na^+ replaces Ca^{2+} at extracellular binding sites of cells (Hu et al., 2005) and it inhibits the transport of Ca^{2+} to the meristematic regions in growing leaves often resulting in the plant developing a calcium

deficiency (Maas et al., 1999). This was displayed in a study by Loupassiki et al. (2002) where six olive cultivars were exposed to varying salt concentrations (0 – 200 mM NaCl) and they found that for the root, young and mature leaf tissue types, Ca^{2+} dropped significantly when compared to the control for each tissue type. A study performed by Bernstein et al. (1995) exhibited a similar result. Sorghum (*Sorghum bicolor* [L.] Moench, cv. 'NK 265') was grown in a Hoagland nutrient solution in addition to being exposed to 1 or 100 mM NaCl (Bernstein et al., 1995). The spatial distribution of Ca was determined 24 hours after the emergence of leaf 6 and they found that salinity significantly reduced leaf Ca concentrations, with the basal area containing of the leaf 25% of the Ca found in the control treatment and the distal area containing 40% of the Ca found in the control treatment (Bernstein et al., 1995). A lot more work performed in this area supports the above-mentioned studies, thus attesting to the fact that saline soils have the potential to induce calcium deficiencies in many crop species.

Globally, the number of croplands affected by saline stress continues to increase, thus increasing the likelihood of more and more crops likely to be affected by Ca deficiency disorders (Grattan et al., 1999). A few studies have been performed assessing the viability of Ca supplementation for plants grown on saline soils. Kaya et al. (2002) demonstrated the benefits of Ca supplementation in strawberries (*Fragaria* x *ananassa* Duch) cvs. 'Oso Grande' and 'Camarosa'. The experiment consisted of three treatments, namely: 1) nutrient solution, 2) nutrient solution with 35 mM NaCl and 3) nutrient solution with the addition of 35 mM NaCl and 5 mM $CaCl_2$ (Kaya et al., 2002). Their findings show the NaCl decreased plant dry weight, fruit yield, chlorophyll content and water usage (Kaya et al., 2002). They also found an increase in membrane permeability associated with saline conditions (Kaya et al., 2002). Interestingly, they found that the addition of Ca to the nutrient solution ameliorated the negative effects of NaCl on plant growth, water usage, fruit yield and membrane permeability (Kaya et al., 2002). Similar results were found in tomato (*L. esculentum* Mill.) cv. "Target F1 (Tuna et al., 2007), soybean (*G. max* L. Merill cv. Fukuyutaka) and cucumber (*C. sativus* L. cv. Choujitu Ochiai Nigou) (Ikeda et al., 2005). However, the benefits of Ca supplementation are highly species and crop cultivar specific (Grattan et al., 1998). Schmidt et al. (1993) found that supplemented Ca (10 mM) did not improve the growth of whole plants and calluses of *Brassica* species grown under saline conditions (8 $ds.m^{-1}$ NaCl). As such, the benefits of calcium supplementation are limited to a number of crop species as not all species respond in the same fashion with the same beneficial outcomes.

1.3.5 Magnesium

Unlike the above-mentioned mineral nutrients, little studies have been conducted on the relations between magnesium (Mg) and soil salinity in crop plant species (Grattan et al., 1999; Hu et al., 2005). However, it is known that the competitiveness between Ca_2^+ and Mg_2^+ for the binding sites along the root plasma membrane is very strong, with Ca_2^+ having a higher affinity than that of Mg_2^+ (Grattan et al., 1999). As such, when the soil substrate consists of high Ca_2^+ concentrations, it is often associated with greater leaf Ca levels and a significant decrease in leaf Mg concentrations (Grattan et al., 1999). Loupassaki et al. (2002) studied the impact of NaCl salinity on magnesium concentrations in the leaf, shoot and root tissues of six olive cultivars. They found that increasing salinities were associated with a marked reduction in root, shoot and leaf magnesium concentrations in all cultivars (Loupassaki et al., 2002). A study conducted by Carvajal et al. (1999) on tomato plants (*L. esculentum*, cv. Mill) grown at different NaCl (1, 20 and 60 mM) and Mg concentrations (0.5, 2.0, 5.0 and 10 mM). Fruit yield, water relations and mineral composition was assessed following the growth period (Carvajal et al., 1999). They found that under non-saline (1 mM NaCl) conditions and saline conditions (20 mM and 60 mM NaCl) fruit yield was reduced at Mg concentrations of 1 mM and 5 – 10 mM, respectively (Carvajal et al., 1999). It was concluded that this owed to the influence of NaCl and Mg on water relations, toxicity and plant nutrient imbalances (Carvajal et al., 1999). As such, the effects of salinity on Mg in plants are highly dependent on the crop cultivar and crop plant species.

1.3.6 Sulphur

Sulphur (S) is a plant nutrient vital to plant growth and development, playing an integral role in the formation of several vitamins, co-enzymes and phytohormones (Nazar et al., 2010). In many organic molecules, such as protein and non-protein molecules, S is incorporated into thiol groups (-SH) (Nazar et al., 2010). However, studies examining the influence of NaCl salinity on plant sulphur nutrition are far and few between (Hu et al., 2005), with most of the focus directed at the comparison of plant response to either sulphate-salinity or chloride-salinity (Grattan et al., 1999). This was often done by exposing different crops to chloride and sulphate salinities with equivalent electrical conductivities, isosmotic potentials or molarities (Grattan et al., 1999). Manchanda et al. (1982) conducted a study on the P requirement of Barley whilst in the presence of chloride and sulphate salts. Their findings suggest that the P

requirement of the barley plants were significantly higher when in the presence of chloride salts in comparison to that of the sulphate salts (Manchanda et al., 1982). In 1962, Bernstein proposed that a great number of vegetable crops were more tolerant of sulphate salts than compared to chloride salts. Furthermore, a study by Mor et al. (1992) displayed the effects of sulphate and chloride salinities on pea sulphur content. They found a reduction in straw sulphur content under the chloride salinity, but an accumulation of S in the roots (Mor et al., 1992). This suggests that sulphate salts are less detrimental to plant growth and development in comparison to chloride salts (Mor et al., 1992; Grattan et al., 1999).

1.3.7 Micronutrients

In recent years, there has been a significant spike in the literature assessing the role of micronutrients in crop production, owing to the greater accessibility to improved analysis techniques and a greater amount of knowledge about the functions of micronutrients in crop plant species (Fageria, 2001). Plant micronutrients include copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo) and zinc (Zn) (Grattan et al., 1999). The availability of micronutrients in the soil is highly dependent on soil pH, pE, the concentration of micronutrients within the soil solution, the organic matter content, the plant species growing within a given region, the composition or type of salts present and the concentration of salts in the soil (Grattan et al., 1999; Fageria et al., 2011). As such, it is rather difficult to ascertain how saline soils may affect the solubility and availability of micronutrients to plants, as plants may experience micronutrient deficiencies, toxicities or may be unaffected completely (Grattan et al., 1999; Fageria, 2001; Fageria et al., 2011). Under saline conditions, barley shoots (*Hordeum vulgare* L.) experience Mn deficiency which has been mediated with the addition of Mn to the soil solution (Grattan et al., 1999; Fageria, 2001). Additionally, salinity induced Mn deficiency in plant shoots have been demonstrated in bean, corn (Fageria, 2001), pea, squash (*Cucurbita pepo* L.) and tomato (Grattan et al., 1999). However, these studies did not examine the effects of added Mn to the growth medium (Grattan et al., 1999, Fageria, 2001). In contrast, Khattak et al. (1989) have found that the addition of NaCl and $CaCl_2$ to the soil medium, increased the Mn available to sugar beet (*B. vulgaris* L. USH 11), thus increasing the Mn content within the shoots of sugar beet (Grattan et al., 1999). The greater part of the literature has provided evidence for salinity-induced increases in shoot Zn content, as seen in bean, citrus, maize (Grattan et al., 1999, Fageria, 2001) and tomato (Al-Karaki, 2000) studies. However, sodicity

imposes greater pressures on Zn availability to plants than salinity does, as it has been demonstrated that shoot Zn content decreases significantly with increasing sodicity (Grattan et al., 1999; Fageria, 2001). Yet, other studies have shown that saline soils reduce plant Zn, as seen in cucumber (Al-Harbi, 1995), whilst others demonstrated no effect at all (Izzo et al., 1991). The addition of Zn to soil have been found to improve plant growth on saline soils, but better results have been found for Zn applications to sodic soils (Grattan et al., 1999; Fargeria, 2001). As incongruous as the salinity studies are for Mn and Zn, so is the research for the influence of saline soils on Fe uptake in plants (Grattan et al., 1999; Fageria, 2001). Saline soils have shown to increase the uptake of Fe in pea shoots (Dahiya et al., 1976), squash (Maas et al., 1972), bean (Bhivare et al., 1984) and tomato (Al-Karaki, 2000); however, the concentration of Fe in barley and corn shoots decreased with increasing salinity (Grattan et al., 1999).

Very little research has been dedicated to the effect of soil salinity on Cu and Mo uptake, and the studies that are available demonstrate how inconsistent the results may be (Grattan et al., 1999; Fageria, 2001). Hassan et al. (1970) studied the effects of salinity on nutrient uptake and distribution in barley and corn and found that increasing salinity decreased Cu levels in both crop species. However, the opposite has been found in hydroponically grown tomatoes, where salinity induced an increase in the leaf Cu concentrations (Grattan et al., 1999). Furthermore, Rahman et al. (1993) found that increasing salinity was positively correlated to Mo concentrations within the maize crops when grown in salt treated soils. Yet, other studies such as that conducted by Izzo et al. (1991), have found that increasing salinity in solution cultures induced no change in Mo concentrations within maize. Thus, it is clear that the effects of salinity on plant micronutrient concentrations and plant micronutrient uptake is uncertain and highly dependent on the mineral nutrient, soil pH, pE, type of salt and plant species.

1.4 Effects of salinity on plant hormone regulation

Crop plants are often subjected to a number of environmental conditions that may be detrimental to plant growth and yield, including drought, saline soils and saline irrigated waters (Kaya et al., 2009). A plant's ability to withstand or tolerate salt stress amasses a number of mechanisms, including genetic, physiological and biochemical systems (Babu et al., 2012). Phytohormones are endogenous organic growth regulators produced in various areas within the plant, from where it is translocated to regions where it is necessary (Kaya et al., 2009; Iqbal et

al., 2014). These hormones often occur at low concentrations, however, even at low levels they are able to induce physiological response (Kaya et al., 2009). These physiological responses include the regulation of plant growth, germination, metabolism and mechanisms necessary for the tolerance of environmental stressors (Iqbal et al., 2014). To alleviate biotic or abiotic stressors acting upon the plant, many phytohormones are up or downregulated (Javid et al., 2011). Thus, these phytohormones play a significant role in response to external stressors (Javid et al., 2011).

1.4.1 Gibberellic Acid

Gibberellins (GA) are plant hormones involved in plant growth, development, flowering, stem elongation leaf expansion and it controls the germination times of seeds (Maggio et al., 2010; Javid et al., 2011; Iqbal et al., 2014). Gibberellins or gibberellic acid metabolism and signalling is tightly controlled by the homeostasis of GA (Javid et al., 2011). Yet, the mechanism behind this homeostasis remains largely unclear (Javid et al., 2011). Furthermore, there is longstanding knowledge that GA and other plant hormones are constantly in a state of cross-talk, whereby the interactive signalling between GA and the other plant hormones control the development and growth of plants (Maggio et al., 2010; Javid et al., 2011).

DELLA proteins have been suggested to act as transcriptional regulators of GA, as they play a significant role in the negative regulation of GA signalling (Javid et al., 2011; Fahad et al., 2015). Additionally, there is a definitive correlation between GA and DELLA proteins with regards to the survival of plants grown in saline conditions (Fahad et al., 2015). The DDF1 (dwarf and delayed flowering 1) gene is induced when plants are experiencing salt stress, and it reduces plant growth in part through decreased GA levels, thus improving plants tolerance to salinity (Magome et al., 2008; Fahad et al., 2015). In *Arabidopsis*, bioactive GA is reduced when plants are grown in a high-salt environment, resulting in the increase of DELLA proteins which promotes plant growth retardation in saline conditions (Magome et al., 2008).

With regards to plant response to abiotic and biotic stressors, a lot of research has been dedicated to GA regulation under stress (Kaya et al., 2009; Javid et al., 2011). When plants are experiencing any biotic or abiotic stressors, GA accumulates rapidly (Kaya et al., 2009; Javid et al., 2011). It is known that GA is involved in plant salt stress response (Iqbal et al., 2014), by alleviating the effects of salinity on plant water use efficiency by improving plant water

relations (Fahad et al., 2015). Maggio et al. (2010) found that GA application to tomato plants grown under saline conditions improved stomatal resistance and increased plant water use at lower salinities. Additionally, their findings demonstrated that under supplementary GA, crop growth and yield increased even under salt stress (Maggio et al., 2010). A similar result was found in a study performed by Prakash et al. (1990), whereby the application of exogenous GA to salt stressed rice (*Oryza sativa* L. var GR-3) reduced the accumulation of Na^+ and Cl^- ions, it increased chlorophyll production and improved the growth and yield of the rice crops. Thus, proving that the application of exogenous GA may be beneficial to crops grown under saline conditions (Kaya et al., 2009; Fahad et al., 2015).

1.4.2 Abscisic Acid

Abscisic acid (ABA) is an important plant hormone which is thought to be involved with plant stress responses, including drought and salt stress (Javid et al., 2011; Iqbal et al., 2014; Fahad et al., 2015). ABA regulates seed maturation, dormancy and responses to abiotic stressors (Gurmani et al., 2013; Fahad et al., 2015). Additionally, ABA is able to induce drought and salt tolerance by means of controlling leaf expansion and initiation as well as stomatal movement (Gurmani et al., 2013). When plants are exposed to saline conditions, an increase in plant ABA concentrations can be found and is likely accompanied by an increase in leaf and soil water potential (Javid et al., 2011; Fahad et al., 2015). This is done by ABA-mediated signalling through the expression genes sensitive to salt, as well as regulating plant water status (Fahad et al., 2015). Increasing concentration of ABA is considered essential for plants grown in saline soils as they play a significant role in protective measures and mechanisms to avoid saline-induced damage (Fahad et al., 2015). An example of this can be seen in a study by Mäkelä et al. (2003) where both ABA-deficient mutants (*sitiens*) and wild-type cultivars (Rheinlands Rhum) of tomato were exposed to moderate saline conditions under moderate and high relative humidity. They found that at both moderate and high relative humidity, the degree of injury to the older leaves in *sitiens* was always significantly larger than the Rheinlands Rhum cultivar, which sustained no visible injury in the older leaves (Mäkelä et al., 2003). These results displayed the importance of ABA in the maintenance and preservation of older plant tissues when under saline stress (Mäkelä et al., 2003). These results are mirrored in conducted by Mulholland et al. (2003) on ABA-deficient mutants (*notabilis*) and wild-type (Ailsa Craig) genotypes of tomato plants grown under a range of salinities. They found that leaf area reduced

and root:shoot ratio and ABA concentrations increased under rising salinity (Mulholland et al., 2003). However, under moderate salinity (90 mM NaCl), higher ABA concentrations in the wild-type genotype ameliorated the impact of salinity on leaf area and root growth so much so that it exhibited near control levels; whereas in the ABA-deficient mutant, considerably low leaf areas remained (Mulholland et al., 2003). Thus, these findings suggest that under moderate salinities, higher ABA concentrations are able to mitigate some of the effects of salinity on leaf and root development (Mulholland et al., 2003).

It had been reported that the application of exogenous ABA has the capacity to mitigate many of the negative effects of salt stress (Fahad et al., 2015). This is done by delaying the deleterious impacts of NaCl, such as Cl^- ion accumulation, as seen in citrus leaves, in so doing inhibiting ETHY release and leaf abscission under salt stress (Gomez-Cadenas et al., 2002; Fahad et al., 2015). Additionally, a study led by Gurmani et al. (2013) demonstrated the effects of ABA seed pre-treatment on the growth of rice (*O. sativa* indica) grown under saline conditions. ABA treatment reduced Na^+ concentrations in the leaf sheaths and blades, increased the K^+/Na^+ ratio, increased the net assimilation rate as well as the stomatal conductance of salt stressed rice seedlings (Gurmani et al., 2013). Thus, demonstrating the potential for enhancing seedling tolerance to salinity by means of ABA seed pre-treatment (Gurmani et al., 2013).

As priorly mentioned, ABA, in its most generic form, is upregulated when plants are experiencing salt stress (Javid et al., 2011; Fahad et al., 2015). When salinity-induced upregulation of ABA occurs, it induces the expression of genes involving salt and osmotic amelioration (Javid et al., 2011; Fahad et al., 2015). Shi et al. (2002) performed a study demonstrating the effects of salt stress and ABA on the expression of the *A. thaliana AtNHX1* gene, which encodes for the vacuolar Na^+/H^+ antiporter that is essential for tolerating salt stress. Their findings established that both salt and ABA induces the upregulation of *AtNHX1* at a transcriptional level (Shi et al., 2002). Thus, it suggests a complex interplay, whereby saline stress stimulates *AtNHX1* expression transcriptionally, but the upregulation is in part also reliant on the biosynthesis of ABA and ABA signalling (Shi et al., 2002). Additionally, Fukuda et al. (2006) examined the influence of ABA on the expression of the genes *HvVHA-A* (involved in the catalytic subunit, subunit A, of vacuolar H+ - ATPase, EC 3.6.1.3) and *HVP1* and *HVP10* (involved in vacuolar H+-inorganic pyrophosphate, EC 3.6.1.1) in barley (*H. vulgare* L.) grown under salt stress. This was done by means of transcript level quantification. Both H^+-inorganic pyrophosphatases and H^+ - ATPases found in the vacuolar membranes translocate H^+ from the cytosol into the vacuoles (Fukuda et al., 2006). This is essential,

particularly for salt stressed plants (Fukuda et al., 2006). Their findings demonstrated that ABA treatment stimulated Na^+/H^+ antiport activity, increased the translocating activities by means of H^+-PPase and H^+-ATPase as well as increasing the amount of V-PPase protein of the tonoplast vesicles (Fukuda et al., 2006). Furthermore, these findings suggest that ABA plays a significant role in regulating the expression of the genes encoding for the H^+-pump and Na^+/H^+ antiport, thus signifying its important role in the expression of genes essential for salt stress mitigation (Fukuda et al., 2006).

1.4.3 Indole Acetic Acid

Within the realm of plant hormones, Indole acetic acid (IAA) was the first plant hormone to be identified (Fahad et al., 2015). It belongs to the class of hormones called auxins and it plays a significant part in regulating plant growth and development (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). These processes involve cell elongation, development of vascuolar tissue and apical dominance (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). IAA has also been linked to plant growth response when stressed (Fahad et al., 2015). Though, little literature can be found assessing the relationship between plant auxin levels and salinity stress, variations in IAA content under stressful conditions have demonstrated similarities to that of ABA (Kaya et al., 2009), with greater concentrations of IAA being associated with reduced plant growth (Javid et al., 2011). This increase in IAA under abiotic stress suggests variations in plant hormone homeostasis (Kaya et al., 2009; Fahad et al., 2015). Veselov et al. (2008) provide evidence for this in their study on maize (*Z. mays* L.) grown with a saline nutrient solution. They assessed the expression of the expansin gene, *ZmEXPA1*, and leaf growth of maize and found that auxins accumulated in the maize leaves under salinity treatment (Veselov et al., 2008). Furthermore, they displayed that the application of exogenous IAA enhanced *ZmEXPA1* gene expression (Veselov et al., 2008). However, others have found the opposite, with IAA levels declining with rising salinity (Kaya et al., 2009; Javid et al., 2011). Thus, different plant species may have varying IAA responses in the presence of a saline environment.

1.4.4 Cytokinins

Cytokinins (CK) are phytohormones that play an integral role in plant growth and development procedures (Fahad et al., 2015). These include flowering (Kaya et al., 2009), apical dominance, cell division, leaf senescence, chloroplast biogenesis, shoot differentiation, vascular differentiation nutrient mobilization, photo-morphogenic development and anthocyanin production (Javid et al., 2011; Fahad et al., 2015). It is able to enhance plants' tolerance to saline conditions and high temperatures (Javid et al., 2011; Fahad et al., 2015). This is done through the antagonistic effect of CK on ABA (Iqbal et al., 2014) and the IAA synergistic/antagonistic effect in different plant processes (Iqbal et al., 2006; Fahad et al., 2015). The antagonistic relationship between ABA and CK is due to the opposing effects that either have on plant developmental processes such as cotyledon expansion, stomatal opening and seed germination (Javid et al., 2011). An early response seen in plants after exposure to saline conditions is the decrease in CK concentration (Fahad et al., 2015). In a study conducted by Walker et al. (1981), they exposed tomato plants to salt stress in a balanced nutrient solution. They discovered that by day two, salt stress was accompanied by a peak in ABA and zeatin riboside (CK), but a decline in cis and trans zeatin (CK) (Walker et al., 1981). Thereafter, a sharp decline in ABA was found and even when moved to a non-stressful environment, ABA levels remained low (Walker et al., 1981). However, zeatin levels rose significantly and plant growth continued normally after being moved to a non-stressful environment (Walker et al., 1981).

Exogenous applications of CK have demonstrated its importance in a number of different plant growth and developmental processes (Javid et al., 2011). CK application has the capacity to reverse leaf and fruit abscission due to ABA or osmotic stress (Fahad et al., 2015). Mathew et al. (1995) displayed significant increases in rice yield when plants were supplied with exogenous CK. Additionally, priming seeds with CK has been shown to improve plant salinity tolerance (Iqbal et al., 2006). In functional assays assessing CK stress responses in plants, it was found that all three CK receptors in *Arabidopsis* perform a negative regulatory effect in ABA signalling as well as in osmotic stress responses (Tran et al., 2010; Javid et al., 2011; Fahad et al., 2015). However, even though it is known that CK is involved in osmotic stress responses, the mechanism behind this is not well understood (Tran et al., 2010; Javid et al., 2011; Fahad et al., 2015).

1.4.5 Jasmonates

The term jasmonates is used collectively for jasmonic acid (JA) as well as methyl jasmonates (MeJA) (Javid et al., 2011; Iqbal et al., 2014). JA plays a pivotal role as a cellular regulator and is involved in a number of plant developmental processes (Javid et al., 2011; Fahad et al., 2015). These include seed germination, fertility, root growth, senescence, fruit ripening (Javid et al., 2011), callus growth, flowering, formation of gum and bulb (Fahad et al., 2015). Recently, they have received quite a bit of attention owing to their capacity for plant protection under saline conditions, by means of plant signal transduction (Iqbal et al., 2014). Jasmonates are involved in plant defence responses to both biotic and abiotic stressors (Fahad et al., 2015), including insect-driven wounding, pathogen infections, drought, salinity and low temperatures (Kaya et al., 2009; Javid et al., 2011). In 1993, Sembdner et al. reported that plant wounding or pathogenic organisms can induce JA synthesis by means of the production of fatty acids by cell membranes, which metabolizes through lipoxygenase to JA. Additionally, abiotic stress has also been associated with the synthesis of JIP proteins which become abundant when JAs are synthesized (Sembdner et al., 1993; Moons et al., 1997). Wang et al. (2001) demonstrates the influence of salinity on JA production in *Iris hexagona*. They found that JA increased in response to salinity in the leaves, stalks, fruits and seeds of *I. hexagona* (Wang et al., 2001). Additionally, Walia et al. (2007) found a substantial overlap between saline-responsive genes and JA-regulated genes (arginine decarboxylase, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase and apoplastic invertase) in barley (*H. vulage* L.). Furthermore, Pedranzani et al. (2003) displayed that steady-state levels of JA in salt-tolerant tomatoes (cv. Pera) were higher than in salt-sensitive tomatoes (cv. Hellfrucht Frühstamm (HF)). Thus, demonstrating that varying endogenous JA levels in response to salt stress differs among genotypes with opposing saline-tolerant capacities (Pedranzani et al., 2003).

The exogenous application of JA has been associated with increased tolerance to salinity (Kaya et al., 2009; Javid et al., 2011). Walia et al. (2007) performed a study on barley, *H. vulgare* L., pre-treating plants with JA before exposing them to salt stress. They found that pre-treated plants accumulated low levels of sodium ions in the shoots when compared to the untreated treatment (Walia et al., 2007). Additionally, pre-treatment somewhat mitigated the effects of salinity on photosynthetic inhibition (Walia et al., 2007). A similar result was found in a study by Tsonev et al. (1998) conducted on barley seedlings exposed to salinity. JA pre-treatment for 4 days before exposing seedlings to salinity decreased the negative impacts of high salinity on plant growth and photosynthesis (Tsonev et al., 1998). Thus, JA in the presence of salinity has demonstrated its protective function in plants.

1.4.6 Brassinosteroids

Brassinosteroids (BR) comprise of a group of novel steroidal phytohormones which consist of brassiniloide, castasetrone and a number of their derivatives (Bajguz et al., 2009; Fahad et al., 2015). These phytohormones have properties beneficial for plant growth and development (Javid et al., 2011; Fahad et al., 2015) including seed germination, pollen tube growth, vascular differentiation, leaf bending and epinasty, photosynthesis, reproductive growth, flowering, senescence, fruit production and leaf abscission (Bajguz et al., 2009; Javid et al., 2011; Fariduddin et al, 2014; Fahad et al., 2015). They are able to mitigate the negative impacts of salinity on plant growth (Iqbal et al., 2014; Fahad et al., 2015). An example of this was determined in a study conducted by Anuradha et al. (2001) on rice seedlings (*O. sativa* L.) grown under saline conditions with or without the application of 24-epibrassinolide and 28-homobrassinolide. Their findings demonstrated the benefits of BR supplementation, as it was able to reverse the inhibitory impacts of salinity on seed germination and seedling growth (Anuradha et al., 2001). Additionally, an increase in nucleic acids soluble proteins was associated with BR treatment under salinity (Anuradha et al., 2001). Additionally, a study conducted on maize, *Z. mays* L., grown under salinity stress, with or without the application of 28-homobrassinolide, was performed and seedling growth, lipid peroxidation and antioxidative enzyme activity was assessed (Arora et al., 2008). It was found that the 28-homobrassinolide treatment reduced the toxic effects of salinity on seedling growth (Arora et al., 2008). Furthermore, the 28-homobrassinolide treatment reduced oxidative damage and improved antioxidative enzyme activity in comparison to the saline-only treatment (Arora et al., 2008). Thus, BR have a protective function in plants, allowing them to tolerate salinity.

1.4.7 Triazoles

Triazoles (TR) are a known group of compounds which are used as fungicides and plant growth regulators (Fletcher et al., 2000; Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). TRs are able to protect plants from both biotic and abiotic stresses (Fletcher et al., 2000; Fahad et al., 2015). TRs have been shown to protect plants from a number of environmental stresses, such as anoxia, drought, extreme temperatures, air pollutants, salinity and ultra-violet light (Kaya et al., 2009; Javid et al., 2011). Jaleel et al. (2008) assessed the effects of salinity and

propiconazole (a group of triazoles with fungicide and plant growth regulating properties) on the Madagascan periwinkle (*Catharanthus roseus*). Their findings provided evidence for the mitigation of salt stress in *C. roseus*, as propiconazole increased plant root length, dry and fresh weight as well as the antioxidant activity of superoxide dismutase (SOD), polyphenol oxidase (PPO) and peroxidase (POX) (Jaleel et al., 2008). A similar result was found in a study conducted by Jaleel et al. (2007) on *C. roseus* grown under salt stress and the plant growth regulating triazole, paclobutrazol. Their results provide evidence for increased salt tolerance under paclobutrazol treatment, as the triazole treatment improved components of antioxidant activity (Jaleel et al., 2007). Triadimefon treatment in peanut seedlings (*Arachis hypogaea*) mitigated the effect of salinity on plant dry weight, root growth, carotenoid and chlorophyll production and protein and glycine betaine contents (Muthukumarasamy et al., 1997). Additionally, it decreased proline accumulation, proline oxidase concentrations and ATPase (Muthukumarasamy et al., 1997). Hexaconazole decreased the inhibitory effects of salinity in canola plants (*Brassica napus* L.) by increasing root and shoot growth, plant dry weight, chlorophyll and protein content and antioxidant enzymatic activity (Akbari et al., 2011). Therefore, triazoles are able to significantly improve plants' salt tolerance.

1.4.8 Salicylic acid

Salicylic acid (SA) is a phenolic component found in plants (Iqbal et al., 2015) which has growth regulatory properties (Fahad et al., 2015). SA is involved in physiological processes including plant growth, flowering, ethylene production, nitrate metabolism and plant defences to abiotic and biotic stressors (Fahad et al., 2015). Additionally, its role in seed germination, fruit yield, glycolysis, stomatal conductance, ion uptake, ion transport, transpiration, nodulation, senescence and thermo-tolerance is rather evident (Javid et al., 2011). In the presence of biotic and abiotic stresses, SA improves plants' tolerance to the stresses by stimulating the antioxidant defence system, as seen in salinity studies (Iqbal et al., 2015). This was demonstrated in a study by Karlidag et al. (2009) on strawberry (*Fragaria x ananassa Duch*.) plants grown under saline conditions (0 and 35 mM NaCl) with or without exogenous application of SA (0.0, 0.25, 0.50 and 1.00 mM). Salinity reduced plant growth, chlorophyll content and plant mineral uptake (Karlidag et al., 2009). However, strawberry plants treated with SA had higher fresh and dry weights as well as greater chlorophyll contents when compared to the saline only treatments (Karlidag et al., 2009). Additionally, leaf water relative

content (LWRC) decreased under saline stress, whilst electrolyte leakage increase, whereas in the SA treatments, LWRC was significantly higher than the salt stress treatments as well as electrolyte leakage which was significantly reduced under SA treatment (Karlidag et al., 2009). These results suggest that the negative effects of salt stress on strawberry plants may be mitigated by the application of exogenous SA (Karlidag et a., 2009). A similar result was seen in wheat plants, where SA application reduced the negative effects of salt stress on seedling growth and development (Sakhabutdinova et al., 2003). Additionally, Sakhabutdinova et al. (2003) found that SA treatment reduced variations in plant phytohormone levels in salt stressed wheat. Wheat treated with SA displayed no decreases in IAA and CK levels, thus reducing the likelihood of stress-induced plant growth inhibition (Sakhabutdinova et al., 2003). Salt stressed maize plants (*Z. mays* L.) displayed significant reductions in plant shoot and root lengths, shoot and root fresh and dry weights, leaf area, Rubisco activity, sugar contents, photosynthetic efficiency and pigments (Khodary, 2004). However, the exogenous application of SA thwarted the negative effects of NaCl on maize, by enhancing plant root and shoot lengths, fresh weights, dry weights and leaf area. Additionally, SA treatment was able to improve plant Rubisco and photosynthetic activity under salt stress (Khodary, 2004). Thus, SA has the potential to mitigate the negative effects of salt stress on plant growth and metabolism.

1.5 Glycophytic versus Halophytic plants

On agricultural lands in arid and semi-arid regions, secondary salinization owing to poor irrigation management is a growing concern particularly in the face of climate change and global warming (Horie et al., 2012). This poses particular threats towards food security in countries which experience frequent droughts and high temperatures, which promote the need for extensive irrigation and the likelihood of secondary salinization (Etesami et al., 2018). Exposure of plants to saline soils may impact plant growth, crop yield and under extreme conditions, plant mortality (Kosová et al., 2013). A considerably large amount of crop plants is derived from a group of plants known as glycophytes (Glenn et al., 1999). Glycophytic (salt-sensitive) and halophytic (salt-tolerant) plants are plants which differ in their tolerance to soil salinity, where glycophytic plants lack the genetic basis for tolerating high salinities as compared to halophytic plants (Horie et al., 2012).

A lot of uncertainty remains regarding the true definition of halophytic plants which make up only 1% of the global flora (Flowers et al., 2008). Flowers et al. (2008) suggests the idea of

"natural halophytes", whereby the plant species are capable of completing their life cycles at 200 mM NaCl and under similar conditions to that encountered in their natural habitats (Kosová et al., 2013). This allows for the categorical separation of true or natural halophytes from other plant species that are able to tolerate higher concentrations of salt, which do not naturally inhabit saline environments (Flowers et al., 1986; Flowers et al., 2008). Additionally, halophytes can be divided into obligate or facultative halophytes, whereby the former is dependent on highly saline conditions to function optimally, and the latter which does not require high salinity to complete its life cycle (Mucina et al., 2005). Furthermore, halophytic species have developed a number of physiological adaptations which have allowed them to occupy saline environments and often these species can be categorised by 'physiotype' (Mucina et al., 2005). These physiotypes are seen as strategies used to cope in an environment with high salt concentrations (Mucina et al., 2005).

Naturally occurring halophytic plant species can be found in coastal areas (rocky headlands, sand dunes, beaches), salt marshes as well as estuarine environments (Rozema et al., 1985; Mucina et al., 2005). The osmotic, ionic (Na^+, Cl^- and SO_4^{2-}) and oxidative stresses seen in glycophytic plants after exposure to high soil salinities are avoided in halophytic species owing to a number of different mechanisms which protect the plant from increasing salinities (Jha et al., 2012). These include the physiological adaptations (Mucina et al., 2005), expression of specific genes (Wilson et al., 2005; Jha et al., 2012), the meticulous uptake and compartmentalization of sodium (Na^+), potassium (K^+) and chloride (Cl^-) ions (Flowers et al., 2008; Etesami et al., 2018) as well as the synthesis of organic compatible solutes (Flowers et al., 2008).

1.5.1 Ecophysiological adaptations

Halophytic plants may employ a number of different strategies which allow them to tolerate environments with high salinities (Mucina et al., 2005). These strategies or physiotypes include (1) 'salt regulators', (2) 'salt accumulators' and (3) 'root salt excluders' (Mucina et al., 2005). 'Salt regulators' have the capacity to control the salt concentrations within the internal tissues by means of sequestering the salt ions to salt glands or by increasing the succulence of the leave and stem tissues, thereby desalinizing the internal salt concentrations of the plants (Mucina et al., 2005). *Aegialitis*, a genus belonging to the Plumbaginaceae family of mangroves, regulates leaf salt concentrations by excreting excess salt ions through salt glands on the abaxial leaf

surface (Atkinson et al., 1967). Another strategy utilised is the accumulation of salts (Mucina et al., 2005). 'Salt accumulators' are unable to regulate the continuous influx of salt and as such have adopted a number of mechanisms which hyper-concentrate salts into particular tissues and then discarding the oversaturated tissues or increasing the succulence of said tissues (Mucina et al., 2005). An example of this strategy can be seen in *Populus euphratica* which demonstrates increased leaf succulence when exposed to 400 mM NaCl (Ottow et al., 2005). *P. euphratica* is able to regulate its internal salt concentration by sequestering Na^+ ions apoplastically, thereby limiting damage to the cytosolic structures and maintaining osmotic potential (Ottow et al., 2005). Lastly, 'root excluders', as the name implies, exploit the roots capacity to exclude salts during the uptake of water and minerals from the soil by means of the root membranes (Mucina et al., 2005). *Rhizophora*, a genus of the mangrove family Rhizophoraceae, is an example of a typical root salt excluder (Atkinson et al., 1967). Medina et al. (2015) demonstrates this clearly in their comparison between the salt-excluding species, *R. mangle*, and the salt-secreting species, *Laguncularia racemosa*. Over a 4-month period, they studied the concentration of the elements N, P, S, K, Mg, Ca, Mn, Fe and Na in the young, adult (mature), old and senescent leaves of the two species (Medina et al., 2015). They concluded that the concentration of Na^+ ions remained significantly similar in the young, adult and old leaves of *R. mangle* in comparison to the *L. racemosa*, which demonstrated significant increases in leaf Na^+ concentration as the leaf age increases (Medina et al., 2015). Thus, reinforcing the idea of *R. mangle* as salt-excluding species (Atkinson et al., 1967; Medina et al., 2015). This strategy, however, is not exclusive to halophytes, but can be seen in non-halophytic grasses, too (Mucina et al., 2005; Ottow et al., 2005).

1.5.2 Compartmentalization of salt ions

Under saline conditions, plants are required to adjust their osmotic potential owing to the low external water potential induced by the presence of salt ions (Gorham, 1992; Flowers et al., 2008). The maintenance of shoot osmotic and turgor pressure in halophytes is highly reliant on the use of inorganic ions when grown under salinity, whereas glycophytes do this by increasing the synthesis of organic solutes (Shabala, 2013). The osmotic pressure of the cell sap in halophytes are made up of 80 to 95% of Na^+, K^+ and Cl^- (Shabala, 2013). Whereas, in glycophytes this typically made up of 50 to 70% of the cell sap osmotic pressure (Shabala, 2013). When taking electrical charges on plant membranes into consideration in plants grown

on moderately to highly saline soils, the internal (plant cells) and external ion concentrations of Na^+, K^+ and Cl^- suggests that the uptake of K^+ is an active process, with energy required for it to happen, whereas the influx of Na^+ and Cl^- is mainly passive (Gorham, 1992). In glycophytic plant species, Na^+ leakage into plants occurs by means of the symplasm of root cortial cells, whereby Na^+ competes for binding sites with K^+ for K^+ transporters and cation channels (Glenn et al., 1999). However, in halophytes, the uptake of Na^+ and K^+ appear to be unlinked, which allows halophytes to widely maintain K^+ uptake rates even in the presence of various ranges of external Na^+ concentrations (Glenn et al., 1999). As such, it is dubious that Na^+ leakage into halophyte cells occurs by means of K^+ transporters and carriers (Glenn et al., 1999). However, owing to the high rates of NaCl uptake into halophyte roots, it is safe to assume that its uptake occurs by means of gated cation and anion channels (Glenn et al., 1999).

Owing to toxicity of monovalent ions at the concentrations that are necessary for osmotic adjustment, it is assumed that Na^+ and Cl^- would be compartmentalized in vacuoles, in order to maintain the tolerable concentrations within the cytoplasm (Zhu, 2001; Parks et al., 2002; Flowers et al., 2008; Shabala, 2013). Metabolically, toxic Na^+ concentrations are thought to be similar between halophytes and glycophytes (Flowers et al., 2008), as such it is necessary that Na^+ is separated from metabolic pathways that are sensitive (Parks et al., 2002; Shabala, 2013). The standard interpretation is that Na^+ is translocated into vacuoles by means of tonoplast Na^+/H^+ antiporters belonging to the CPA family of cation/proton antiporters (Shabala, 2013). These need to be energized by vacuolar H^+ pumps and tonoplast H^+- ATPases and PP-ases (Ayala et al., 1996; Glenn et al., 1999; Zhu, 2001; Parks et al., 2002; Shabala, 2013). Halophytes have a greater capacity for Na^+ vacuole sequestration than glycophytes owing to the constitutive expression of tonoplast Na^+/H^+ antiporters which are constantly activated, when in the presence or absence of salt (Ayala et al., 1996; Glenn et al., 1999; Shabala, 2013). Halophyte physiological data provides evidence for the capacity of halophytes to rapidly scavenge Na^+ and sequester it into cell vacuoles even when plants are grown at low salinities, however optimal growth often occurs at moderate to high concentrations of NaCl (Glenn et al., 1999; Ayala et al., 1996). Ayala et al. (1996) demonstrated this with *Salicornia bigelovii* Torr. grown under 5 mM NaCl and 200 mM NaCl. Their findings showed increased growth under greater NaCl concentrations and higher ATPase activity under higher salinities (Ayala et al., 1996). In salt-tolerant glycophytes, these antiporters need to be activated by NaCl, however, in salt-sensitive glycophytes, the expression of these Na^+/H^+ antiporters are very low and thus are not inducible by salt (Shabala, 2013).

Additionally, the role that Cl^- plays in osmoregulation and plant salt tolerance is as important as that of Na^+ (Glenn et al., 1999). Plants that are salt tolerant often compartmentalize Cl^- ions into the vacuole in vast concentrations (Maathuis et al., 1992; Glenn et al., 1999; Flowers et al., 2008). Changes in the cytoplasmic and vacuolar concentrations of Cl^- have demonstrated the regulatory effect it has on the transport of other anions into the vacuole (Glenn et al., 1999). Passive ion movement channels found in the tonoplast have been found for the transport of Cl^- (Glenn et al., 1999). These channels provide a mechanism of uniport transport of Cl^- into the vacuole (Glenn et al., 1999). These channels are tightly controlled by membrane potential, as seen in intravacuolar Cl^- concentrations which regulate the anion channel activity of the vacuole (Glenn et al., 1999). In cases where Cl^- concentrations in the vacuole are high, the influx of nitrate and phosphate into the vacuole is favoured; and the influx of additional anions other than Cl^- into the vacuole was accompanied by an efflux of Cl^- out of the vacuole and into the cytosol (Glenn et al., 1999). As such, halophytes have been estimated to have cytoplasmic Cl^- concentrations between 25 to 150 mM (Glenn et al., 1999). However, in their vacuoles they would be able to amass concentrations between 200 and 1000 mM of Cl^- without any additional cellular energetic expenditure (Glenn et al., 1999).

There is potential for Na^+ leaking out of the vacuole and into the cytoplasm owing to sharp contrasts between the concentrations between the two compartments (Glenn et al., 1999; Flowers et al., 2008). If this were to occur, the potential for Cl^- leakage increases and would proceed to occur if the vacuole loses its positive charge relative to the cytoplasm (Maathius et al., 1992; Glenn et al., 1999). However, in halophytes, the tonoplast of the vacuoles is composed of highly saturated fatty acids and other lipid properties which decrease the permeability to NaCl (Glenn et al., 1999; Flowers et al., 2008). However, in comparison to glycophytic tonoplasts, the protein content was lower in halophytic tonoplasts and the polypeptide composition differed (Glenn et al., 1999). Additionally, in the halophyte, *Suaeda maritima*, cation channels found in the tonoplast which have the potential to allow Na^+ leakage back into the cytoplasm were found to be closed at significant physiological concentrations of Na^+ (Glenn et al., 1999; Flowers et al., 2008). As such, not much H^+ ATPase activity would be required to maintain the compartmentalization of NaCl, thus decreasing the energy expenditure dedicated to this process (Ayala et al., 1996; Glenn et al., 1999). This, therefore, allows halophytes to maintain high yields even under high salinities (Glenn et al., 1999; Flowers et al., 2008).

1.5.3 Genetic expression

A key mechanism that has long been recognized for its role in Na^+ exclusion and ion homeostasis at the cellular level is the activation of the SOS signalling pathway (Yokoi et al., 2002; Ji et al., 2013). The isolation and characterization of *sos3, sos2* and *sos1* mutants in *Arabidopsis* showed root hypersensitivity to saline conditions, thus leading to the identification of this pathway ([b]Zhu, 2001; Chakraborty et al., 2012; Horie et al., 2012; Ji et al., 2013). The general idea for many years was that excess Ca^{2+} had the capability to enhance plant Na^+ tolerance (Ji et al., 2013). However, evidence suggests that salinity induces a spike in cytoplasmic Ca^{2+} in root cells which then activates the SOS signal transduction cascade to protect the cells from the damage caused by Na^+ accumulation ([b]Zhu, 2001; Zhu, 2002; Ji et al., 2013). As Ca^{2+} increases in the cytosol owing to excess Na^+ in the cytoplasm, it stimulates SOS3 to encode for a myristoylated calcium-binding protein which functions as a primary calcium sensor ([a]Zhu, 2001; [b]Zhu, 2001; Zhu, 2002; Ji et al., 2013). Once bound to Ca^{2+}, SOS3 activates the serine/threonine protein kinase SOS2 which belongs to the sucrose non-fermenting-1-related protein kinase-3 family (SnRK3) ([a]Zhu, 2001; [b]Zhu, 2001; Zhu, 2002; Chakraborty et al., 2012; Ji et al., 2013). Recent findings have shown that alternatively, a SOS3-like Calcium Binding Protein 8 (SCaBP8) has been able to regulate SOS2 activity in the shoots of *Arabidposis*, which differs to SOS3 which is more active in plant roots ([a]Zhu, 2001; Zhu, 2002; Chakraborty et al., 2012; Ji et al., 2013). When SOS2 phosphorylates SCaBP8, it stabilizes the protein complex (Ji et al., 2013). SOS2 and SOS3 provide a pathway for regulating Na^+ and K^+ homeostasis as well as plants' ability to tolerate saline conditions (Chakraborty et al., 2012). Interactions between SOS3-SOS2 or SCaBP8-SOS2 mobilizes SOS2 to the plasma-membrane leading to SOS1 activation which is an Na^+/H^+ antiporter responsible for sodium exclusion in the apoplast ([b]Zhu, 2001; Zhu, 2002; Zhu, 2003; Chakraborty et al., 2012; Horie et al., 2012; Ji et al., 2013). SOS1 is a protein with 10 to 12 transmembrane domains which has a long tail which are expected to reside in the cytoplasm (Zhu, 2002; Zhu, 2003). This process leads to the efflux of Na^+ from the cytosol to surrounding intercellular spaces (Horie et al., 2012; Ji et al., 2013). However, the activity of SOS1 can also be regulated by SOS4, which mobilizes the formation of pyridoxal-5-phosphate, a cofactor capable of serving as a ligand for SOS1 due to the latter containing a reputed binding sequence for this cofactor (Chakraborty et al., 2012).

Of the SOS loci, the role of SOS1 is the most significant in regard to the tolerance of plants to salinity when compared to SOS2 and SOS3 (Chakraborty et al., 2012). When assessing *sos1*,

sos2 and *sos3* mutants, *sos1* mutants displayed higher sensitivity to ionic stresses such as Na^+ and Li^+ ([a]Zhu, 2001; Chakraborty et al., 2012). Whereas, overexpression of SOS1 appeared to lower shoot Na+ concentrations as well as improving the salt tolerance of *Arabidopsis* plant and callus tissue ([b]Zhu, 2001; Zhu, 2003). Additionally, the overexpression of the plant gene, *AtNHX1*, displayed enhanced salt tolerance in *A. thaliana* ([b]Zhu, 2001; Zhu, 2003; Ma et al., 2004; Horie et al., 2012). *AtNHX1* is found in the tonoplast and functions primarily in Na^+ compartmentalization (Zhu, 2003).

Thellungiella salsuginea, known prior as *Thellungiella halophila*, provides a great model to study the genetic systems of plant salt tolerance (Ali et al., 2017). *T. salsuginea* demonstrates extremophile properties and displays extreme tolerance to high salinities (Ali et al., 2017). *A. thaliana* and *T. salsuginea* are two species which are closely related, sharing 92% of its sequence homology (Ali et al., 2017). As such, comparative studies have been able to take advantage of the similarities between the two and thus are able to demonstrate significant differences between their salinity tolerances (Ali et al., 2017). The SOS pathway plays an essential role in the plant salt tolerance of *T. salsuginea* (Ali et al., 2017). *T. salsuginea* exhibits increased transcript levels, with regards to genes involved salt tolerance (SOS1, SOS2 and SOS3), even in non-saline environments (Bartels et al., 2013). A great number of these genes remain unexpressed in *A. thaliana* when in non-saline environments, suggesting that the continuous expression of conserved stress-related genes is fundamental to the salt tolerance of *T. salsuginea* (Bartels et al., 2013). Under salt stress, the expression of these genes are induced in *A. thaliana*, whereas, it is upregulated further in *T. salsuginea* (Bartels et al., 2013). This clearly demonstrates the differences in gene expression between halophytes (*T. salsuginea*) and glycophytes (*A. thaliana*) (Nikalje et al., 2017).

1.5.4 Organic solutes as Osmoprotectants

Glycophytes growing in highly saline soils are not only faced with salinity stress, but also the hyperosmotic stress imposed on them owing to high concentrations of NaCl in the soil ([b]Zhu, 2001; Yokoi et al., 2002). As such, the physiological response to this is osmotic adjustment (Yokoi et al., 2002). Both glycophytes and halophytes share a similar cytosolic and organellar sensitivity to Na^+ and Cl^-, therefore, to achieve osmotic adjustment, these compartments accumulate compatible solutes and osmoprotectants ([a]Zhu, 2001; [b]Zhu, 2001; Yokoi et al., 2002; Zhu, 2002; Horie et al., 2012). The majority of compatible osmolytes are organic solutes,

however a few are elemental ions, including K^+ (Yokoi et al., 2002; Horie et al., 2012). The organic solutes that make up the majority of compatible osmolytes include simple sugars, such as fructose and glucose, sugar alcohols, glycerol and methylated inositols, quaternary amino acid derivates (proline, glycine betaine, β-alanine betaine, proline betaine), complex sugars, trehalose, raffinose and fructans, , tertiary amines 1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrmidine), and sulfonium compounds (Choline 0-sulfate, dimethyl sulfonium propironate (Yokoi et al., 2002; Rhodes et al., 2002). Additionally, several osmolytes are considered to be 'osmoprotectants' as the concentrations at which they accumulate are not significant enough to facilitate osmotic adjustment (Yokoi et al., 2002; Rhodes et al., 2002). Furthermore, osmoprotectants have the potential to improve plant stress tolerance when expressed as products of transgenes (Yokoi et al., 2002). An example of this can be found in plants undergoing hyperosmotic and ionic stresses, where some osmoprotectants perform the biochemical functions allowing for the scavenging of reactive oxygen species to prevent them from inflicting damage on cellular structures and organelles ([b]Zhu, 2001; Yokoi et al., 2002; Horie et al., 2012).

An important feature of organic compatible solutes is that they are able to accumulate in the cells in high concentrations yet inflicting no damage on the intracellular biochemistry of the cells (Yokoi et al., 2002; Zhu, 2002; Horie et al., 2012). Additionally, they are able to maintain enzymatic activity in saline solutions with minimal effects on cellular pH and charge balance in the cytosol (Yokoi et al., 2002). Stress triggers and signals often induce the synthesis of compatible osmolytes by the metabolic diversion of intermediary metabolites into unique biochemical reactions (Yokoi et al., 2002). An example of this is found in higher plants where the synthesis of glycine betaine is produced from choline via two reactions which are catalysed in a particular sequence by choline monooxygenase and betaine aldehyde dehydrogenase (Yokoi et al., 2002).

1.6 Strategies for alleviating salt stress in plants

1.6.1 Salt-tolerant crops and transgenic lines

The use of salt-tolerant crops are becoming increasing important to help alleviate the problem with saline soils globally (Shrivastava et al., 2015). Crop plants with greater tolerance to salinity allows for the use of irrigated water high in salt ions (Shrivastava et al., 2015). Owing

to the negative implications of salinity on growing crops (i.e. reduction in plant growth and yield, with an increase in the senescence of mature leaves), there has been a great incentive to produce and develop crops with increased salt tolerance, so as to allow increased crop yield on saline cropland soils (Flowers et al., 2010). However, owing to the complexity of halophytic salt tolerance, the development of successful salt-tolerant breeding crop lines has been hindered (Flowers et al., 2010; Shrivastava et al., 2015). As such, though a significant amount of resources has been directed toward the creation and development of salt tolerant crop lines, extending over a wide range of transgenics, a small number of salt-tolerant varieties have been successfully produced (Flowers et al., 2010; Shrivastava et al., 2015). Experiments assessing the salt tolerance of transgenic lines have mainly been carried out on a limited number of seedlings (smaller sample sizes) and these experiments were mainly carried out within greenhouse conditions (Shrivastava et al., 2015). These results do not always coincide with field experiments, as all the other factors which prevail in the presence of saline soil (high pH soils, higher daytime temperatures, low moisture levels and the presence of other sodic ions) are not found in laboratory experiments (Shrivastava et al., 2015). As such, the salt tolerance of crop plants in the field needs to be evaluated on a scale of function of yield, owing to the variability of soil factors including saline conditions, soil fertility, temperature, soil moisture and light intensity (Shrivastava et al., 2015). As such, greenhouse and laboratory experiments which assess the tolerance of crops have little correlation to field studies (Shrivastava et al., 2015).

Model plants, including tobacco, *Arabidopsis* and rice, have displayed much success in developing salt stress tolerant mutants (Shrivastava et al., 2015). It was well known that the SOS signalling cascade is important for improving plant tolerance to saline conditions. Liu et al. (2000) displayed this in a study investigating the importance of the SOS gene in salt tolerance of *A. thaliana*. The characterization of *Arabidopsis sos* (salt overly sensitive) mutants demonstrated increased salt sensitivity owing to ineffective regulation of intracellular Na^+ and K^+ (Liu et al., 2000). Liu et al. (2000) determined that SOS2 is necessary for the homeostasis of intracellular Na^+ and K^+ in *A. thaliana*, as *sos2* mutants developed significant imbalances in Na^+ and K^+, which decreased plant growth and development. Additionally, Apse et al. (1999) were able to confer that the overexpression of vacuolar Na^+/H^+ antiport in *A. thaliana* sustained plant growth even when exposed to 200 mM NaCl. Thus, providing evidence for increased salt tolerance in *A. thaliana* mutants with higher *AtNHX1* transcripts, protein and vacuolar Na^+/H^+ antiport activity (Apse et al., 1999). Furthermore, the late embryogenesis abundant (LEA)

protein gene, *HVA1*, taken from barley (*H. vulgare* L.) and introduced into *O. sativa* L. to create transgenic rice plants in a study by Xu et al. (1996). Xu et al. (1996) found that transgenic plants maintained higher growth rates, delayed onset of salt damage symptoms and improved recovery when removed from the saline conditions, when compared to that of the non-transformed control rice plants. Thus, providing evidence for the use of LEA genes in transforming plants to combat saline stress. Lastly, Holmström et al. (2000) transformed tobacco (*Nicotiana tabacum*) using the *betB* gene taken from *Escherichia coli* to induce the accumulation of glycine betaine in tobacco. Transgenic tobacco lines were found to display increased salt tolerance and enhanced recovery from photoinhibition (Holmström et al., 2000). Thus, demonstrating that the use of transgenics in agriculture could potentially improve the problem faced with rising soil salinities.

However, there is an urgent need to introduce salt tolerant mutants from other crop species (Shrivastava et al., 2015). A number of technical and financial difficulties are associated with transforming crop plants, particularly monocots (Shrivastava et al., 2015). Firstly, the transformation of monocots (other than rice) is not done as often and to establish a series of independent homozygous lines is financially costly and takes a longer period of time (Shrivastava et al., 2015). Secondly, screening for salt tolerance will need to include field experiments, as most salt stress tolerance assays are involve using nutrient-rich media, which is unlikely to display similar results to field trials (Shrivastava et al., 2015). Thirdly, saline soils are often a complex combination of different salts ($CaCl_2$, NaCl, Na_2SO_4, $CaSO_4$), contain high concentrations of boron and are alkaline (Shrivastava et al., 2015). Thus, plants which show promising characteristics will have to be evaluated in these sorts of environments (Shrivastava et al., 2015).

1.6.2 Plant growth promoting bacteria (PGPB)

A number of different strategies have been established to aid the decrease in deleterious effects of salinity on plant development including both the genetic engineering of plants as well as the use of plant growth-promoting bacteria (PGPB) (Shrivastava et al., 2015). The importance of microorganisms has been well established and documented whereby they are capable of promoting plant growth, nutrient management and controlling plant pathogens (Shrivastava et al., 2015). Microorganisms play a vital role in soil ecosystems, whereby they contribute to nutrient turnover and sustainable crop production (Ahemad et al., 2014). These PGPB colonize

the rhizosphere (rhizobacteria) or the internal plant tissues (endophytic bacteria/endophytes) where they promote plant growth either through direct or indirect mechanisms (Rashid et al., 2012; Ahemad et al., 2014; Ali et al., 2014; Shrivastava et al., 2015; Numan et al., 2018). PGPB that belong to the rhizobacterial group include *Azospirillum, Azotobacter, Arthrobacter, Achromobacter, Burkholderia, Bacillus, Enterobacter, Klebsiella, Microbacterium, Pseudomonas, Pantoea, Paenibacillus, Streptomyces, Serratia*, etc (Ahemad et al., 2014; Numan et al., 2018; Singh et al., 2018). Endophytic PGPB are capable of colonizing healthy plant tissues without the plant displaying any disease symptoms (Rashid et al., 2012; Ali et al., 2014). Thus, the use of PGPB in managing plant growth offers an exciting alternative to the current management of plant stressors, including high salinities (Ahemad et al., 2014; Shrivastava et al., 2015).

PGPB induce physical and chemical alterations that result in increased plant tolerance to a particular stressor (Shrivastava et al., 2015). The term proposed for this is coined Induced Systemic Tolerance (Shrivastava et al., 2015). PGPB promote plant growth by reducing plant pathogens (antibiotic and lytic enzyme production), facilitating nutrient uptake through the production of phytohormones such as auxin, cytokinin and gibberellins, lowering plant ethylene levels through the production of ACC (1-aminocyclopropan-1-carboxylate) deaminase (Glick, 2005), nitrogen fixation, ammonia production, solubilizing mineral nutrients, siderophore production (Rashid et al., 2012; Ali et al., 2014; Shrivastava et al., 2015; Tsukanova et al., 2017; Etesami et al., 2018; Numan et al., 2018) and the sequestration of toxic heavy metals (Ahemad et al., 2014; Fan et al., 2018). Additionally, high-stress environments possess bacterial communities capable of tolerating those particular stressors (Shrivastava et al., 2015), such as coastal marine plants which are colonized by halophytic bacteria (Etesami et al., 2018). Plants inoculated with PGPB taken from high-stress environments often display increased root and shoot length, increased biomass and enhanced biochemical levels in comparison to control treatments for the same stressor (Shrivastava et al., 2015; Etesami et al., 2018). Plants grown in high-stress environments, such as highly saline soils, often display a rise in ethylene biosynthesis, which is counterintuitive to plant growth (Ali et al., 2014). In a study conducted by Ali et al. (2014), ACC deaminase containing endophytic PGPB (*Pseudomonas fluorescens YsS6* and *Pseudomonas migulae 8R6*) as well as their deficient mutants were used to evaluate their capacity for plant growth promotion in tomato plants grown under saline conditions (0 mM, 165 mM and 185 mM). ACC (1- aminocyclopropan-1-carboxylate) deaminase cleaves ACC to α-ketobutyrate and ammonia, and in doing so it

decreases ethylene levels in the host plants (Glick, 2005; Rashid et al., 2012; Ali et al., 2014). It was found that *P. fluorescens YsS6* and *P. migulae 8R6* were able to promote tomato plant growth even in the absence of salinity (Ali et al., 2014). Plants pre-treated with the ACC deaminase containing endophytes exhibited increased dry and fresh weight, higher chlorophyll levels and more flowers than those treated with the mutant strains and the control (no bacteria) treatment (Ali et al., 2014). Thus, providing evidence for the use of ACC deaminase containing PGPB in the facilitation of crop plants grown on saline soils (Glick, 2005; Ali et al., 2014). Ribaudo et al. (2006) reported positive interactions between *Azospirillum brasilense FT326*, a nitrogen fixing bacterium, and tomato plants. They found that *A. brasilense FT326* increased root weight, shoot weight and root hair length, owing to higher phytohormone secretion levels (IAA) (Ribaudo et al., 2006). *A. brasilense* was able to generate a similar result in sweet pepper (*Capsicum annuum* L.) where it increased plant dry weight and maintain stomatal conductance, thus sustaining photosynthetic levels (del Amor et al., 2012).

Therefore, the utilization of a select group of PGPB can be useful in facilitating sustainable agriculture on saline soils (Ali et al., 2014; Shrivastava et al., 2015; Etesami et al., 2018; Etesami et al., 2018), as it is a cost effective tool that can be used to promote plant growth and yield on saline conditions (Numan et al., 2018).

1.7 Application of PGPB in agriculture

The human population currently sits at 7.8 billion individuals and is projected to increase to 10 billion within the next 43 years (Glick, 2014). In order to feed all of those individuals, a significant increase in the global agricultural productivity is absolutely necessary for the upcoming decades (Glick, 2014). To do this, a number of different approaches and strategies will need to be implemented in order for it to be successful (Glick, 2014). These will include expanding the range of agricultural land, irrigation and drainage practices, increase the use of chemicals such as herbicides, pesticides and fertilizers, soil aeration, increasing farm mechanization, electroreclamation, increasing the utilization of transgenic crops, phytoremediation and increasing the use of plant growth promoting microorganisms into mainstream production (Glick, 2014; Shin et al., 2016). However, the use of chemical fertilizers, herbicides and pesticides has reportedly increased the rate of soil acidification, weakening plant root systems and increasing their susceptibility to disease (De Souza et al., 2015; Yimwe et al., 2019). Additionally, the cost of energy, poor quality water used for

irrigation and inefficient resources used for the reformation of saline soils (Shin et al., 2016) demands the need for determining effective, long term sustainable solutions (De Souza et al., 2015). Thus, plant growth promoting bacteria (PGPB) provides us with promising technology to overcome these problems whilst remaining relatively environmentally friendly (Glick, 2004; De Souza et al., 2015).

The current literature offers great insight and detail into the effectiveness of PGPB in facilitating plant growth against abiotic and biotic stressors (Glick, 2014). PGPB do this by facilitating nutrient uptake through the production of phytohormones such as auxin, cytokinin and gibberellins, lowering plant ethylene levels through the production of ACC (1-aminocyclopropan-1-carboxylate) deaminase (Glick, 2005; Glick, 2014) and sequestrating toxic heavy metals Ahemad et al., 2014; Fan et al., 2018) as well as reducing plant pathogens (antibiotic and lytic enzyme production) (Glick, 2005). PGPB can be used as an alternative to chemical fertilizers owing to growth promoting characteristics and thus can be introduced to plants as a 'biofertilizers' (De Souza et al., 2015; Yimwe et al., 2019). Biofertilizers consist of organic fertilizer into which PGPB have been incorporated (Yimwe et al., 2019). These biofertilizers include nitrogen-fixing, siderophore-, phytohormone-, ACC deaminase-producing and potassium and phosphorus solubilizing bacteria (Yimwe et al., 2019). Examples of microorganisms that perform well under both greenhouse and field conditions are *Trichoderma* and *Azospirillum* species (Yimwe et al., 2019). Both have shown to improve mineral uptake, mobilize minerals in the soil, induce plant hormone production, increase systematic resistance mechanisms and thus, they have been useful in biofungicides and biofertilizers (Yimwe et al., 2019).

Rising soil salinity is severely affecting microbial diversity, biomass and enzymatic activity on agricultural land, which in turn negatively impact plant growth (Shin et al., 2016). However, owing to the ability of halotolerant microorganisms to adapt to a range of saline conditions, the use of halotolerant PGPB in salt-affected agricultural areas holds much promise (Shin et al., 2016; Li et al., 2017). These interactions could provide a cost-effective, sustainable method for the amelioration of saline soils as well as improving crop growth and yield production on these salt-affected soils (Shin et al., 2016). This technology has already been successful in several countries, albeit on a smaller scale than mainstream agriculture (Glick, 2014). Thus, owing to the potential of PGPB in agricultural practices, more field studies need to be performed involving promising PGPB strains in salt-affected soils.

1.8 Justification

The human population is estimated to increase to 10 billion within the next 43 years (Glick, 2014). This will be put significant strain on global food security, particularly in developing African nations already suffering from shortages (Etesami et al., 2018). This problem is further escalated owing to the rise in salt-affected agricultural land which reduces crop growth and crop yield (Ali et al., 2014; Shrivastava et al., 2015; Etesami et al., 2018). Thus, the need for alternative strategies with minimal environmental impacts and long-term sustainability is highly necessary (Glick, 2014, Shin et al., 2015). Here, plant growth promoting bacteria provide promising technology which can be used to overcome problems faced of rising salinity on crop lands whilst remaining environmentally friendly and cost effective (Glick, 2014; De Souza et al., 2015).

1.9 Aims and Objectives

Thus, this study will aim to isolate, characterize and screen endophytic bacteria taken from the coastal plant, *Arctotheca calendula* (the Capeweed) for plant growth promoting properties. Thereafter, the crop species *P. vulgaris* cv. Star 2000 will be inoculated with the isolated bacteria to examine their plant growth promoting capabilities under saline stress. Additionally, if positive plant growth promotion is demonstrated, this study will aim to identify the mechanisms involved.

CHAPTER 2: METHODS AND MATERIALS

2.1 Surface sterilization of *Arctotheca calendula* leaf and root tissue

Whole plant *A. calendula* samples were harvested from Sunset Beach, Muizenberg (34°06'15.6"S, 18°28'42.1"E) on the West Coast of South Africa. The species is native to South Africa, preferring full sun and sandy soil areas (Brundu et al., 2015). However, it is a very stress tolerant plant, able to tolerate drought and salt stress conditions (Brundu et al., 2015). It is highly invasive globally, particularly in areas with Mediterranean climates (Brundu et al., 2015). The plant material was rinsed with water until it was free from any sand after which it was patted dry using tissue paper. The leaf and root material were then separated into distinct sterile 50 ml Greiner tubes for surface sterilization of the plant tissues. Owing to the presence of the epiphytic bacteria on the plant tissue surfaces, the root and leaf tissue were surface sterilized using different protocols. The leaf material was submerged and shaken in 0.35% sodium perchlorate for 2 min, followed by 70% ethanol for 5 min and 0.35% sodium hypochlorite for 1 min. The root material was submerged and shaken in 2% sodium perchlorate for 2 min, 70% ethanol for 5 min and 2% sodium hypochlorite for 1 min. Thereafter, the plant tissue samples were washed 10 times with sterile distilled water for a total of 30 seconds per wash. Upon completing the sterile distilled water wash, 500 µl of the last wash for each tissue sample was plated onto R2A agar plates to assess whether the plant tissue had been sterilized effectively.

Figure 1. A. calendula plant species at Sunset Beach, Muizenberg, South Africa.

2.2 Endophyte extraction

After the surface sterilization of the *A. calendula's* leaf and root tissues, the samples were ground up in 0.9% NaCl solution using a mortar and pestle and stored in 50 ml tubes also filled with 0.9% NaCl solution. The ground up samples were stored and incubated overnight in the salt solution at room temperature (21°C), encouraging endophytic bacterial growth. Thereafter, a ten-fold serial dilution was performed, and every dilution step was plated onto R2A agar of varying NaCl concentrations, namely 0 mM, 300 mM and 600 mM. The plates were then incubated at 27°C for 7 days and the cultures were purified by means of isolating individual endophytic bacterial colonies and streaking plating them onto R2A agar plates.

2.3 Extraction of bacterial DNA

The boiling lysis method was used to extract the bacterial DNA. Overnight cultures were prepared in LB liquid media, thereafter 2 ml of the overnight cultures were centrifuged at 13000 rpm for 10 minutes. The pellet was suspended in 200 ul of TE buffer [Tris-HCL (10 mM), EDTA (1mM)] and the pellets were heated for 15 min on a heating block. Thereafter, the samples were placed on ice for an additional 15 min. Following this, the tubes were

centrifuged at 13000 rpm for a further 10 min and 100 ul of the DNA-containing supernatant was transferred to a 2 ml tube.

2.4 Identification of endophytes

To prepare the PCR products, the 16S forward and reverse primers used were E9F (GAGTTTGATCCTGGCTCAG) and 517R (ATTACCGCGGCTGCTGG), respectively. The PCR was performed in a 25 µl reaction volume that consisted of 2.5 µl bacterial DNA, 2.5 µl 10 X PCR buffer, 0.5 µl dNTPs (10 mM), 1 µl $MgCl^2$ (50 mM), 1 µl forward and reverse primers each, 0.25 µl Taq polymerase and made up to 25 µl with nuclease free water (ThermoFischer Scientific). The PCR products were then viewed on a 1% agarose gel. Thereafter, an amplified rDNA (Ribosomal DNA) restriction analysis was performed using Hae III and RsaI as restriction enzymes. The restriction digests were incubated at 37°C for a period of 2 hours. Thereafter, the digested products were viewed on a 3% agarose gel. Following this, 16s rDNA amplicons for each isolate was sent to the Central Analytical Facility (CAF, Stellenbosch University) for genome sequencing. Thereafter, the isolates were identified using the National Centre for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) and EZBioCloud.

2.5 Bacterial Assays

2.5.1 Nitrogen fixation assay

To assess nitrogen fixation, an adaptation of Burk's nitrogen free medium (0.0025 g Sodium Molybdate, 0.005 g Iron Sulphate, 0.05 g Sodium Sulphate, 0.1 g Magnesium Sulphate, 0.2 g Calcium Chloride, 0.41 g Monopotassium Phosphate, 0.52 g Dipotassium Phosphate, 10.0 g Glucose, 15.0 g agar in 1000 ml distilled water) as followed by Park et al. (2005) and augmented with the addition of NaCl at the concentrations 0 mM, 300 mM and 600 mM. The pH was adjusted to 6.9 – 7.1 before autoclaving the media at 121°C for 20 min. The bacterial isolates were spot inoculated on the media and incubated at 30°C for 7 days. Nitrogen fixation was assumed by means of luxuriant growth on the nitrogen free medium (Park et al., 2005).

2.5.2 Phosphate solubilization assay

Pikovskayas agar was used to test the phosphate solubilization efficiency of the endophytic bacteria. The plates were prepared using 0.5 g Yeast extract, 10.0 g Dextrose, 5.0 g Calcium phosphate, 0.5g Ammonium sulphate, 0.2 g Potassium chloride, 0.1 g Magnesium sulphate, 0.0001 g Manganese sulphate, 0.0001 g Ferrous sulphate and 15.0 g Agar (Himedia, 2015) supplemented with NaCl at varying concentrations, namely 0 mM, 300 mM and 600 mM, in 1000 ml of distilled water. The media was autoclaved at 121°C for 20 minutes before use. Single endophytic bacterial colonies were spot inoculated onto the plates and incubated at 30°C for 7 days. A halo clearing around the colony indicated the endophytes capacity for phosphate solubilization.

2.5.3 Zinc solubilization assay

A basal medium (10.0 g Glucose, 1.0 g Ammonium sulphate, 0.2 g Potassium chloride, 0.1 g Dipotassium phosphate, 0.2 g Magnesium sulphate and 15.0 g Agar in 1000 ml distilled water) supplemented with 0.2% insoluble Zinc oxide (ZnO) was prepared to assess the zinc solubilization capacity of the endophytic bacteria (Dinesh et al., 2018). The media was augmented by the addition of NaCl to produce the concentrations 0 mM, 300 mM and 600 mM. The media was sterilized at 121°C for 20 minutes before use. Thereafter, the endophytic bacterial isolates were spot inoculated onto the plates and incubated at 28°C for 48 hours. A zone of clearance surrounding the single colonies was taken as an indication for solubilization capacity of the isolates.

2.5.4 Siderophore activity

Siderophore production was determined by means of the Chrome Azurol S agar method as described by Fan et al. (2018) and augmented with the addition of NaCl at concentrations 0 mM, 300 mM and 600 mM. Following a 5-day incubation at 28°C, the formation of a zone of clearing often identified by the presence of orange halo rings surrounding the bacterial colonies was taken as evidence of siderophore excretion (Fan et al., 2018).

2.5.5 Ammonia production

The endophytic bacterial isolates were incubated in 10 ml LB broth (0 M, 0.3 M and 0.6 M NaCl) at 30°C for 24 hours. Subsequently, 20 ul of the overnight bacterial cultures were added to 10 ml of Peptone water (20.0 g Yeast extract and 30.0 g NaCl in 1000 ml of distilled

water) in a colorimetric test. The mixture was subjected to continuous shaking (140 rpm) at 30°C for 5 days. Following the incubation period, an admixture of 0.2 ml culture supernatant and 0.5 ml of Nessler's reagent was prepared. Indication of yellow colouration was indicative of ammonia production (Rashid et al., 2012). Thereafter, the optical density of the mixture was measured by means of a spectrophotometer at 430 nm to ammonia production. A faint yellowish colour change was taken as the production of ammonia.

2.5.6 Indole Acetic Acid production

Indole acetic acid (IAA) production was established by means of a colorimetric test. The bacterial isolates were incubated in YEM broth (0.5 g Dipotassium Phosphate, 0.2 g Magnesium Sulphate, 10 g Mannitol, 0.1 g Sodium Chloride and 1 g Yeast Extract) with 1 g Tryptophan in 1000 ml distilled water for 5 days at 28°C as described by Mohite (2013). Thereafter, 2 ml of the culture was centrifuged at 13000 rpm for 10 minutes. 1 ml of the supernatant was then added to 2 ml Salkowski reagent and incubated for 30 minutes in the dark. Lastly, the optical density was measured at 530 nm.

2.6 Plant growth trial

2.6.1 Without Salt

Phaseolus vulgaris cv. Star 2000 seeds were surface sterilized using 0.35% NaOCl bleach and 70% ethanol, for a period of 1 min and 30 seconds respectively. The seeds were then rinsed thoroughly and imbibed in a bacterial culture that was grown overnight for 20 - 30 min. Thereafter, the seeds were planted in 500 ml plant pots filled with an autoclaved soil mixture (potting soil 1:2 silica sand). The seedlings were grown in a greenhouse with 16:8 hr light-dark cycle, at 25°C. They were watered twice weekly with 100 ml of water.

2.6.2 With Salt

The same procedure as above was repeated where *P. vulgaris* cv. Star 2000 seeds were surface sterilized using 0.35% NaOCl bleach and 70% ethanol, for a period of 1 min and 30 seconds respectively. The seeds were then rinsed thoroughly and imbibed in a bacterial culture that was grown overnight for 20 - 30 min. Thereafter, the seeds were planted in 500 ml plant pots filled with an autoclaved soil mixture (potting soil 1:2 silica sand). The seedlings were grown in a greenhouse with 16:8 hr light-dark cycle, at 25°C. They were watered twice weekly

with 100 ml of water until the plants reached the first trifoliate. After reaching the first trifoliate, the plants were treated with 100 mM NaCl divided over a period of a 3 days as to avoid the plants going into osmotic shock. Thereafter, which the plants were watered with 100 ml water again.

2.7 Plant biomass determination (Dry mass)

After harvesting the plants from their pots, three leaves as well as the roots of each plant were removed, and the samples were placed in individual foil packaging with small holes in it to allow for the escape of moisture. Thereafter, the samples were dried overnight at 80°C and weighed the next day.

2.8 Determination of Cell Death (Evans Blue Assay)

The Evans Blue assay was adapted from that of Egbichi et al. (2014) to assess cell death in *P. vulgaris*. Plant material was thoroughly washed with water to remove any debris. Thereafter, 1 cm^3 blocks were excised from the leaf material and 2 cm were excised from the root tips. The excised material was then placed in 1.5 ml Eppendorf tubes containing 1 ml of 0.25% Evans Blue solution and incubated for one hour at room temperature in the dark. After the incubation period, the Evans Blue was decanted out of the Eppendorf tubes and the samples were rinsed with water. Subsequently, the samples were incubated in distilled water for 12 hours. Thereafter, the water was decanted and 1 ml of 1% SDS solution was added to the Eppendorf tubes which were heated to 65°C for an hour. Next, the samples were crushed in 1% SDS solution and centrifuged at 13000 rpm for 5 minutes. Lastly, the supernatant was loaded in triplicate on a microtitre plate and it was measured at 600 nm on a spectrophotometer.

2.9 Determination of superoxide concentration

Superoxide determination was adapted from Qiu et al. (2014). 8 blocks, each 1 cm^3 in size, were prepared from the leaf samples and 4 cm from the tip of the root samples were taken and added to 800 µl of 50 mM potassium Phosphate buffer (pH = 7.0) in 1.5 ml Eppendorf tubes. The samples were then incubated for 20 min at room temperature (21°C), thereafter which they were crushed using a mortar and pestle. Subsequently, the samples were centrifuged at 13000 rpm for 5 min. Lastly, 200 µl of the supernatant was loaded onto a microtitre plate in triplicate and read at 600 nm using a spectrophotometer.

2.10 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Plant material was divided into the respective tissue types namely leaf, bean pod and root for each treatment (0mM NaCl and 100 mM NaCl) and isolate group. The samples were then ground up in liquid nitrogen using a mortar and pestle. Thereafter, 200 mg of each sample was transferred to clean 2 ml Eppendorf tubes. Nitric acid (6%) was then added to each sample and the tubes were tightly wrapped in parafilm after which they were heated to 90°C for 3 hours. Subsequently, using a syringe, 9 ml of 2% nitric acid and 1 ml of the sample was taken up and filtered into a Greiner tube using a 0.45 um filter. Lastly, ICP-OES analyses were performed on the samples.

2.11 Statistical Analysis

The analyses were performed using R Studio (R Core Team, 2017). Normality and homoscedasticity tests will be used to assess the data. For parametric data, an ANOVA and a Tukey HSD Multiple Comparisons test were used to assess the differences between groups. Whereas for non-parametric data, a Kruskall-Wallis one-way ANOVA and a Mann-Whitney U test were performed. The significance value was set to ≤ 0.05

CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL ENDOPHYTES ISOLATED FROM THE HALOPHYTIC COASTAL SPECIES, *ARCTOTHECA CALENDULA*

Abstract

Aim: The aim of this chapter was to isolate and characterize bacterial endophytes isolated from the halophytic coastal plant species, *A. calendula*.

Methods: Endophytes were harvested from leaf and root material of *A. calendula* specimens, taken from Sunset Beach, Muizenberg. Subsequently, the endophytes were subjected to four plate assays (phosphate solubilization, zinc solubilization, siderophore production and nitrogen fixation assays) and two colorimetric assays (ammonia and indole acetic-acid production). Additionally, the isolates were identified using 16S rDNA sequence analysis.

Conclusion: Plant growth promoting traits had been demonstrated in all the endophytes isolated from *A. calendula*, under conditions of 0 mM, 300 mM and 600 mM NaCl. The isolates captured belonged to the genera *Erwinia, Bacillus, Ochrobactrum* and *Microbacterium*. *E. persicina* NBRC 102418^T, revealed N_2-fixation, ammonia production, phosphate solubilization, zinc solubilization, siderophore and IAA production. The isolates belonging to *Bacillus* species (*B. marisflavi* JCM 11544^T and *B. zhangzhouensis* DW5-4^T) had both demonstrated ammonia production, phosphate solubilization, zinc solubilization and siderophore production. However, *Bacillus marisflavi* JCM 11544^T had also exhibited N_2-fixation which *B. zhangzhouensis* DW5-4^T did not. *O. rhizosphaerae* PR17^T had shown ammonia production, zinc solubilization and siderophore activity under saline conditions as well as IAA production (under non-saline conditions). And lastly, *M. gubbeenense* DSM 15944^T had demonstrated ammonia production, zinc solubilization (only within the 300 mM NaCl treatment) and siderophore production under saline conditions. As such, each isolate demonstrated characteristics which could be construed as plant growth promoting properties.

3.1 Introduction

The importance of microbes has been well established and well documented in literature, especially in their capacity to promote plant growth, soil nutrient management and pathogen control (Shrivastava et al., 2015). Soil microorganisms are essential to soil ecosystems, as they contribute significantly to nutrient turnover (Ahemad et al., 2014). This is done through the breakdown of organic materials and solubilization of inorganic compounds, making nutrients and minerals available to plants for uptake (Ahemad et al., 2014). These organisms either colonize the rhizosphere (rhizobacteria or fungi) or they colonize the internal plant tissues (endophytic bacteria) without the plant displaying symptoms of infection or disease (Rashid et al., 2012; Ali et al., 2014). These microbes are often referred to as plant growth promoting (PGP) microorganisms as they are capable of promoting plant growth either through direct or indirect mechanisms (Rashid et al., 2012; Ahemad et al., 2014, Ali et al., 2014; Shrivastava et al., 2015; Numan et al., 2018), including reducing plant pathogens (antibiotic and lytic enzyme production), assisting plant nutrient uptake by means of phytohormone production (auxins, cytokinins and gibberellins), decreasing plant ethylene levels through the production of ACC (1-aminocyclopropan-1-carboxylate) deaminase (Glick, 2005), nitrogen fixation, ammonia production, the solubilization of inorganic mineral nutrients, siderophore production (Rashid et al., 2012; Ali et al., 2014; Shrivastava et al., 2015; Tsukanova et al., 2017; Etesami et al., 2018; Numan et al., 2018) and the sequestration of toxic heavy metals (Ahemad et al., 2014; Fan et al., 2018). Thus, the use of PGP microorganisms offers an exciting alternative to current management of plant growth under abiotic or biotic stressors (Ahemad et al., 2014; Shrivastava et al., 2015; Sharma, Kulkarni and Jha, 2016).

Globally, food security has been under threat owing to rising soil salinity on cultivated agricultural lands (Etesami et al., 2018). Saline soils have played a large role in the decline of the crop production of major agricultural crops such as rice, wheat, barley and maize displaying significant declines in crop yield globally (Shrivastava et al., 2015; Etesami et al., 2018). As such, it is essential that alternative and sustainable practices be used to mitigate the effect of rising salinity on crop growth and yield, including the use of plant growth promoting bacteria (PGPB) as biofertilizers (De Souza et al., 2015; Yimwe et al., 2019). These biofertilizers include nitrogen-fixing, siderophore, phytohormone, ACC deaminase-producing and potassium and phosphorus solubilizing bacteria (Yimwe et al., 2019). Bacteria taken from saline environments, such as coastal environments, salt marshes and salt pans, are often capable of tolerating or even thriving in those conditions (Shrivastava et al., 2015). Thus, plants

inoculated with PGPB taken from saline environments often display increased root and shoot length, biomass and enhanced biochemical levels in comparison to control treatments with added salinity and no inoculate (Shrivastava et al., 2015; Etesami et al., 2018). Therefore, in this study we hoped to identify halophilic or salt-tolerant endophytes with PGP characteristics, including the ability to fix nitrogen (N), produce ammonia, solubilize phosphorus (P) and zinc (Zn), displaying siderophore activity as well as producing Indole Acetic acid (IAA).

One of the mineral elements required by plants in large amounts is nitrogen (N) (Grattan et al., 1998; Hu et al., 2005). Nitrogen makes up the fundamental building blocks of a number of cellular components including amino acids and nucleic acids (Hu et al., 2005). Plants take up nitrogen in the forms of nitrate (NO_3^-) and ammonium (NH_4^+) (Grattan et al., 1998; Gorham, 1999). The interaction between salt ions and nitrogen remains highly complex as the most abundant nitrogen ions (NO_3^- and NH_4^+) are affected differently by salt ions (Na^+, Cl^-, Ca_2^+ and K^+) (Grattan et al., 1998; Hu et al., 2005). An increase in soil Cl^- concentration has been shown to decrease shoot NO_3^- concentration and is known as the chloride-nitrate antagonism (Grattan et al., 1998, Hu et al., 2005), whereas the opposite has been seen for NH_4^+, which displays increased concentrations under saline conditions (Min et al., 2016). Interestingly, studies have provided evidence for decreased plant sensitivity to salinity with increased soil NH_4^+ (Flores et al., 2001), but plants supplemented with NO_3^- and NH_4^+ in equal concentrations responded better than other ratios of the two nitrogen ions (Shaviv et al., 1991; Arshad et al., 1999). As such, finding PGPB with the ability to fix N and produce ammonia in saline conditions for plant uptake is necessary.

Another essential plant mineral element is phosphorus (P). P is an essential plant nutrient as it forms the building blocks of many compounds including phospholipids, phosphoproteins, dinucleotides and adenosine triphosphate (ATP) (Hu et al., 2005; Sharma et al., 2013). Additionally, it is highly necessary for processes such as photosynthesis, enzyme regulation, carbohydrate transportation and energy storage and transfer (Hu et al., 2005; Sharma et al., 2013). P deficiency in some plants and reduced ionic strength of P in the soil has been attributed to increased soil salinity (Grattan et al., 1998; Shrivastava et al., 2015). Thus, the PGPs capable of solubilizing phosphorus found in this study corroborate observations made by Sharma et al. (2016) and Shukla et al. (2012).

Zn is a trace element or micronutrient essential to plants in small amounts (Cavagnaro, 2008; Alloway, 2009; Khanghahi et al., 2018). It forms part of the structural constituents and

regulatory co-factors of many enzymes and proteins which are involved in biochemical pathways (Cavagnaro, 2008; Alloway, 2009). These enzymes play significant roles in carbohydrate metabolism, protein synthesis, regulation and maintenance of gene expression, cell membrane integrity, pollen formation and the regulation of auxin synthesis (Cavagnaro, 2008; Alloway, 2009). And yet, Zn deficiency is one of the most omnipresent micronutrient deficiencies seen in today's crops (Cavagnaro, 2008; Alloway, 2009). This in turn allows for rising Zn deficiencies in human populations, with one third of the world's population currently suffering from inadequate Zn nutrition (Cavagnaro, 2008; Alloway, 2009). Furthermore, the rise in soil salinity and soil pH (owing to rising salinity) on agricultural lands has further exacerbated the decrease in the phytoavailability of Zn in croplands (Mehrotra et al., 1986; Khashgoftarmanesh et al., 2005; Alloway, 2009; Khanghahi et al., 2018). PGP bacteria and fungi provide an alternative solution to Zn fertilizer supplementation on agricultural soils, as they mine the soil for Zn (Cavargnaro, 2008; Khanghahi et al., 2018). The observation and indication of an isolate with Zn-solubilizing capabilities was thus noteworthy for this study.

Another essential micronutrient for plant growth is iron (Fe) which plays an integral role in promoting plant growth (Sagar et al., 2018). Although a large component of soil is made up of Fe, its availability to plants is still limited, as it exists in the high insoluble ferric oxides (Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). PGPB and rhizobacteria often solubilize Fe from their environments by secreting low molecular weight, iron-binding molecules called siderophores which have a high affinity for Fe^{3+} and form a ferric-siderophore complex (Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). In doing so, Fe becomes soluble for plant uptake and Fe nutrition (Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). In saline soils and soils with a high pH, Fe uptake becomes even more limiting owing to ionic changes in the soil (Hoffman et al., 2002). Evidence suggests that depending on the strain of bacteria, the PGPB may increase or decrease its siderophore activity under saline conditions (Sadeghi et al., 2012; Deshwal et al., 2013). Thus, finding an isolate with the capacity to produce siderophores under saline conditions is important.

Lastly, auxins are a group of plant phytohormones which are an integral part of plant growth and development (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). One such auxin is Indole Acetic acid (IAA) (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). IAA is involved in the processes of cell elongation, vascular development, apical dominance all of which play a significant role in plant growth and development (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). IAA has also been associated with plant growth response to stressors

(Fahad et al., 2015), where variations in IAA content have been demonstrated in different plant species, though little literature can be found on the effects of salinity on plant IAA content (Kaya et al., 2009). Some studies have found that IAA content increases under saline stress (Veselov et al., 2008), whereas others have found decreasing IAA levels with rising salinity (Kaya et al., 2009; Javid et al., 2011). However, the literature has noteworthy evidence for improved plant growth under saline stress when inoculated with IAA-producing PGPB (Egamberdieva, 2009; Sadeghi et al., 2012 The isolation and description of an endophytic bacterium with IAA biosynthetic potential as noted in this study adds to the body of knowledge and the field in general.

3.2 Results

3.2.1 Application of endophytes isolated from A. calendula

Table 1. Morphological characteristics of bacterial isolates captured and extracted from *A. calendula.*

Isolate code	Colour	Form	Elevation	Margin
P3L1	Opaque White	Circular	Flat	Entire
P2L3	Yellow	Circular	Flat	Entire
P5L3	Beige-Orange	Circular	Flat	Entire
P3RB	Bright Yellow	Circular	Flat	Entire
P3RC	Cream-orange	Circular	Flat	Undulate

Three isolates were obtained from *A. calendula* leaves (P5L3, P3L1 and P2L3), and the other two were isolated from the root material (P3RB and P3RC). All five isolates were able to grow on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl. Table 1 provides morphological characteristics of each isolate.

3.2.2 Identification of endophytic isolates

Table 2. The identification of endophytic isolates taken from *A. calendula* using 16S rDNA.

Isolate Code	Taxon name	Strain name	Similarity (%)	Accession number	Source	Site
P3L1	*Erwinia persicina*	NBRC 102418 ^{T}	99,6	BCTN01000053	Hao et al. 1990	EZBioCloud
P2L3	*Bacillus marisflavi*	JCM 11544 ^{T}	100	LGUE01000011	Yoon et al. 2003	EZBioCloud
P5L3	*Ochrobactrum rhizosphaerae*	PR17^{T}	97,0	NNRK01000031	Kämpfer et al. 2008	EZBioCloud
P3RB	*Microbacterium gubbeenense*	DSM 15944 ^{T}	97,2	AUGQ01000019	Brennan et al. 2001	EZBioCloud
P3RC	*Bacillus zhangzhouensis*	DW5-4^{T}	100	JOTP01000061	Liu et al. 2016	EZBioCloud

Five bacterial endophytes were isolated from *A. calendula*, three from the leaves and two from the roots. The molecular identification of 16S rDNA gene fragments of these isolates showed that 2 isolates were *Bacillus* species (Table 2). P2L3 had been recognized as *B. marisflavi* strain JCM 11544 (LGUE01000011) and P3RC was identified as *B. zhangzhouensis* strain DW5-4 (JOTP01000061), both of which demonstrated a 100% likeness (Table 2). *B. marisflavi* strain JCM 11544 had been isolated from the leaf tissue of *A. calendula*, whereas *B. zhangzhouensis*

strain DW5-4^{T} had been isolated from the root material (Table 2). The two other endophytes isolated from the leaf material, were P3L1 and P5L3. P3L1 had demonstrated a 99.6% similarity with *Erwinia persicina* strain NBRC 102418 (BCTN01000053) and P5L3 displayed a 97% likeness with *Ochrobactrum rhizosphaerae* strain PR17^{T} (NNRK01000031) (Table 2). Lastly, the only other endophyte isolated from the root of *A. calendula* was P3RB, which had shown a 97.2% resemblance with *Microbacterium gubbeenense* strain DSM 15944 (AUGQ01000019) (Table 2).

3.2.3 Characterization of endophytes extracted from A. calendula

3.2.3.1 Bacterial growth on N_2-free media

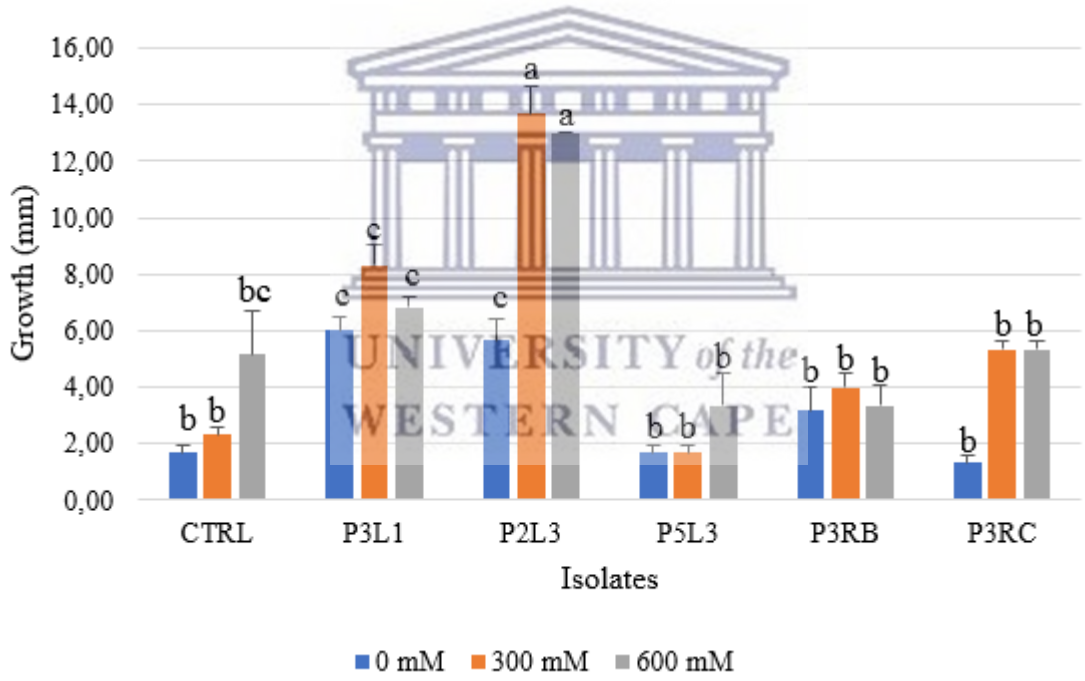

Figure 2. The colony diameter (mm ± SE) of endophytic isolates taken from *A. calendula* on a modification of Burk's N-free medium supplemented with 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significance is symbolized by superscript [a].

A modification of Burk's N-free medium (Park et al., 2005) was used to determine whether the isolates were capable of fixing nitrogen, where luxuriant growth was used to determine nitrogen fixation. P2L3 significantly had the greatest colony diameter at the 300 mM and 600 mM NaCl treatment when compared to all the other endophytes, thus providing evidence for nitrogen fixation ($p < 0.05$) (Figure 2). Additionally, P3L1 and P2L3 (0 mM NaCl) displayed

significantly greater colony diameters when compared to the rest of the isolates namely P3RC, P3RB, P5L3 and the control, *E. coli* KRX ($p < 0.05$) (Figure 2).

3.2.3.2 Production of ammonia

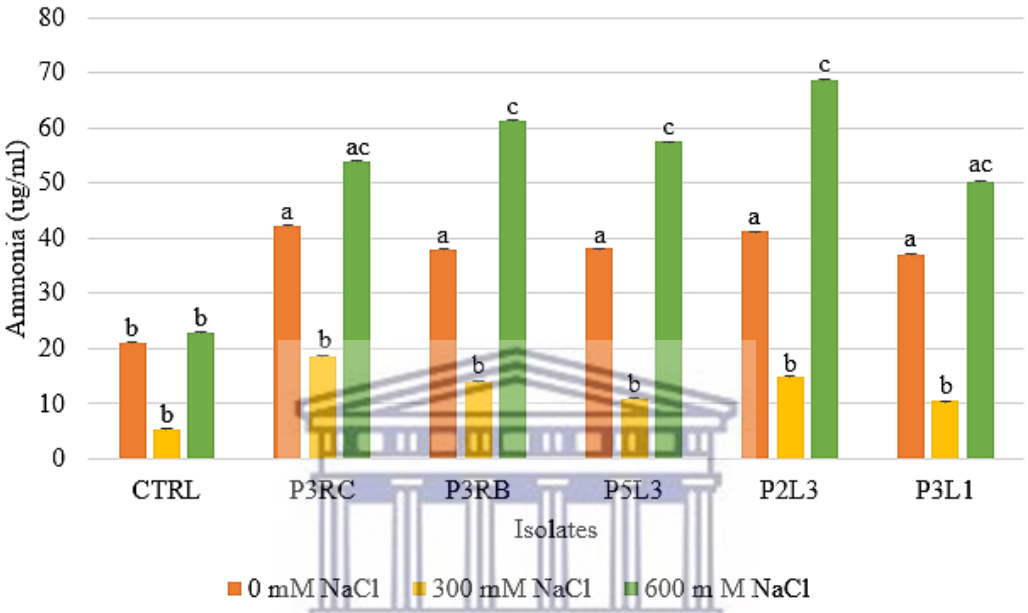

Figure 3. Ammonia production (ug/ml ± SE) of the endophytic isolates taken from *A. calendula* and the control (*E. coli* KRX) grown at 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significance is symbolized by a superscript [a].

The control, *E. coli* KRX, had produced the least amount of ammonia of all isolates ($p < 0.05$) (Figure 3). Interestingly, in the 300 mM NaCl treatments, all isolates produced significantly less ammonia when compared to their 0 mM and 600 mM NaCl treatments ($p < 0.05$) (Figure 3). All isolates, excluding the control, had demonstrated the greatest production of ammonia in the 600 mM NaCl treatment. However, only the isolates P2L3, P5L3 and P3RB displayed significantly greater ammonia production in their 600 mM NaCl treatment when compared to their performance in the 0 mM NaCl treatment ($p < 0.05$). This contrasts with P3L1 and P3RC which exhibited no significant differences between their 0 mM and 600 mM NaCl treatments (Figure 3).

3.2.3.3 Phosphorus solubilization activity

Table 3. Total diameter and colony diameter (millimeter ± SE) of the endophytic isolates taken from *A. calendula* grown on Pikovskayas media supplemented with 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significant differences are symbolized by superscript [a].

	Total Diameter (mm ± SE)			Colony Diameter (mm ± SE)		
Isolate	**0 mM**	**300 mM**	**600 mM**	**0 mM**	**300 mM**	**600 mM**
Control	1.33 ± 0.88^{bc}	1.00 ± 1.00^{bc}	2.33 ± 1.20^{abc}	1.33 ± 0.88^{abc}	1.00 ± 1.00^{abc}	2.33 ± 1.20^{abc}
P3L1	11.17± 2.95^{a}	10.33 ± 0.67^{abc}	10.33 ± 2.60^{abc}	10.00 ± 2.75^{abc}	8.50 ± 1.32^{abc}	8.50 ± 2.47^{abc}
P2L3	10.17 ± 0.73^{abc}	10.50 ± 0.24^{abc}	9.00 ± 0.00^{abc}	8.00 ± 1.53^{abc}	9.17 ± 0.73^{abc}	7.83 ± 0.73^{abc}
P5L3	3.17 ± 0.44^{abc}	5.33 ± 3.84^{abc}	4.17 ± 0.61^{abc}	3.17 ± 0.44^{abc}	5.33 ± 2.11^{abc}	4.17 ± 2.92^{abc}
P3RB	2.17 ± 0.17^{abc}	3.00 ± 0.58^{abc}	2.00 ± 0.76^{abc}	2.17 ± 0.17^{abc}	3.00 ± 0.58^{abc}	2.00 ± 0.76^{abc}
P3RC	5.33 ± 1.86^{abc}	8.00 ± 2.52^{abc}	6.17 ± 3.19^{abc}	3.67 ± 0.67^{abc}	7.67 ± 2.33^{abc}	6.17 ± 3.19^{abc}

Table 4. The phosphorus solubilization activity (%) of the endophytic isolates taken from *A. calendula* grown on Pikovskayas media supplemented with 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significant differences are symbolized by superscript [a]. P solubilization activity was calculated by ((total diameter – colony diameter)/colony diameter) x 100.

	P solubilization activity (%)		
Isolate	**0 mM**	**300 mM**	**600 mM**
Control	0.0^{d}	0.0^{d}	0.0^{d}
P3L1	11.7^{c}	21.5^{bc}	21.5^{bc}
P2L3	27.1^{b}	14.5^{c}	14.9^{c}
P5L3	0.0^{d}	0.0^{d}	0.0^{d}
P3RB	0.0^{d}	0.0^{d}	0.0^{d}
P3RC	45.2^{a}	4.3^{d}	0.0^{d}

All isolates were capable of growing on the Pikovskayas media, with and without NaCl supplementation (Table 3). However, only the isolates P3L1, P2L3 and P3RC were capable of solubilizing phosphate (P) from the media (Table 4). P3L1 had a colony diameter of 10 mm (which was significantly different to the control, *E. coli* KRX, $p < 0.05$), 8.50 mm and 8.50 mm for the salt treatments 0 mM, 300 mM and 600 mM NaCl, respectively; whereas a solubilization activity (SA) [measured as the ((total diameter – colony diameter)/colony

diameter) x 100] of 11.7%, 21.5% and 21.5% for the respective treatments (Table 4). Thus, P3L1 was able to solubilize P significantly better in the salt treatments than when compared to the control treatment (0 mM NaCl) ($p < 0.05$) (Table 4). Additionally, P3RC had a colony diameter of 3.67 mm, 7.67 mm and 6.17 mm for the treatments 0 mM, 300 mM and 600 mM NaCl, respectively. However, unlike P3L1, P3RC performed significantly better on the control treatment (0 mM NaCl) ($p < 0.05$) when compared to the salt treatments, where it had a SA of 45.2% in the 0 mM NaCl treatment, whereas it only had a SA of 4.3% in the 300 mM NaCl treatment and no activity was found in the 600 mM NaCl treatment (Table 4). Furthermore, P2L3 had a colony diameter of 8.00 mm, 9.17 mm and 7.83 mm at salt concentrations 0 mM, 300 mM and 600 mM NaCl with a SA of 27.1%, 14.5% and 14.9%, respectively. Thus, the SA of P2L3 in the 0 mM NaCl treatment was significantly higher than that of the 300 mM and 600 mM NaCl treatments ($p < 0.05$) (Table 4). Lastly, the SA of P3L1 (for all three treatments), P3RC (0 mM NaCl treatment) and P2L3 (all three treatments) were significantly higher than that of P3RB, P5L3 and the control, *E. coli* KRX, which displayed no P solubilization activity (Table 4).

3.2.3.4 Zinc solubilization activity

Table 5. Total diameter and colony diameter (millimeter ± SE) of the endophytic isolates taken from *A. calendula* grown on media supplemented with 0 mM, 300 mM and 600 mM NaCl as well as 0.2% insoluble zinc oxide (ANOVA, Tukey HSD, $p < 0.05$). Significance is symbolized by superscript [a].

	Total Diameter (mm ± SE)			**Colony Diameter (mm ± SE)**		
Isolate	**0 mM**	**300 mM**	**600 mM**	**0 mM**	**300 mM**	**600 mM**
Control	1.00 ± 0.00 [a]	1.67 ± 0.33 [a]	0.33 ± 0.33 [a]	1.00 ± 0.00 [a]	1.67 ± 0.33 [a]	0.33 ± 0.33 [a]
P3L1	3.00 ± 1.53 [a]	4.67 ± 3.71 [a]	13.33 ± 13.33 [a]	1.67 ± 0.33 [a]	3.00 ± 2.08 [a]	12.67 ± 12.67 [a]
P2L3	7.00 ± 3.21 [a]	6.00 ± 5.51 [a]	8.33 ± 6.01 [a]	5.00 ± 1.53 [a]	3.67 ± 3.18 [a]	6.33 ± 4.10 [a]
P5L3	2.67 ± 1.20 [a]	2.67 ± 1.76 [a]	3.33 ± 1.33 [a]	2.67 ± 1.20 [a]	2.33 ± 1.45 [a]	2.67 ± 0.67 [a]
P3RB	2.67 ± 0.67 [a]	4.67 ± 1.20 [a]	2.67 ± 1.76 [a]	2.67 ± 0.67 [a]	3.67 ± 0.67 [a]	2.67 ± 1.76 [a]
P3RC	3.33 ± 1.20 [a]	8.33 ± 1.67 [a]	6.67 ± 3.71 [a]	2.50 ± 0.87 [a]	3.67 ± 0.67 [a]	2.83 ± 0.93 [a]

Table 6. The zinc solubilization activity (%) of the endophytic isolates taken from *A. calendula* grown on media supplemented with 0 mM, 300 mM and 600 mM NaCl as well as 0.2% insoluble zinc oxide (ANOVA, Tukey HSD, $p < 0.05$). Significant differences are symbolized by superscript [a]. Zn solubilization activity was calculated using the formula: ((total diameter – colony diameter)/colony diameter) x 100.

	Zn solubilization activity (%)		
Isolate	**0 mM**	**300 mM**	**600 mM**
Control	0.0^{a}	0.0^{a}	0.0^{a}
P3L1	80.0^{f}	55.6^{e}	5.3^{a}
P2L3	40.0^{d}	63.6^{e}	31.6^{cd}
P5L3	0.0^{a}	14.3^{b}	25.0^{bc}
P3RB	0.0^{a}	27.3^{c}	0.0^{a}
P3RC	33.3^{cd}	127.3^{g}	135.3^{g}

All of the endophytic isolates and the control were capable of growing on the media supplemented with 0 mM, 300 mM and 600 mM NaCl as well as 0.2% insoluble zinc (Zn) oxide (Table 5). However, the control, *E. coli* KRX, was unable to solubilize Zn at any concentration of NaCl (Table 6). Zn solubilization activity (%) was measured using the formula ((total diameter – colony diameter)/colony diameter) x 100. There were no significant differences in total diameter and colony diameter for all isolates ($p < 0.05$) (Table 5). However, there were significant differences in Zn solubilization activity (SA) (Table 6). P3RB had a colony diameter of 2.67 mm, 3.67 mm and 2.67 mm for the treatments 0 mM, 300 mM and 600 mM NaCl, respectively (Table 5). However, P3RB was only able to solubilize Zn in the 300 mM NaCl treatment, with a SA of 27.3% (Table 6). The isolate P5L3 had a colony diameter of 2.67 mm, 2.33 mm and 2.67 mm for the treatments 0 mM, 300 mM and 600 mM NaCl (Table 5). Yet, it was only able to solubilize Zn in the two salt treatments, where it had a SA of 14.3% and 25% for the 300 mM and 600 mM treatments (Table 6). Thus, displaying increasing Zn solubility with increasing salinity. The opposite was seen for the isolate P3L1 which displayed decreasing Zn SA with increasing salinity. P3L1 had a colony diameter of 1.67 mm, 3.00 mm and 12.67 mm for the three treatments 0 mM, 300 mM and 600 mM NaCl, respectively (Table 5). For said treatments, the Zn solubilization activity was 80%, 55.6% and 5.3%, thus demonstrating a reduction in Zn SA as the salinity increased (Table 6). Furthermore, P3RC revealed significant up-regulation in Zn solubilization activity as salinity increased, with the greatest Zn SA out of all isolates within the salt treatments ($p < 0.05$). P3RC had a colony diameter of 5.0 mm, 3.67 mm and 6.33 mm for the three salt treatments (Table 5) and a Zn SA

of 33.3 %, 127.3% and 135.3% for said treatments (Table 6). Lastly, P2L3 had a colony diameter of 5.00 mm, 3.67 mm and 6.33 mm for the treatments 0 mM, 300 mM and 600 mM NaCl (Table 5). The Zn solubilization activity for P2L3 for the NaCl treatments (0 mM, 300 mM and 600 mM) were 40%, 63.6% and 31.6%, thus exhibiting the greatest Zn SA for the mid-range salinity treatment (Table 6).

3.2.3.5 Siderophore activity

Table 7. Total diameter and colony diameter (millimeter ± SE) of the endophytic isolates taken from *A. calendula* grown on Chrome Azurol S media supplemented with 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significance is symbolized by superscript [a].

	Total Diameter (mm ± SE)			**Colony Diameter (mm ± SE)**		
Isolate	**0 mM**	**300 mM**	**600 mM**	**0 mM**	**300 mM**	**600 mM**
Control	4.00 ± 1.00^{b}	3.33 ± 0.86^{b}	1.67 ± 0.88^{b}	2.33 ± 0.88^{b}	1.67 ± 0.67^{b}	0.67 ± 0.33^{b}
P3L1	23.33 ± 0.88^{bc}	25.33 ± 1.67^{bc}	25.00 ± 1.00^{bc}	9.33 ± 0.33^{bc}	11.33 ± 1.76^{ac}	11.67 ± 1.20^{ac}
P2L3	20.33 ± 1.76^{bc}	25.33 ± 1.86^{bc}	19.67 ± 0.67^{bc}	14.00 ± 2.08^{ac}	13.67 ± 1.45^{ac}	13.33 ± 0.33^{ac}
P5L3	15.67 ± 3.28^{bc}	15.83 ± 10.83^{bc}	13.33 ± 7.36^{bc}	6.00 ± 1.53^{bc}	5.67 ± 1.76^{bc}	6.67 ± 2.33^{bc}
P3RB	18.17 ± 13.67^{bc}	11.00 ± 5.00^{bc}	22.00 ± 14.50^{bc}	6.33 ± 3.84^{bc}	7.67 ± 3.18^{bc}	10.00 ± 4.00^{bc}
P3RC	32.33 ± 4.67^{ac}	34.67 ± 0.88^{ac}	35.67 ± 4.67^{ac}	8.00 ± 0.58^{bc}	12.00 ± 0.58^{ac}	15.67 ± 1.76^{ac}

Table 8. The Siderophore solubilization activity (%) of the endophytic isolates taken from *A. calendula* grown on Chrome Azurol S media supplemented with 0 mM, 300 mM and 600 mM NaCl (ANOVA, Tukey HSD, $p < 0.05$). Significant differences are symbolized by superscript [a]. Siderophore solubilization activity was calculated by ((total diameter – colony diameter)/colony diameter) x 100.

	Siderophore Solubilization Activity (%)		
Isolate	**0 mM**	**300 mM**	**600 mM**
Control	100.0^{b}	122.2^{b}	150.0^{b}
P3L1	151.1^{b}	139.9^{b}	117.4^{b}
P2L3	52.2^{a}	87.3^{ab}	32.4^{a}
P5L3	166.5^{bc}	127.8^{b}	79.3^{a}
P3RB	130.6^{b}	40.0^{a}	77.8^{a}
P3RC	311.0^{d}	189.5^{c}	129.7^{b}

All endophytes including the control *E. coli* KRX grew on the Chrome Azurol S media supplemented with 0 mM, 300 mM and 600 mM NaCl (Table 7). However, the total diameter of P3RC was significantly larger than that of the control for all three salt treatments ($p < 0.05$) (Table 7). Additionally, the colony diameters of P3RC and P2L3 for all three treatments were significantly greater than the control's colony diameter ($p < 0.05$) (Table 7). Furthermore, the colony diameters for P3L1 for the 300 mM and 600 mM NaCl treatment were significantly larger than the control's ($p < 0.05$) (Table 7).

P3RC had the greatest siderophore solubilization activity (SA) across all isolates and treatments in the 0 mM NaCl, with a SA of 311% ($p < 0.05$) (Table 8). P3RC displayed significant decreases in siderophore SA with increasing salinity ($p < 0.05$) (Table 8). The control, *E. coli* KRX, demonstrated no significant differences in siderophore SA across the three salt treatments ($p < 0.05$) (Table 8). However, it had a significantly greater siderophore SA when compared to that of P3RB (300 mM and 600 mM NaCl treatments), P3RB (600 mM NaCl) and P2L3 (0 mM and 600 mM NaCl) ($p < 0.05$) (Table 8). The highest siderophore SA for P3RB was seen in the 0 mM NaCl treatment with a SA of 130.6%, which was significantly larger than that of the 300 mM and 600 mM NaCl treatments which displayed no significant differences to each other (Table 8). Furthermore, the isolates P3L1 and P2L3 displayed no significant differences between the salt treatments, respectively (Table 8).

3.2.3.6 Production of Indole Acetic Acid

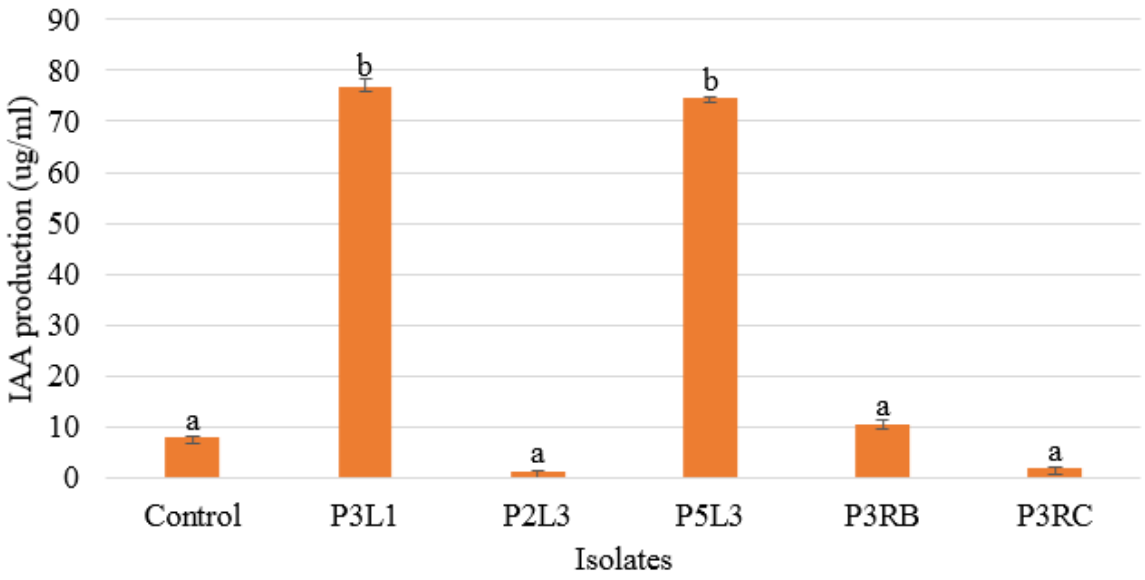

Figure 4. Indole acetic acid (IAA) production (ug/ml) of the endophytic isolates taken from *A. calendula* and the control, *E. coli* KRX. Significance is indicated by a superscript [a]. (*ANOVA, Tukey HSD*, $p < 0.05$).

The isolates P5L3 and P3L1 significantly produced the greatest amount of IAA when compared to the other isolates and the control ($p < 0.05$) (Figure 4). Furthermore, the control, P3RC, P3RB and P2L3 displayed no significant differences in IAA production ($p < 0.05$) (Figure 4).

3.3 Discussion

Saline soils are one of the most detrimental environmental stressors globally in agriculture, as they cause ionic imbalances in the soil which results in reduced crop growth and yield (Numan et al., 2018; Singh et al., 2018). This is especially seen in arid and semi-arid regions where frequent irrigation is used (Numan et al., 2018; Singh et al., 2018). As such, it is imperative that a long-term and sustainable solution to mitigating the effects of salinity be found (Singh et al., 2018). The use of plant growth promoting bacteria (PGPB) offers a safe, environmentally friendly and biological means to managing agricultural practices (Singh et al., 2018). A means to do this could be isolating endophytic PGPB from bacterial communities in saline environments, such as coastal marine plants colonized by halophilic or salt-tolerant endophytes (Shrivastava et al., 2015; Etesami et al., 2018). Furthermore, when compared to rhizospheric and phyllospheric bacteria, endophytes interact the closest with the host plant and owing to their close interaction, are able to directly or indirectly promote plant growth and health under abiotic stresses such as salinity (Zhao et al., 2016)

A. calendula, often called the cape dandelion or cape weed, is a native South African species belonging to the Asteraceae family (Campos et al., 2004). It is often found in coastal areas and disturbed areas, as it is highly invasive, particularly in coastal Mediterranean areas globally (Campos et al., 2004). The five strains of endophytic bacteria were isolated from *A. calendula* taken from Sunset Beach, Muizenberg, Cape Town, South Africa (34°06'15.6"S, 18°28'42.1"E) with all isolates capable of growing luxuriously on R2A agar media supplemented with 0 mM, 300 mM and 600 mM NaCl. As such, it was determined that the isolates were all halo-tolerant species with the capacity to grow on media up to 600 mM NaCl. Table 1 provides morphological characteristics about each isolate.

Identification of endophytes isolated from *A. calendula*

Using 16S rDNA amplicons and molecular analyses, the endophytes P3L1, P2L3, P5L3, P3RB and P3RC isolated from the *A. calendula* were identified. The endophyte P3L1 had been isolated from the leaf material of *A. calendula* had demonstrated a 99.6% similarity to the plant pathogen *Erwinia persicina* strain NBRC 102418 ^{T} (Table 2). *E. persicina*, priorly known as *E. persicinus* (Hao et al., 1990), belongs to the family Enterobacteriaceae (Zhang et al., 2014; Orel, 2020). Microorganisms of the genus *Erwinia* are motile (peritrichous flagella), Gram-negative, rod-shaped (Bottone et al.,1972), oxidase negative, catalase positive, fermentative, facultatively anaerobic (Dickey, 1979; Santos et al., 2009) which form pink (Zhang et al., 2014), peach (Hao et al., 1990) and beige (Orel, 2020) pigmented colonies. Members of this genus are mostly pathogenic, but can also be non-pathogenic, epiphytes and opportunistic human pathogens (Burroso et al., 2017). *Erwinia* species tend to infect susceptible plants through wounds and natural openings, where they enter into and damage vascular tissue before spreading to other plant tissue types (Zrelovs et al., 2020). *E. persicina* was first isolated from tomato, banana and cucumber (Hao et al., 1990). It is a pink or beige pigmented, opportunistic phytopathogen which tends to colonize priorly damaged or wounded plant tissues (Nechwatal et al., 2019). However, in this study, *E. persicina* NBRC 102418 ^{T} isolated from *A. calendula* was opaque-white in colour (Table 1). Furthermore, *E. persicina* is capable of enduring harsh environment conditions including arid, saline and alkaline environments as well as surviving under a wider range of pH (Zhang et al., 2014). Here, *E. persicina* NBRC 102418 ^{T} was able to grow on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl. *E. persicina* infection has been found in a variety of vegetable cultivars, including parsley which develops pink rot when infected (Nechwatal et al., 2019), common bean which show symptoms of generalized chlorosis and necrosis in the leaves and tendrils (leaf spot disease) (Santos et al., 2009). *E. persicina* infection has also been found in tomato, banana, cucumber (Hao et al., 1990), soybean and alfalfa crops which had shown seedling and floral wilt as well as the development of necrotic spots on the leaves, stems and flowers (Zhang et al., 2014). When infected, lettuce crops developed large water-soaked spots on the leaves as well as arduous rot (soft rot) (Orel, 2020). When infected with *E. persicina*, enoki mushrooms (Yan et al., 2019) and garlic demonstrated pink soft rot (Gálvez et al., 2015). *E. persicina* infections had been reported in the Anatolia peninsula, Turkey (Orel, 2020), Zhangzhou, China (Yan et al., 2019), Bavaria, Southern Germany (Nechwatal et al., 2019), Toledo, Spain (Gálvez et al., 2015). It is likely that the sample source, *A. calendula*, had been colonized by *E. persicina* NBRC 102418

^{T}, however, no signs of disease had been detected. This may suggest that *E. persicina* has specific host plant species which are susceptible to its infection, whereas other species are not. In Shen et al. (2012) *E. persicinus* RA2 (*E. persicina*) had demonstrated positive plant growth in tomatoes (*Lycopersicon esculentum* Mill.). Thus, specific strains of *E. persicina* may be phytopathogenic, whereas others may be PGPB.

P2L3 had been identified as the endophyte *Bacillus marisflavi* strain JCM 11544 ^{T} with a 100% similarity (Table 2). *B. marisflavi* had first been isolated from a tidal flat in the Yellow Sea, Korea by Yoon et al. (2003). In 2014, it had also been isolated from the rhizosphere of mustard plants in Haryana, India (Hariprasad et al., 2014). It is Gram-positive, or Gram-variable, aerobic, rod-shaped and motile (single polar flagellum) microorganism, which have central and subterminal ellipsoidal endospores in swollen sporangia (Yoon et al., 2003). Colonies are yellow in colour (Table 1), circular to slightly irregular in shape, have an optimal pH for growth at 6.0 to 8.0, however, growth had been observed at a pH of 4.5 (Yoon et al., 2003). Furthermore, it is capable of growing in the presence of 0 – 16% (w/v) NaCl, however, its optimal range had been determined as 2 – 5% (w/v) NaCl) (Yadav et al., 2016). In this study, *B. marisflavi* JCM 11544 ^{T} had been able to grow luxuriantly on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl. Furthermore, it is catalase-positive and oxidase-negative (Yoon et al., 2003). Most *Bacillus* species have been categorized with halophilic or halotolerant properties (Yoon et al., 2003) and are yellow-, orange- or pink-pigmented (Khaneja et al., 2010; Akayli, et al., 2016).

The third endophyte which had been isolated from *A. calendula* is P5L3, which had been established as *Ochrobactrum rhizosphaerae* strain PR17^{T} with a 97% similarity using 16S rDNA amplicons (Table 2). *O. rhizosphaerae* PR17^{T} had first been identified by Kämpfer et al. (2008) which had isolated it from the rhizosphere of a potato plant in Austria. The genus *Ochrobactrum* belongs to the Brucellaceae family, Alphaproteobacteria class (Huber et al., 2010; Imran et al., 2010) and was first introduced by Holmes et al. in 1988. Members of this genus have been recovered from vastly different sources, including soil, plants, rhizospheres, industrial environments, animals, humans and sludge (Imran et al., 2010; Li et al., 2016; Sigida et al., 2020; Szpakowska et al., 2020). However, some *Ochrobactrum* species, including O. anthropic, are opportunistic human pathogens, which are able to cause induce infections in immunocompromised individuals (Sigida et al., 2020). *Ochrobactrum* sp. are characterized as Gram-negative, motile, obligate aerobic oxidase- and catalase-positive as well as displaying a nitrate reduction activity (Li et al., 2016). *O. rhizosphaerae* are non-spore forming rods which

have shown good growth on R2A agar (Kämpfer et al., 2008). In this study, *O. rhizosphaerae* PR17^{T} was able to demonstrate good growth on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl. It was beige-orange-pigmented, circular, flat with an entire margin (Table 1). These results correspond with that of Kämpfer et al. (2008).

With a 97.2% similarity, the endophyte P3RB, isolated from the root of *A. calendula* was identified as *Microbacterium gubbeenense* strain DSM 15944 ^{T} (Table 2). The genus *Microbacterium* belongs to the Microbacteriaceae family, class Actinobacteria (Anand et al., 2012; Meng et al., 2016). Members of this genus are Gram-positive, catalase-positive, oxidase-negative (Chen et al., 2016), salt tolerant, non-spore-forming, rod-shaped and yellow, yellow-brown or yellow-white in colour (Brennan et al., 2001; Chen et al., 2016). They have been isolated from vastly different environments including soil, deep sea sediments (Shivaji et al., 2007), industrial waste sites, plants, insects, dairy products and humans (Anand et al., 2012; Yan et al., 2015; Chen et al., 2016). *M. gubbeenense* was first described by Brennan et al. (2001), where it was isolated from the surface of smear-ripened cheese, Gubbeen. Surface-ripened, or smear-ripened cheeses, e.g. Tilsiter, Limburger, Münster, Livarot and Gubbeen, are economically important in Europe and are characterized by the development of microbial communities which are largely undefined (Brennan et al., 2001) which give rise to a "red-orange smear" which consists of a viscous, microbial mat of yeasts and bacteria (Mounier et al., 2007; Mounier et al., 2009). The initial ripening of the cheese occurs by means of yeasts colonizing the cheese surface (Mounier et al., 2007; Mounier et al., 2009). The yeasts produce lactase and ammonia which recues the acidity of the cheese surface, which in turn allows for the colonization of bacterial communities which are less tolerant to lower pHs (Mounier et al., 2007; Mounier et al., 2009). In this study, *M. gubbeenense* DSM 15944 ^{T} was brightly yellow pigmented and able to grow on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl (Table 1). These characteristics are similar to that of Brennan et al. (2001).

The last endophyte which had been isolated from the roots of *A. calendula* was P3RC. Using 16S rDNA, P3RC demonstrated a 100% similarity with microbe *Bacillus zhangzhouensis* strain DW5-4^{T} (Table 2). Species belonging to the genus *Bacillus* are Gram-positive, aerobic or facultatively anaerobic, spore-forming and rod-shaped (Wu et al., 2019). They are ubiquitous in a vast majority of both terrestrial and marine environments, having been found in stratospheric air, soil, plants, humans and deep-sea sediments (Liu et al., 2016). *B. zhangzhouensis* DW5-4^{T} had first been isolated by Liu et al. (2016) from the aquaculture water of a shrimp farm in Zhangzhou, China. Omar et al. (2020), had also isolated B. zhangzhouensis,

from the wastewater effluent from a petroleum factory (Panki, Kanpur, India). *B. zhangzhouensis* DW5-4^T are Gram-positive, strictly aerobic, rod-shaped, motile (subpolar flagella), catalase- and oxidase positive and capable of surviving in 0 – 12% NaCl (w/v) (Liu et al., 2016). Furthermore, colonies are cream-white in colour and circular (Liu et al., 2016). Grown on R2A agar supplemented with 0 mM, 300 mM and 600 mM NaCl, *B. zhangzhouensis* DW5-4T was cream-orange pigmented and circular shaped (Table 1).

Characterization of endophytes isolated from *A. calendula*

A variety of assays were performed to establish whether the isolated endophytes were capable of plant growth promotion. These include nitrogen fixation, ammonia production, phosphorus and zinc solubilization, siderophore production as well as indole acetic acid production. Firstly, nitrogen (N) is an essential element required in large amounts (Grattan et al., 1998) as it makes up the integral building blocks of cellular components such as amino acids and nucleic acids (Hu et al., 2005). Plants take up nitrogen in the forms of nitrate (NO_3^-) and ammonium (NH_4^+) (Grattan et al., 1998; Gorham, 1999; Orhan, 2016). Park et al. (2005) developed a modification of the Burk's N-free medium which was used to determine the nitrogen fixation capacity of bacteria, where luxuriant growth was used to determine nitrogen fixation. The isolate *B. marisflavi* JCM 11544^T (P2L3) displayed a significantly greater colony diameter in the saline treatments, when compared to all the other isolates (Figure 2), which suggests that it is able to fix nitrogen at higher salinities. Furthermore, the isolate *B. marisflavi* JCM 11544^T (non-saline treatment) and *E. persicina* NBRC 102418^T displayed significantly greater colony growth when compared to the rest of the isolates and the control (0 mM and 300 mM NaCl) (Figure 2). Nitrogen fixation has long been attributed as part of the capabilities of several *Bacillus* species (Wahab, 1975; Seldin et al., 1984). Ding et al. (2005) isolated and identified nitrogen-fixing *bacilli* species from plant rhizospheres in Beijing, China, based on their growth on N_2-free media. One of these species was *B. marisflav*i and it had been the first demonstration of nitrogen fixation in *B. marisflavi* (Ding et al., 2005). In this study, we had demonstrated luxuriant growth of *B. marisflavi* JCM 11544^T on N_2-free media under saline conditions, where its growth increased with increasing salinity. Additionally, nitrogen fixing *Erwinia* species had been isolated by Neilson et al. (1976), Neilson (1979) Papen et al. (1979). Acetylene reduction as a means to fix nitrogen is widely distributed throughout the Enterobacteriaceae family (Neilson, 1979). One of the *Erwinia* species which had been identified with the capacity for

nitrogen fixation is *E. herbicola* (Nielson, 1979; Papen et al., 1979). However, little to no literature is available on nitrogen fixation in *E. persicina* NBRC 102418^T. This is one of the first studies which has demonstrated the potential of nitrogen fixation in *E. persicina* NBRC 102418T.

However, ammonia production is as vital as nitrogen fixation, owing to the fact that plants take up ammonia as a nitrogen source (Orhan, 2016). Plants cannot utilize molecular nitrogen without it being converted to ammonia by means of biological nitrogen fixation (Colnaghi et al., 1997; Richard et al., 2018; Rosenblueth et al., 2018). This process is carried out by diazotrophs, particularly rhizobacteria, which employ nitrogenase enzymatic systems that reduce dinitrogen to ammonia (Colnaghi et al., 1997; Mus et al., 2016; Rosenblueth et al., 2018). When assessing ammonia production, all were capable of producing ammonia (NH_3) (µg/ml) to some degree, however, the control, *E. coli* KRX, produced significantly less ammonia than all the other isolates in the 0 mM and 600 mM NaCl treatments (Figure 3). Interestingly, all the endophytic isolates displayed significantly less ammonia production in the mid-range saline treatment, when compared to the non-saline treatment and the highly saline treatment (Figure 3). This may owe to the fact that they come from an environment with an average salinity of 600 mM NaCl (marine salinity) and as such, it is what they are adapted to. It may also explain why the isolates produced greater quantities of ammonia in the highly saline treatment, when compared to the non-saline treatment (Figure 3). It is well established that genus *Bacillus* contains species with the capacity for nitrogen fixation (Mus et al., 2016), as such, it is unsurprising that the species *B. marisflavi* JCM 11544^T and *B. zhangzhouensis* DW5-4^T were capable of ammonia production (Figure 3). In 1963, Billing et al. had discovered the production of ammonia in an *Erwinia* sp. *E. amylovora*, however, it is not a phytopathogen. In the present study, we were able to demonstrate ammonia production under both saline and non-saline conditions for *E. persicina* NBRC 102418T. Furthermore, *Ochrobactrum* sp have demonstrated ammonia production (Ngom et al., 2004; Imran et al., 2014; Abraham et al., 2016; Singh et al., 2019) and *Microbacterium* nitrogen-fixing activity (Gtari et al., 2012; Puri et al., 2018), yet the literature remains lacking for nitrogen fixation and ammonia production by *O. rhizosphaerae* PR17^T as well as *M. gubbeenense* DSM 15944^T. Here, this study has provided evidence for ammonia production by *O. rhizosphaerae* PR17^T and *M. gubbeenense* DSM 15944^T in non-saline and saline conditions (Figure 3). Often, the intensive use of chemical fertilizers is used to compensate for nitrogen-poor soils (Etesami et al., 2018). However, the excessive addition of these inorganic fertilizers to agricultural land may further

degrade soils and the environment (Agbenin et al., 1997) by increasing salinity, altering the microbial flora of the soil and increasing the amount of toxic heavy metals in the soil (Doni et al., 2014; Etesami et al., 2016; Etesami et al., 2018). This study provides evidence for the use of salt-tolerant PGPB with the potential to be introduced as biofertilizers in the form of nitrogen fixing and ammonia producing microbes.

It was essential to find phosphorus (P) solubilizing salt-tolerant endophytes as soils with greater salinities often impose major limitations to plants in terms of P nutrition. P is one of the major macronutrients essential to plant growth (Rodríguez et al., 1999; Son et al., 2006; Etesami et al., 2018). Soils deficient in soluble P are highly likely to restrict crop growth and yield (Son et al., 2006). Intensive cultivation on both saline and non-saline soils deplete soil's mineral elements and as such often the use of inorganic NPK fertilizers are applied to the soils (Agbenin et al., 1997; Doni et al., 2014; Etesami et al., 2018). However, often the exogenously applied P fertilizers are easily precipitated into its insoluble forms, thus becoming unavailable for plant uptake and assimilation and leading to an excess of P on the crop land (Rodríguez et al., 1999; Son et al., 2006). When unmanaged, the damage incurred on the environment, owing to runoff and soil erosion, may be severe (Son et al., 2006). Additionally, the frequent use of inorganic fertilizers may increase soil salinity and therefore finding a biofertilizer with the potential to provide crops with P (Emami et al., 2019), without affecting soil salinity is imperative. In this study, three of the five endophytes taken from *A. calendula* displayed P solubilization activity, namely *E. persicina* NBRC 102418^T, *B. marisflavi* JCM 11544^T and *B. zhangzhouensis* DW5-4^T. The mechanisms used by bacteria for P solubilization consist of the production of organic acids and acid phosphatases (Rodríguez et al., 1999). It has already been established that some members of the genera *Erwinia* and *Bacillus* demonstrated P solubilization (Ahemad et al., 2014). *E. persicina* NBRC 102418^T, displayed increasing P solubilization activity when comparing the non-saline treatment to the two saline treatments, 300 mM and 600 mM NaCl (Table 3). Prabhu et al. (2018) had demonstrated P solubilization by *B. marisflavi* FA7. In contrast, the opposite trend was seen for *B. zhangzhouensis* DW5-4^T and *B. marisflavi* JCM 11544^T which both displayed significant decreases in P solubilization with increasing salinity (Table 3). *Pseudomonas* strains in a study by Deshwal et al. (2013) all displayed optimum P solubilization under 0 to 1.25% NaCl, which decreased significantly with increasing salinity. However, Tank et al. (2010) found PGPB (*Pseudomonas* spp.) with P solubilization capacity up to 6% NaCl. As such, all three isolates possess the ability to promote plant growth by means of P solubilization, however, *E. persicina* NBRC 102418^T possesses the greatest potential for

P solubilization under saline conditions. This result is interesting, as *E. persicina* NBRC 102418^T has been described as an opportunistic, weak phytopathogen in the literature, yet displays characteristics which make it a favorable plant growth promoting species.

The trace element zinc (Zn) is essential to plants in small amounts and it forms part of important structural and regulatory co-factors of a substantial number of enzymes and proteins (Cavagnaro, 2008; Alloway, 2009; Khanghahi et al., 2018). Zn deficiency is one of the most widespread micronutrient deficiencies in plants globally, causing nutritional and health problems in a third of the world's human population (Dinesh et al., 2018). Zn deficiency is not linked to the low concentrations of Zn in the soil, but rather the unavailability of soluble Zn in the soil (Gontia-Mishra et al., 2017). This is further exacerbated by increasing salinity on agricultural lands (Dinesh et al., 2018). This has led to the need for synthetic fertilizers to enhance the availability of Zn in soils on crop lands (Dinesh et al., 2018). However, of the Zn fertilizers that are applied exogenously, only 1.0 – 4.0 % of the applied Zn remains available for plant uptake, as 96.0 – 99.0% is converted back to unavailable Zn pools owing to the precipitation of Zn with carbonates, oxides, phosphatases, etc (Dinesh et al., 2018). As such, the need for Zn solubilizing microorganisms become imperative for agricultural soils. Zn solubilizing bacteria are bacteria that can transform insoluble Zn into its soluble forms, which then become available to plants for uptake (Sarvanan et al., 2011). The majority of Zn solubilizing bacteria belong to the genera *Pseudomonas, Bacillus, Enterobacter, Xanthomonas, Stentrophomonas* (Khanghahi et al., 2018). Interestingly, almost all the isolates found in this study displayed different trends in Zn solubilization for the three treatments (Table 5). *M. gubbeenense* DSM 15944^T only displayed Zn solubilization at the mid-range saline treatment (300 mM NaCl) and no solubilization activity was seen for the other treatments (Table 5). It is not yet clear why Zn solubility had only been shown in the 300 mM NaCl treatment. Evidence regarding *M. gubbeenense* Zn solubilization capacity is lacking and as such, this may be the first set of data available for *M. gubbeenense* Zn solubilization. The isolate *O. rhizosphaerae* PR17^T displayed increasing Zn solubilization with increasing salinity (Table 5). Other *Ochrobactrum* species, such as *O. anthropi* have also shown solubilization of zinc sulphate and zinc oxide (Imran et al., 2014). In contrast to *O. rhizosphaerae* PR17^T, *E. persicina* NBRC 102418^T demonstrated exceptional declines in Zn solubilization with increasing salinity (Table 5). Few studies have assessed the capacity of Zn solubilization in the genus *Erwinia*, as such this may be the first study to have addressed Zn solubilization in *E. persicina* under non-saline and saline conditions. Additionally, the isolate *B. marisflavi* JCM 11544^T displayed unusual

results, with its greatest Zn solubilization activity seen in the mid-range salinity treatment, whereas the least activity was seen in the highly saline treatment (600 mM NaCl). *B. zhangzhouensis* DW5-4^T was the best performing isolate in terms of Zn solubilization, as it exhibited significantly higher Zn solubilization under salinity, outperforming all the other isolates in the saline treatments (Table 5). *B. megaterium* and *B. edaphicus* are two *Bacillus* species which have also been found to increase the bioavailability of Zn in soil (Wu et al., 2006). Intriguingly, *B. zhangzhouensis* had demonstrated no Zn solubilization in a study by Kushwaha et al. (2020), which contrasts with the results we have observed in this study. This study offers insight into the Zn solubilization capacity of halo-tolerant endophytic bacteria and the potential it holds as biofertilizers for the agricultural sector.

The availability of the essential micronutrient, iron (Fe), is often limited in the natural environment, owing to the fact that it exists in highly insoluble ferric oxides (Alexander et al., 1991; Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). The dissolution of these insoluble Fe forms liberates the soluble forms of Fe, Fe^{2+} and Fe^{3+} which can be taken up by plants and microorganisms (Alexander et al., 1991). As such, the evolution of Fe scavenging mechanisms had evolved in microorganisms under Fe-limited conditions (Alexander et al., 1991) as well as a means to combat the colonization of pathogenic microorganisms (Loaces et al., 2011). Many PGPB and rhizobacteria secrete low molecular weight, iron-binding molecules called siderophores (Alexander et al., 1991; Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). These siderophores have a high affinity for Fe^{3+} and from ferric-siderophore complexes, which makes Fe soluble and in doing so, Fe becomes available for plant uptake (Alexander et al., 1991; Bashan et al., 2005; Ahemad et al., 2014; Sagar et al., 2018). Additionally, siderophore secretion helps facilitate plant-bacteria associations which contribute to leaf, root and stem colonization (Loaces et al., 2011). Siderophore production have been demonstrated in members of the genera *Azotobacter*, *Pseudomonas* and *Bacillus* (Tian et al., 2009).

In this study, the isolate, *B. zhangzhouensis* DW5-4^T, had the greatest siderophore solubilization activity of all the isolates captured from *A. calendula*, however demonstrated significant decreases in siderophore solubilization activity with increasing salinity (Table 7). It is well known in the literature that some members of the genus *Bacillus* produce siderophores to promote its colonization in plants and their rhizospheres through the antagonistic activity of siderophores on plant pathogens (Yu et al., 2011) as seen in *B. subtilis* CAS15 (Yu et al., 2011) and *Bacillus* sp. J119 (Sheng et al., 2008). Whereas, the isolates *E. persicina* NBRC 102418^T,

B. marisflavi JCM 11544^T and the control, *E. coli* KRX, displayed no significant differences in siderophore solubilization activity across the three treatments (0 mM, 300 mM and 600 mM NaCl) (Table 7). In spite of these results, it is important not to categorize *E. persicina* NBRC 102418^T and *B. marisflavi* JCM 11544^T with the control and claiming them to have similar results for their solubilization activities (SA), as *E. persicina* NBRC 102418^T (for the saline treatments) and *B. marisflavi* JCM 11544^T had significantly greater colony diameters to the control, *E. coli* KRX (Table 6). Other *Erwinia* species which have demonstrated siderophore activity include *E. chrysanthemi* (*Dickeya dadantii*) (Persmark et al., 1989; Münzinger et al., 2000; Expert et al., 2008) and *E. amylovora* (Smits et al., 2011), both of which are also plant pathogens. The presence of Fe-chelating factors in plant pathogens are important for its virulence owing to its antagonistic effect on other microorganisms (Expert et al., 2008), as such it is unsurprising that *E. persicina* NBRC 102418^T demonstrates siderophore activity as it is a recognized phytopathogen. However, owing to the opportunistic pathogenic nature of *E. persicina* NBRC 102418^T, the presence of siderophore activity may be beneficial for plant species which are not susceptible to the *E. persicina* infection, but may tolerate its colonization. Furthermore, members of the Actinobacteria phylum are known to produce siderophores as secondary metabolites and display other plant growth promoting properties (Corretto et al., 2020). As such, the presence of siderophore activity by *M. gubeenense* DSM 15944^T was expected. *M. gubbeenense* DSM 15944^T and *O. rhizosphaerae* PR17^T had both displayed siderophore activity, however, exhibited significant decreases in siderophore SA with increasing salinity (Table 8). Other siderophore-secreting *Microbacterium* sp. include *Microbacterium* sp. F10a (Sheng et al., 2009), *M. oxydans, M. phyllosphaerae, M. foliorum, M. ginsengisoli, M. hydrocarbonoxydans, M. ketosireducens, M. oleivorans, M. yannicii* (Coreeto et al., 2020), *M. paraoxydans* (Kaur et al., 2011) and *M. metallidurans* TL13 (Ouertani et al., 2020). Additionally, members of the genus *Ochrobactrum* have also displayed siderophore activity, including *Ochrobactrum* sp. SP18 (Martin et al., 2006), *O. intermedium* CP-2 (Saini et al., 2017) and *O. anthropic* TRS-2 (Chakraborty et al., 2009). These results obtained by *M. gubbeenense* DSM 15944^T and *O. rhizosphaerae* PR17^T were somewhat expected, as rising salinity does place saline stress on the endophytes, as was found in Tank et al. (2010) and Deshwal et al. (2013). Interestingly, the isolates *E. persicina* NBRC 102418^T and *B. marisflavi* JCM 11544^T displayed no significant differences in their siderophore SA. However, *B. zhangzhouensis* DW5-4^T still demonstrated that greatest SA under the highest salinity treatment (Table 8). Thus, *E. persicina* NBRC 102418T, *B. marisflavi* JCM 11544^T and *B. zhangzhouensis* DW5-4^T displayed positive results for siderophore activity under rising

salinity. However, only *B. marisflavi* JCM 11544^T and *B. zhangzhouensis* DW5-4^T should be considered for plant inoculation, owing to pathogenic capacity of *E. persicina* NBRC 102418^T.

Lastly, indole-3-acetic acid (IAA) is a phytohormone involved in plant growth and development (Kaya et al., 2009; Javid et al., 2011; Fahad et al., 2015). It plays an important role in controlling vascular tissue development, apical dominance and cell elongation (Wang et al., 2001). Under stressful conditions, IAA has been associated with plant growth response and significant evidence has been found for improved plant growth and development under salt stress when inoculated with IAA-producing PGPB (Egamberdieva, 2009; Sadeghi et al., 2012). Prokaryotes make use of several IAA biosynthetic pathways which are classified based on their intermediatory constituents, including indole-3-acetamide (IAM), indole-3-pyruvate (IPyA), tryptamine and indole-3-acetonitril pathways (Manulis et al., 1998; Yang et al., 2007).This study identified two endophytes, namely *O. rhizosphaerae* JCM 11544^T and *E. persicina* NBRC 102418^T, with the capacity to produce significantly greater quantities of IAA than the control, *E.coli* KRX, and the other isolates (Figure 4). Other IAA-producing *Ochrobactrum* species which have been identified includes *O. intermedium* CP-2 (Saini et al., 2017), *Ochrobactrum* sp. MGJ11 (Yu et al., 2016) and *O. cicero* (Imran et al., 2010). This is a positive result, as other studies including Tiwari et al. (2011) demonstrated increased wheat growth and higher IAA content in the rhizosphere of IAA-producing salt tolerant *Halomonas* sp. A similar study performed by Li et al. (2017) displayed a similar result when inoculating maize seedlings with salt-tolerant, IAA-producing *B. aquimaris* DY-3, where *B. aquimaris* DY-3 alleviated salt stress in the crop species, maize. However, the presence of IAA synthesis in *E. persicina* NBRC 102418^T needs to be studied further, as a result of the phytopathogenic tendencies *E. persicina* had demonstrated in other studies (Santos et al., 2009; Zhang et al., 2014; Gálvez et al., 2015; Nechwatal et al., 2019; Yan et al., 2019; Orel, 2020). IAA production in phytopathogens are involved in the spread of infection (Yang et al., 2007). Mechanisms implemented in pathogenesis include epiphytic colonization, promotion of host cell division, inhibition of pathogenic defenses by host and the stimulation of pathogen growth within the plant tissues (Kunkel et al., 2018). In cases such as these, the production of IAA is seen as a virulence factor (Kunkel et al., 2018). An example of this can be found in *E. herbicola* which produces substantial amounts of IAA on plant surfaces, enough to affect the physiology of the plant (Brandl et al., 1998). As such, a clear distinction between pathogenic and non-pathogenic strains of *E. persicina* need to be made before inoculating host plants with it.

3.4 Conclusion

All the endophytes isolated from *A. calendula* had displayed some plant growth promoting characteristics under both saline and non-saline conditions. *Erwinia persicina* NBRC 102418^T had revealed N_2-fixation, ammonia production, phosphate and zinc solubilization as well as siderophore and IAA production. However, owing to the pathogenic nature of certain *E. persicina* strains, these properties may not necessarily benefit plant growth, but instead add to its virulence factors, helping to establish colonies more easily in susceptible host plants. However, *E. persicina* NBRC 102418^T may belong to the group of *E. persicina* strains which are non-pathogenic. *Bacillus marisflavi* JCM 11544^T had exhibited N_2-fixation, ammonia production, phosphate solubilizing, zinc solubilizing and siderophore producing properties. *Ochrobactrum rhizosphaerae* PR17^T had shown ammonia production, zinc solubilization under saline conditions, siderophore and IAA production. *Microbacterium gubbeenense* DSM 15944^T had demonstrated ammonia production, zinc solubilization (only under 300 mM NaCl) and siderophore production. *Bacillus zhangzhouensis* DW5-4^T had presented ammonia production, phosphate solubilization, zinc solubilization and siderophore production.

CHAPTER 4: ALLEVIATION OF SALT STRESS ON THE GROWTH OF *PHASEOLUS VULGARIS* USING PLANT GROWTH PROMOTING ENDOPHYTIC BACTERIA ISOLATED FROM *ARCTOTHECA CALENDULA*

Abstract

Aims: The aim of this chapter was to inoculate *P. vulgaris* cv. Star 2000 with salt-tolerant, plant growth promoting bacteria isolated from the halophyte, *A. calendula* and determine whether plant growth promotion is occurring under saline stress.

Methods: P. vulgaris cv. Star 2000 seeds were imbibed in bacterial cultures containing the PGPB, *Erwinia persicina* NBRC 102418^T, *Bacillus marisflavi* JCM 11544^T, *Ochrobactrum rhizosphaerae* PR17^T, *Microbacterium gubbeenense* DSM 15944^T and *Bacillus zhangzhouensis* DW5-4^T, before being planted. The seedlings were exposed to 0 mM and 100 mM NaCl once they had reached the first trifoliate. After harvesting, plant dry mass, NaCl-induced cell death, superoxide concentration and nutrient content of *P. vulgaris* cv. Star 2000 was determined.

Conclusion: Salinity reduced *P. vulgaris* cv. Star 2000's dry mass, enhanced cell death, superoxide concentration and reduced plant nutrient uptake. However, the PGPB were able to alleviate the burden of salt stress on *P. vulgaris* cv. Star 2000 by improving plant growth and nutrient uptake and decreasing salinity-induced programmed cell death and superoxide concentration.

4.1 Introduction

The demand for greater food production rates has increased significantly owing to an increasing global human population (Shrivastava et al., 2015), which is currently displaying a growth rate of approximately 1.05% each year (Kumar et al.,2020). It is estimated that the world population is to reach 10 billion by 2050 and thus, grave foot shortages are expected (Rani et al., 2019). A 70% rise in food crop productivity is required by 2050 to feed the additional 2.3 billion people globally (Gupta et al., 2014). However, land available for cultivation has reduced which has placed a great deal of stress on the sustainability of agriculture (Shrivastava et al., 2015; Kumar et al., 2020). Abiotic influences such as temperature, drought, heavy metal contamination and salinity are considered the most environmentally stressful conditions for plant growth and development (Singh et al., 2018). Yet, of these, salinity remains the most widespread and detrimental to plant development on arable land (Isayenkov, 2012; Singh et al., 2018; Numan et al., 2018; Kumar et al., 2020).

It is approximated that by 2050, 50% of arable land will be affected by soil salinity (Shrivastava et al., 2015; Kumar et al., 2020). Currently, salt-affected soils make up 932 million hectares (m ha) across the globe of which 351 m ha is affected by salinity (Rani et al., 2019). Furthermore, soil salinity affects 45 m ha of irrigated land, which produces one-third of the world's food (Rani et al., 2019). Soils are described as saline when the electrical conductivity of the saturation extract in the root zone is greater than 4 dS.m-1 (almost 40 mM NaCl) with 15% exchangeable sodium at 25°C (Shrivastava et al., 2015; Safdar et al., 2019). The accumulation of salt ions in soil owing to anthropogenic effects is called secondary salinization (Isayenkov, 2012) and is often the result of leaching, poor drainage, and the use of irrigated groundwater containing high amounts of salt ions (Numan et al., 2018; Kumar et al., 2020). These salt ions include magnesium (Mg^{2+}), potassium (K^{+}), calcium (Ca^{2+}), bicarbonate (HCO_3^{-}), sulphate (SO_4^{2-}), carbonate (CO_3^{2-}), however the major constituent with the greatest impact on plant growth and soil health is sodium (Na^{+}) and chloride (Cl^{-}) (Numan et al., 2018; Kumar et al., 2020). The impact of secondary salinization is even more detrimental in arid and semi-arid regions demarcated for agricultural practices (Shrivastava et al., 2015). These areas experience high temperatures and low precipitation which encourages the use of irrigated water (Shrivastava et al., 2015). This has been exacerbated even further by global warming (Zhang et al., 2018).

Globally, most crop species are derived from glycophytic species which demonstrate sensitivity to salinity (Glenn et al., 1999; Isayenkov, 2012). Ion toxicity, osmotic stress, nutrient deficiency and oxidative stress are the direct effects of soil salinity on plants (Shrivastava et al., 2015; Orhan, 2016; Torche et al., 2018). Glycophytic plant species experience phytotoxicity owing to the accumulation of Na^+ and Cl^- in the plant tissues, decreased water potential in the root zone resulting in reduced water uptake, nutrient imbalances owing to excess Na^+ and Cl^- in the plant tissues and root zone area and the oxidative damage owing to the production of reactive oxygen species (ROS) (Torche et al., 2018). In turn, this impacts plant germination, growth, development, biochemistry, reproductivity and photosynthesis (Shrivastava et al., 2015; Orhan, 2016). For that reason, research geared towards finding solutions for soil salinity on agricultural land has increased. The strategies examined comprise of the development of salt-tolerant breeding lines (Yokoi et al., 2002; Chaves et al., 2009; Torche et al., 2018), transgenic plants ([b]Zhu, 2001) and the use of plant growth promoting bacteria (PGPB) (Shrivastava et al., 2015; Orhan, 2016; Numan et al., 2018, Singh et al., 2018; ; Moreira et al., 2020). However, the latter approach has become more popular during the last decade as it requires less financial investment, is less labour intensive, less time consuming and does not deploy modified genes into the environment which can affect local ecosystems (Moreira et al., 2020).

The importance of microorganisms in plant growth promotion, disease control and nutrient management is well established (Shrivastava et al., 2015). PGPB possess the ability to promote plant growth either indirectly or directly, by suppressing plant pathogens, production of phytohormones and solubilization of insoluble mineral nutrients (Orhan, 2016; Numan et al., 2018). Indirectly, PGPB can inhibit or decrease the detrimental effects of phytopathogens on plants, by enhancing the resistance of the host against the pathogen (Orhan, 2016). Directly, PGPB can promote plant growth by fixing atmospheric nitrogen (N) into forms plants can take up (NO_3^-), synthesizing phytohormones such as indole acetic acid (auxins), cytokinins, and gibberellins and producing compounds such as siderophores, ACC Deaminase and organic acids (Shrivastava et al., 2015; Orhan, 2016). Siderophore-producing bacteria are valuable for their ability to secrete high affinity iron-chelating compounds (siderophores) which improve the bioavailability of iron in the soil (Shrivastava et al., 2015; Orhan, 2016). Additionally, the secretion of organic acids by PGPB in the rhizosphere acidify soils improving the solubility of mostly insoluble mineral nutrients, making them available for uptake by plants (Shrivastava et al., 2015; Orhan, 2016). Furthermore, Induced Systemic Tolerance (IST), a proposed term for

the physical and chemical changes induced by PGPB, that results in enhanced abiotic stress tolerance in plants has been introduced (Shrivastava et al., 2015). Bacteria occupying highly stressed environments, including highly saline habitats and the rhizosphere of halophytic plants (Etesami et al., 2018), possess traits that allow them to tolerate that environment (Shrivastava et al., 2015). When inoculating wheat (*Triticum aestivum*) with salt-tolerant PGPB, namely *Bacillus pumilus*, *Pseudomonas mendocina*, *Arthrobacter* sp., *Halomonas* sp., and *Nitrinicola lacisaponensis*, increased root and shoot length, biomass, chlorophyll, carotenoid and protein levels were observed in the plants (Tiwari et al., 2011). Tomato plants inoculated with the PGPB *P. fluorescens* YsS6 demonstrated greater fresh and dry biomass, higher chlorophyll concentrations, and developed more floral buds under saline conditions, when compared to those plants which had no been inoculated with the PGPB (Ali et al., 2014). As such, inoculations with PGPB could serve as a powerful tool for the alleviation of salinity stress in glycophytic crop species (Shrivastava et al., 2015).

Phaseolus vulgaris cv. Star 2000, more commonly known as the common bean, is globally significant legume crop species belonging to the Fabaceae family (Kumar et al., 2012; Torche et al., 2018; Padilla-Chacón et al., 2019). It is an herbaceous, self-pollinating, glycophytic, annual species (Graham et al., 1997). It is an inexpensive staple crop species, specifically in low-income countries in Africa, Asia and Latin America (Blair et al., 2003; Kumar et al., 2012; Chekanai et al., 2018; Diana et al., 2018; Padilla-Chacón et al., 2019). It is composed of a number of essential vitamins, minerals, fibre, protein, carbohydrates and phytochemicals (including polyphenolic compounds) which help prevent bodily disorders such as obesity, cardiovascular disease and elevated blood glucose levels (Chekanai et al., 2018; Torche et al., 2018; Chigwedere et al., 2019; Mendoza-Sánchez et al., 2019). On account of the symbiotic relationship it forms with nitrogen-fixing bacteria in root nodules, it plays a significant role in agriculture (Chekanai et al., 2018; Torche et al., 2018). Thus, owing to the importance of *P. vulgaris cv. Star 2000* as a global agricultural crop and its sensitivity to salinity (Torche et al., 2018), it is essential to find strategies for alleviating salinity stress the species.

4.2 Results

4.2.1 Plant dry mass

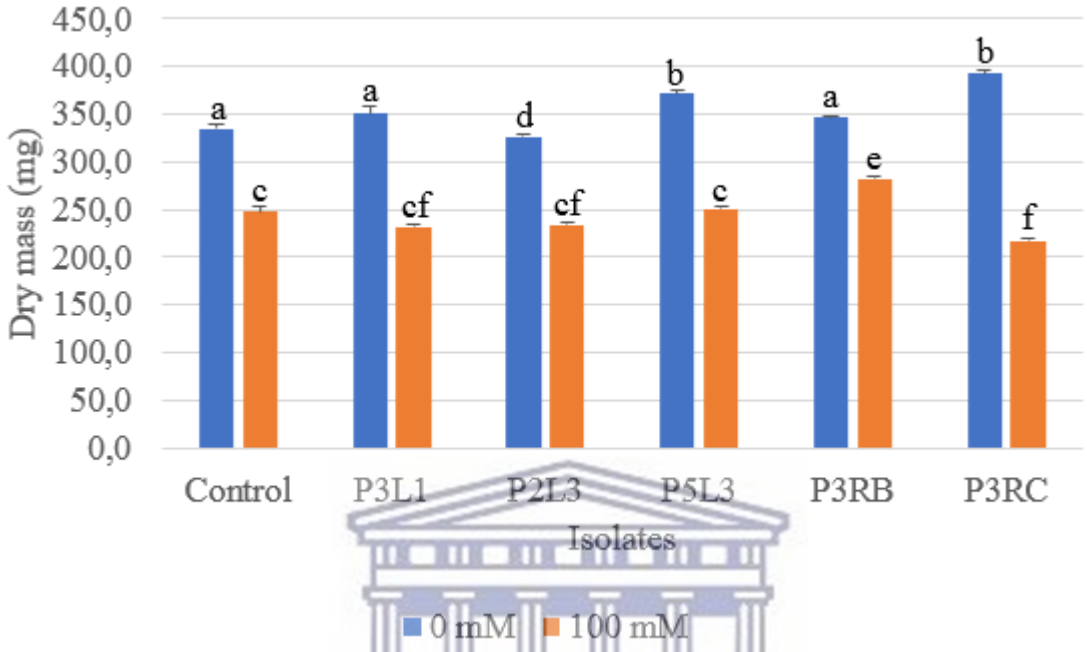

Figure 5. The dry mass (mg) of the leaf material taken from *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl after being inoculated with the bacterial endophytes P3L1, P2L3, P5L3, P3RB and P3RC. The control was not inoculated with any bacterial endophyte. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

Between the two saline treatments, overall, the treatment with the highest dry mass (mg) for each endophytic isolate was the 0 mM NaCl treatment (Figure 5). Within the 0 mM NaCl treatment, the isolates P5L3 (371 mg) and P3RC (392.7 mg) had significantly greater leaf dry masses when compared to all the other isolates and the control treatment ($p < 0.05$). P3L1 (350.7 mg) and P3RB (346 mg) displayed no significant differences to the control (334.3 mg) in the 0 mM treatment, however, P2L3 (326.3 mg) displayed significantly lower leaf dry mass when compared to all the other treatments ($p < 0.05$). Furthermore, in the 100 mM NaCl treatment, P3RB (281.7 mg) had the greatest leaf dry mass when compared to all the other isolates and the control ($p < 0.05$). The isolates P3L1 (232 mg), P2L3 (233.7 mg) and P5L3 (250.3 mg) displayed no significant differences in leaf dry mass in the 100 mM NaCl treatment when compared to the control (247 mg). In contrast, P3RC (217.3 mg) had the lowest dry mass in comparison to the other isolates (100 mM NaCl), however, it was not significantly different from the dry masses of P3L1 and P2L3.

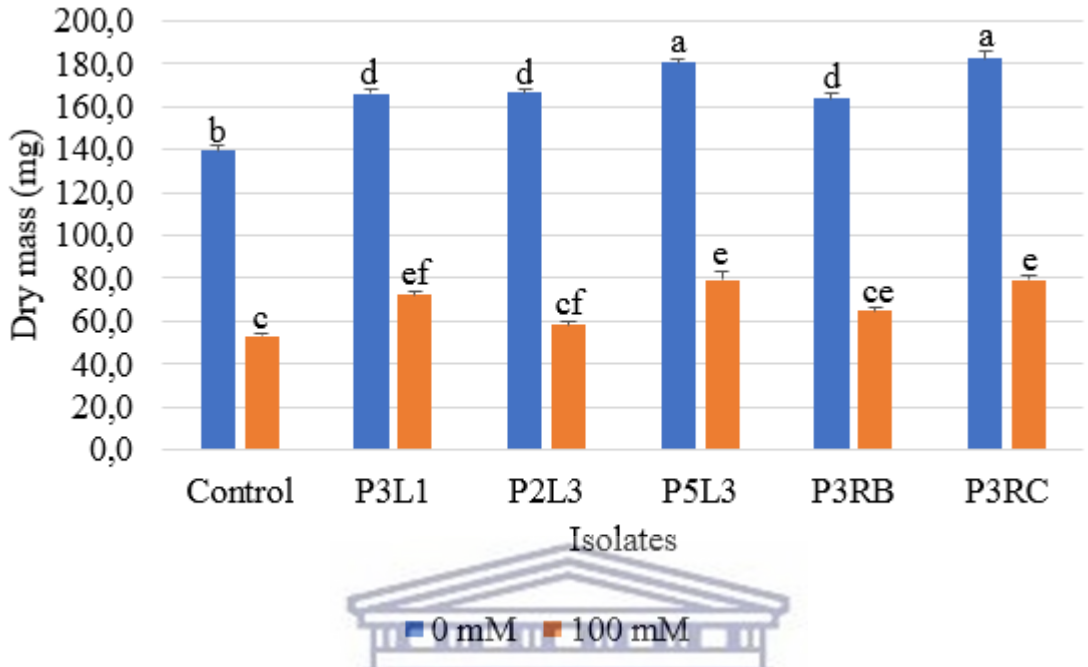

Figure 6. The dry mass (mg) of the root material taken from *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl after being inoculated with the bacterial endophytes P3L1, P2L3, P5L3, P3RB and P3RC. The control was not inoculated with any bacterial endophyte. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

The 0 mM NaCl treatment had root dry masses significantly larger for every isolate in comparison to the 100 mM NaCl treatment ($p < 0.05$) (Figure 6). Within the 0 mM NaCl treatment, the isolates P5L3 (180.3 mg) and P3RC (182.3 mg) had root dry masses significantly higher than all the other isolates, including the control ($p < 0.05$). Whereas the control (0 mM) had a significantly lower root dry mass (139.6 mg) than all the isolates. In addition, whereas the isolates P3L1 (166 mg), P2L3 (166.3 mg) and P3RB (163.7 mg) displayed no significant differences in root dry mass in the 0 mM NaCl treatment. In contrast, the control (139.6 mg) treatment had a root dry mass significantly lower than all the isolates in the 0 mM NaCl treatment ($p < 0.05$).

In the 100 mM NaCl treatment the root dry masses decreased significantly when compared to the 0 mM NaCl treatment. In the saline treatment (100 mM NaCl), the isolates P3L1 (72.3 mg), P5L3 (79.3 mg) and P3RC (79 mg) displayed the highest root dry masses when compared to

the rest of the isolates and as well as the control ($p < 0.05$). However, the isolates P3L1 and P2L3 (58.3 mg) displayed no significant differences in root dry mass ($p < 0.05$). Furthermore, the isolates P2L3, P3RB (64.7 mg) and the control (53 mg) exhibited the lowest root dry masses in the 100 mM NaCl treatment. P2L3, P3RB and the control in the 100 mM NaCl treatment, demonstrated no significant differences in root dry mass.

4.2.2 Determination of cell death

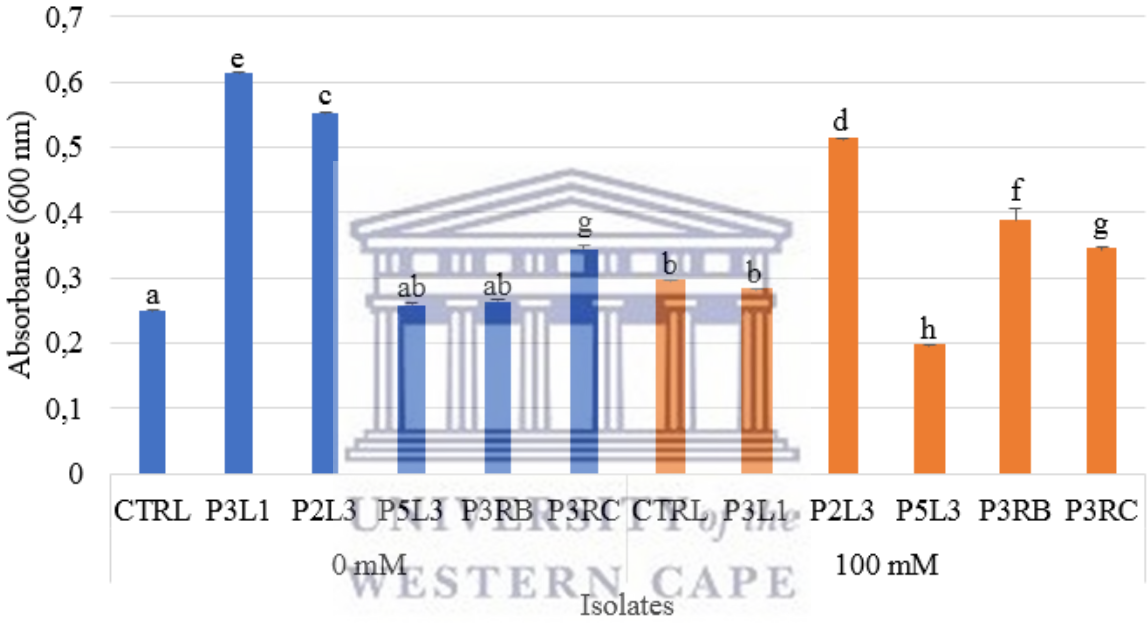

Figure 7. Determination of cell death in *P. vulgaris* cv. Star 2000 leaves after plants were exposed to 0 mM and 100 mM NaCl and inoculated with the isolates P3L1, P2L3, P5L3 and P3RC. Control plants were not inoculated with endophytic bacteria. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

The results for the determination of cell death in *P. vulgaris* cv. Star 2000 leaves after being exposed to 0 mM and 100 mM NaCl varied for each isolate (Figure 7). The control displayed higher cell death in the 100 mM NaCl treatment when compared to the 0 mM NaCl treatment ($p < 0.05$). In the 0 mM NaCl treatment, P3L1 reported the highest cell death observations, closely followed by P2L3. P5L3 and P3RB presented similar results to the control in 0 mM NaCl ($p < 0.05$). In the 100 mM NaCl treatment, significant decreases in leaf cell death had been displayed for P3L1, P5L3 and P2L3 when compared to the non-saline treatment ($p < 0.05$). However, an increase in leaf cell death had been reported for P3RB in the saline

treatment ($p < 0.05$). Lastly, P3RC displayed no significant differences in leaf cell death between the two saline treatments ($p < 0.05$).

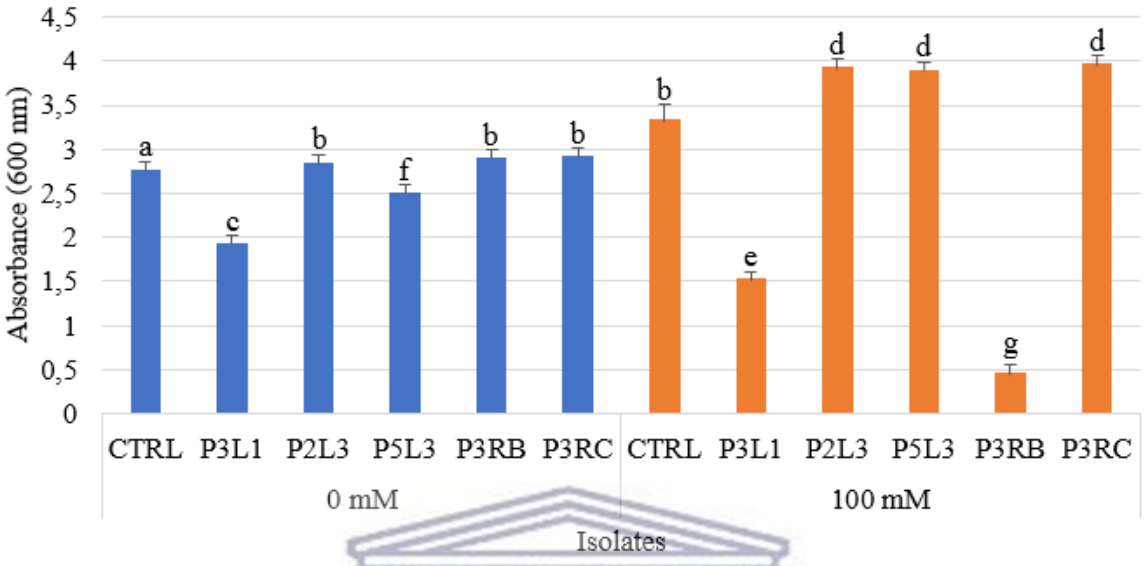

Figure 8. Determination of cell death in *P. vulgaris* cv. Star 2000 roots after plants were exposed to 0 mM and 100 mM NaCl and inoculated with the isolates P3L1, P2L3, P5L3 and P3RC. Control plants were not inoculated with endophytic bacteria. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

Contrasting results can be seen in the determination of root cell death for *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl (Figure 8). A similar trend can be seen in the control treatment for leaf cell death (Figure 7) and root cell death (Figure 8), where a significant increase in root cell death was seen in the 100 mM NaCl treatment, when compared to the 0 mM NaCl treatment ($p < 0.05$). In the 0 mM NaCl treatment, P3L1 reported the least amount of cell death, followed closely by P5L3 ($p < 0.05$). Additionally, no significant difference in root cell death was observed for the isolates P2L3, P3RB and P3RC in the non-saline treatment. The only two groups which had demonstrated significant decreases in root cell death in the 100 mM NaCl treatment, when compared to the 0 mM NaCl treatment, were the plants inoculated with P3L1 and P3RB ($p < 0.05$). The rest of the isolate groups had shown increased root cell death in the 100 mM NaCl treatment ($p < 0.05$).

4.2.3 *Determination of superoxide concentration*

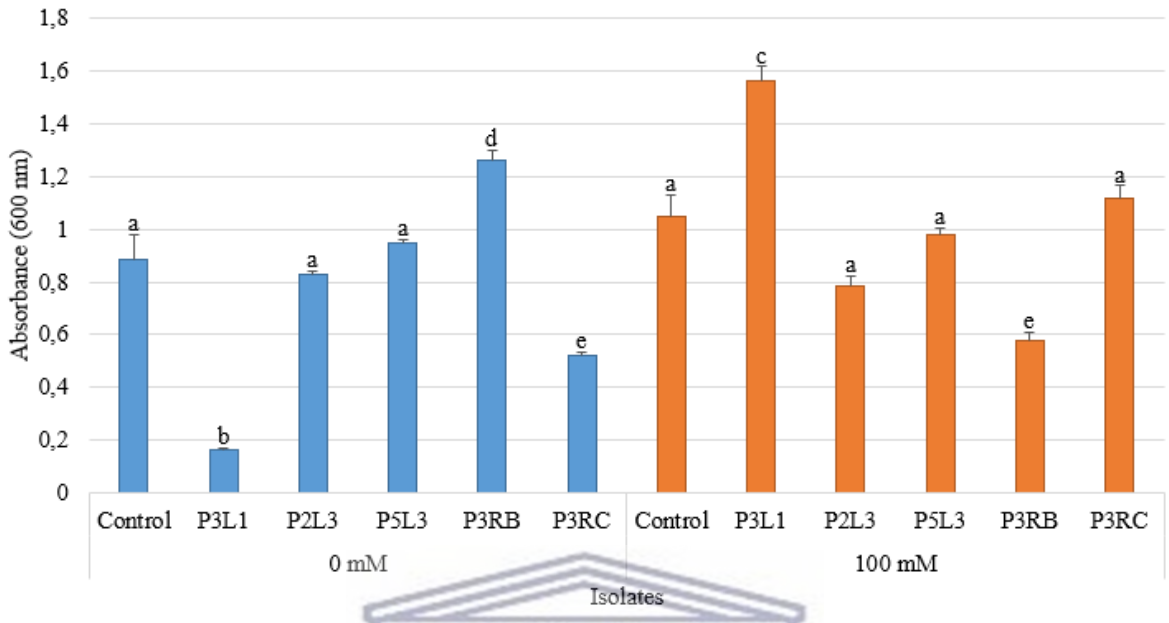

Figure 9. Determination of superoxide concentration in *P. vulgaris* cv. Star 2000 leaves after plants were exposed to 0 mM and 100 mM NaCl and inoculated with the isolates P3L1, P2L3, P5L3 and P3RC. Control plants were not inoculated with endophytic bacteria. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

Superoxide concentration in the leaves after plants were exposed to 0 mM and 100 mM NaCl varied between the endophytic isolates (Figure 9). The control group displayed no significant differences in leaf superoxide concentration between the two salt treatments. Additionally, the two *P. vulgaris* cv. Star 2000 groups inoculated with the isolates P2L3 and P5L3, exhibited no significant differences in superoxide concentration when comparing the two salinity treatments. A sharp increase in leaf superoxide concentration was seen in the *P. vulgaris* cv. Star 2000 group inoculated with the isolate P3L1 in the 100 mM NaCl treatment, when compared the 0 mM NaCl treatment ($p < 0.05$). A similar trend can be seen for the group inoculated with P3RC in the saline treatment, where a significant increase in superoxide concentration in the leaves were observed in relation to the non-saline treatment ($p < 0.05$). In contrast, the *P. vulgaris* cv. Star 2000 group inoculated with P3RB displayed a significant decrease in leaf superoxide concentration with increasing salinity ($p < 0.05$).

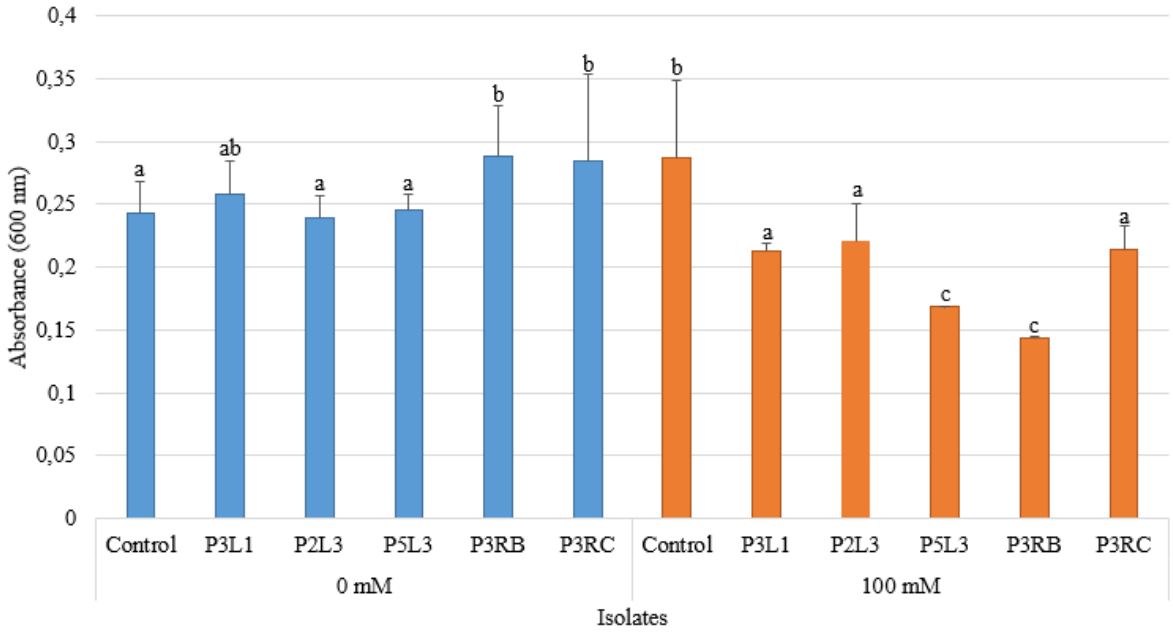

Figure 10. Determination of superoxide concentration in *P. vulgaris* cv. Star 2000 roots after plants were exposed to 0 mM and 100 mM NaCl and inoculated with the isolates P3L1, P2L3, P5L3 and P3RC. Control plants were not inoculated with endophytic bacteria. Superscript [a] displays significance (ANOVA, Tukey HSD, $p < 0.05$).

P. vulgaris cv. Star 2000 plants inoculated with P3L1 and P2L3 displayed no significant difference in root superoxide concentration when comparing the two salinity treatments (Figure 10). Additionally, the pot experiments inoculated with P3RB and P3RC in the 0 mM NaCl treatment exhibited significantly higher root superoxide concentrations when compared to the control group and the isolate groups P2L3 and P5L3 ($p < 0.05$). P3L1 had displayed no significant differences to any isolates nor the control (0 mM NaCl). In the 100 mM NaCl treatment, P5L3 and P3RB had demonstrated the lowest superoxide concentrations of all isolates ($p < 0.05$). P3L1, P2L3 and P3RC had all displayed similar superoxide concentrations in the saline treatment. However, the control group demonstrated significant increases in the 100 mM NaCl treatment, when compared to the non-saline treatment.

4.2.4 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Table 9. Inductively coupled plasma optical emission spectrometry (ICP-OES) of *P. vulgaris* cv. Star 2000 leaves grown under both 0 mM NaCl and 100 mM NaCl and treated with the endophytic isolates P3L1, P2L3, P5L3, P3RB and P3RC. 0 mM NaCl treatment was considered as the baseline for elemental content. Macronutrients presented in the blue blocks and the micronutrients are presented in

the grey blocks. Colour scale depicts the range of least uptake (red) to greatest uptake (green). Significance is depicted by superscript [a] per element row (ANOVA, Tukey HSD, $p < 0.05$). Values are shown as mg/Kg ± SE (N=3).

Element	CTRL		P3L1		P2L3		P5L3		P3RB		P3RC	
	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM
Ca	2,191^{b}	0,894^{ad}	3,068^{bc}	4,168^{c}	2,426^{b}	3,783^{c}	1,027^{a}	1,648^{a}	1,520^{a}	0,474^{d}	1,729^{a}	2,468^{b}
Mg	0,416^{a}	0,192^{a}	0,581^{ab}	0,870^{b}	0,448^{a}	0,803^{b}	0,316^{a}	0,305^{a}	0,263^{a}	0,115^{a}	0,317^{a}	0,370^{a}
K	2,484^{b}	1,025^{a}	3,120^{b}	7,175^{d}	3,062^{b}	6,697^{d}	2,728^{b}	2,750^{b}	2,442^{b}	1,734^{b}	2,117^{b}	2,424^{b}
P	0,159^{a}	0,277^{a}	0,278^{b}	0,453^{b}	0,207^{a}	0,503^{b}	0,148^{a}	0,054^{a}	0,090^{a}	0,162^{a}	0,240^{a}	0,272^{a}
Na	2,738^{c}	4,389^{d}	2,760^{c}	3,196^{c}	1,515^{a}	2,920^{c}	1,702^{a}	1,715^{a}	1,502^{a}	1,599^{a}	0,912^{b}	1,381^{a}
Cu	0,001^{a}	0,002^{b}	0,002^{b}	0,002^{b}	0,001^{a}	0,002^{b}	0,001^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,001^{a}	0,000^{a}
Zn	0,007^{b}	0,048^{c}	0,010^{b}	0,011^{b}	0,008^{b}	0,013^{b}	0,002^{a}	0,002^{a}	0,002^{a}	0,011^{b}	0,005^{b}	0,005^{b}
Mo	0,000^{a}	0,000^{a}	0,001^{c}	0,002^{c}	0,001^{b}	0,002^{c}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,001^{c}
Mn	0,017^{b}	0,047^{d}	0,030^{c}	0,039^{c}	0,029^{bc}	0,049^{d}	0,005^{a}	0,008^{a}	0,007^{a}	0,014^{a}	0,015^{a}	0,023^{b}
Fe	0,008^{b}	0,019^{d}	0,013^{c}	0,013^{c}	0,010^{c}	0,019^{d}	0,000^{a}	-0,001^{a}	0,000^{a}	0,003^{b}	0,005^{b}	0,007^{b}

The leaf tissue of *P. vulgaris* cv. Star 2000 inoculated with the endophytic isolates, control (no endophytic isolate), P3L1, P2L3, P5L3, P3RB and P3RC, were also subjected to ICP-OES analyses for the treatments 0 mM and 100 mM NaCl (Table 9). The saline treatment (100 mM NaCl) in the control group demonstrated significant increases in the Na, Cu, Zn, Mn and Fe with increases of 37.6%, 40.4%, 85.4%, 63.7% and 58.0%, respectively, whereas a notable decrease was observed for the nutrients Ca and K when compared to the non-saline treatment (0 mM NaCl), with a 59.2% and 58.7% decrease each ($p < 0.05$) (Table 9).

Contrasting results were found in the P3L1 group, where only a significant 56.5% rise in K content was found when comparing the 0 mM and 100 mM NaCl treatments ($p < 0.05$) (Table 9). Interesting results were observed when analyzing the results for the endophytic group P2L3. Significant increases in nutrient content were demonstrated for all nutrients assessed, excluding Zn, with a 35.9% increase in Ca, 44.2% in Mg, 54.3% in K, 58.8% in P, 48.1% in Na, 50.9% in Cu, 56.3% in Mo, 40.0% in Mn and 46.2% in Fe with increasing salinity, when comparing the non-saline and saline treatments for the P2L3 group ($p < 0.05$) (Table 9). Conversely, no notable differences were observed for the P5L3 group with increasing salinity (Table 9). Additionally, significant increases in leaf Zn and Fe content were demonstrated in the P3RB group, with an increase of 83.4% and 97.3% each, when comparing the saline treatment to the non-saline treatment ($p < 0.05$) (Table 9). Whereas a 68.8% decrease in leaf Ca was observed with increasing salinity ($p < 0.05$) (Table 9). Lastly, no significant decreases were observed in

the P3RC group, only significant increases in the leaf nutrient content for increasing salinity, with increases in Ca (by 29.9%), Na (34.0%), Mo (42.9%) and Mn (37.1%) ($p < 0.05$) (Table 9).

When comparing the groups in the non-saline treatment (0 mM NaCl), some intriguing results were obtained (Table 9). Only the groups namely, P3L1 and P2L3, had displayed significant increases in a few nutrients when compared to the non-saline control (0 mM NaCl), where notable increases in P, Cu, Mo, Mn and Fe were seen for P3L1 (with 42.8%, 31.5%, 52%, 43.4% and 36.5% increase each) and Mo and Fe for P2L3 (with 59.6% and 22.1% raises in each, respectively) ($p < 0.05$) (Table 9). However, P2L3 had also displayed significant decreases in Na, with a 44.7% decrease in leaf Na content when compared to the non-saline control ($p < 0.05$) (Table 9). For the following groups, P5L3, P3RB and P3RC, only notable reductions in leaf nutrient content between them and the non-saline control were obtained (Table 9). Both P5L3 and P3RB displayed significant decreases in Ca, Na, Zn, Mn and Fe leaf content when compared to the non-saline treatment ($p < 0.05$) (Table 9). P5L3 demonstrated reductions in Ca of 53.1%, Na of 37.8%, Zn 70.2%, Mn 69.4% and Fe 38397% and the observed decreases in P3RB were found to be 30.6% for Ca, 45.1% for Na, 74.5% for Zn, 60.8% for Mn and 99.1% for Fe ($p < 0.05$) (Table 9). Furthermore, significant decreases in leaf Ca, Na and Mn content were found in the P3RC endophytic group when compared to the non-saline control group, with a 21.1%, 66.7% and 14.1% reduction in leaf content for each element, respectively ($p < 0.05$) (Table 9).

The nutrient content of leaves taken from *P. vulgaris* cv. Star 2000 which had been treated with 100 mM NaCl and inoculated with the endophytes P3L1, P2L3, P5L3, P3RB and P3RC were compared in terms of their nutrient content with a saline control treatment (100 mM NaCl) (Table 9). Significant increases in leaf Ca, Mg, K, P and Mo content were observed for the groups P3L1 and P2L3 when compared to the saline control ($p < 0.05$) (Table 9). The percentage increases for P3L1 nutrient content when compared to the saline control were found to be 78.6% for Ca, 77.9% for Mg, 85.7% for K, 38.7% for P and 85.3% for Mo and for the P2L3 group, an increase by 76.4% was seen in Ca, 76.1% for Mg, 84.7% for K, 44.8% for P and 85.3% for Mo ($p < 0.05$) (Table 9). Additionally, a significant decrease in leaf Na and Zn were detected in P3L1 and P2L3, too, however a notable reduction in leaf Fe and Mn content was also detected for P3L1 when compared to the saline control treatment ($p < 0.05$) (Table 9). The percentage decreases of these nutrients are 27.2% % (Na), 76.5% (Zn), 17.2% (Mn) and 30.6% (Fe) for P3L1 and 33.5% (Na) and 72.5% (Zn) for P2L3 ($p < 0.05$) (Table 9).

Furthermore, significant decreases in leaf Na, Cu, Zn, Mn and Fe content were observed for the groups P5L3, P3RB and P3RC ($p < 0.05$) (Table 9). P5L3 had seen a 60.9% decrease in Na content, 99.9% for Cu, 96.8% for Zn, 81.9% for Mn and 3188% for Fe when compared to the saline control treatment ($p < 0.05$) (Table 9). Whereas a 63.6% (Na), 84.4% (Cu), 77.6% (Zn), 69.1% (Mn) and 85.7% (Fe) decrease in leaf nutrient content for the P3RB group when compared to the saline control ($p < 0.05$) (Table 9). Furthermore, a 68.5%, 80.4%, 88.8%, 50.4% and 64.9% decrease was observed for the nutrients Na, Cu, Zn, Mn and Fe in the P3RC group when compared to the saline control ($p < 0.05$) (Table 9). Lastly, an increase in leaf K content was detected for the groups P5L3 and P3RB, where P3RC displayed an increase in leaf Ca, K and Mo content when compared to the saline treatment ($p < 0.05$) (Table 9). The percentage increase in leaf K content for P5L3 was 62.7%, 40.9% for P3RB and 57.7% for P3RC ($p < 0.05$) (Table 9). Additionally, for the P3RC group, a significant increase in 63.8% for Ca and 60.2% for Mo had also been observed ($p < 0.05$) (Table 9).

Table 10. Inductively coupled plasma optical emission spectrometry (ICP-OES) of *P. vulgaris* cv. Star 2000 roots exposed to 0 mM NaCl and 100 mM NaCl and treated with the endophytic isolates P3L1, P2L3, P5L3, P3RB and P3RC. 0 mM NaCl treatment was considered as the baseline for elemental content. Macronutrients presented in the blue blocks and the micronutrients are presented in the grey blocks. Colour scale depicts the range of least uptake (red) to greatest uptake (green). Significance is depicted by superscript [a] per element row (ANOVA, Tukey HSD, $p < 0.05$). Values are shown as mg/Kg ± SE (N=3).

Element	CTRL		P3L1		P2L3		P5L3		P3RB		P3RC	
	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM	0 mM	100 mM
Ca	0,540^{a}	1,350^{c}	0,642^{a}	0,839^{c}	0,558^{a}	0,729^{c}	0,474^{a}	0,327^{a}	0,403^{a}	0,391^{a}	0,558^{a}	0,547^{a}
Mg	0,150^{b}	0,425^{c}	0,174^{b}	0,170^{b}	0,133^{b}	0,160^{b}	0,115^{b}	0,083^{a}	0,106^{b}	0,105^{b}	0,198^{b}	0,144^{b}
K	1,560^{b}	3,093^{c}	1,788^{b}	0,618^{a}	1,618^{b}	0,737^{a}	1,734^{b}	0,842^{a}	1,193^{b}	0,862^{a}	1,532^{b}	0,889^{a}
P	0,331^{b}	0,384^{b}	0,469^{b}	0,271^{b}	0,426^{b}	0,363^{b}	0,162^{a}	0,122^{a}	0,140^{a}	0,119^{a}	0,394^{b}	0,289^{b}
Na	3,348^{b}	2,647^{b}	2,988^{b}	3,882^{b}	1,432^{a}	3,069^{b}	1,599^{a}	2,520^{a}	1,824^{a}	2,552^{a}	0,959^{c}	2,184^{a}
Cu	0,002^{ab}	0,002^{ab}	0,002^{ab}	0,006^{b}	0,002^{ab}	0,002^{ab}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,001^{a}	0,001^{a}
Zn	0,031^{c}	0,006^{a}	0,042^{cd}	0,043^{cd}	0,037^{c}	0,059^{d}	0,011^{b}	0,012^{b}	0,013^{b}	0,013^{b}	0,021^{b}	0,026^{b}
Mo	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{b}	0,000^{a}	0,001^{c}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}	0,000^{a}
Mn	0,023^{b}	0,010^{a}	0,037^{b}	0,030^{b}	0,032^{b}	0,051^{c}	0,014^{a}	0,020^{b}	0,016^{a}	0,013^{a}	0,030^{b}	0,026^{b}
Fe	0,017^{ab}	0,008^{a}	0,024^{b}	0,029^{b}	0,057^{c}	0,025^{b}	0,003^{a}	0,001^{a}	0,004^{a}	0,003^{a}	0,015^{a}	0,014^{a}

When comparing the ICP-OES results of the inoculated *P. vulgaris* cv. Star 2000 plant roots under non-saline (0 mM NaCl) and saline (100 mM NaCl) conditions, interesting results were

obtained (Table 10). The control (which had not been inoculated with any endophyte), displayed significantly greater Ca, Mg and K content in the root material in the 100 mM NaCl treatment, when compared to the non-saline treatment, with a 60.0%, 64.7% and 49.6% increase in Ca, Mg and K respectively. Conversely, it demonstrated significant decreases in Zn and Mn content within *P. vulgaris* cv. Star 2000 roots, with a 79.8% decrease in Zn and a 56.3% reduction in Mn with increasing salinity ($p < 0.05$) (Table 10).

In the endophyte inoculant groups, P3L1 inoculated plants demonstrated significant increases in Ca and Mo content, with Ca displaying a 23.5% increase and Mo, a 46.8% increase with rising salinity in the root tissue ($p < 0.05$) (Table 10). In contrast to this, significant reductions in Mg and K content were observed within the 100 mM NaCl treatment for P3L1 inoculated plants, with a 2.61% and 65.4% decrease nutrient for Mg and K, respectively ($p < 0.05$) (Table 10). The P2L3 inoculant group demonstrated noteworthy increases in nutrient content for Ca, Na, Zn, Mo and Mn in the 100 mM NaCl treatment when compared to the 0 mM NaCl treatment, with a 23.4%, 53.3%, 37.2%, 78.6% and 36.9% increase accordingly ($p < 0.05$) (Table 10). Whereas K and Fe were found with significant declines in nutrient content in the 100 mM NaCl treatment, with 54.4% (K) and 55.3% (Fe) less nutrient content than the 0 mM NaCl treatment ($p < 0.05$) (Table 10). The *P. vulgaris* cv. Star 2000 seedlings inoculated with P5L3 showed a significant increase in root Mn content, with a 26.4% increase in the 100 mM NaCl treatment when compared to the non-saline treatment ($p < 0.05$) (Table 10). However, noteworthy decreases in root Mg, K and Fe content were observed for the saline treatment when compared to the non-saline treatment, with a 27.8% decrease in Mg, a 51.5% decrease in K and a 60.1% reduction in Fe with increasing salinity ($p < 0.05$) (Table 10). The only nutrient that displayed a significant difference when comparing the two salinity treatments (0 mM and 100 mM NaCl) for P3RB was K, which had shown a 27.8% decrease in the 100 mM NaCl treatment ($p < 0.05$) (Table 10). Lastly, in the P3RC inoculant group, Na was the only nutrient which had increased significantly in concentration within the root tissue with increasing salinity, whereas K was the only noteworthy decrease seen with increasing salinity ($p < 0.05$) (Table 10).

When comparing the nutrient content of the root tissue of *P. vulgaris* cv. Star 2000 in the non-saline treatment (0 mM NaCl), no significant differences were found between the control and P3L1 (0 mM NaCl) (Table 10). However, Fe content was found to be significantly greater in the P2L3 inoculant group when compared to the control in the non-saline treatment, whereas Na content demonstrated a notable decrease in the P2L3 group ($p < 0.05$) (Table 10).

Additionally, the inoculant group P5L3 displayed notable decreases in the nutrient content of P, Na, Zn and Mn when compared to the control in the 0 mM NaCl treatment, with a 51.2%, 52.2%, 64.7% and 38.2%, respectively ($p < 0.05$) (Table 10). Likewise, P3RB demonstrated significant reductions in P, Na, Zn and Mn when compared to the control for the 0 mM NaCl treatment, with a reduction in 57.7%, 45.5%, 57.4% and 30.8% accordingly ($p < 0.05$) (Table 10). Lastly, like the P2L3 and P3RB inoculant groups, P3RC displayed notable decreases in Na and Zn content when compared to the non-saline control, with a 71.4% and 31.7% each ($p < 0.05$) (Table 10).

The elemental content of the root tissue belonging to *P. vulgaris* cv. Star 2000 was also compared in a 100 mM NaCl treatment with a control and the five endophyte inoculation groups (P3L1, P2L3, P5L3, P3RB and P3RC) (Table 10). When compared to the control group (100 mM NaCl), the P3L1 group displayed significant increases in Zn, Mn and Fe content within the root tissue with an increase of 85.5%, 66.0% and 72.3% respectively and decreased Mg, K and Mo content at 60.1%, 80.0% and 24.1% each ($p < 0.05$) (Table 10). Similar to P3L1, significantly greater nutrient content in Zn (89.5% increase), Mn (80.2%) and Fe (68.0%) were seen in P2L3, however Mo (37.1%) content had also seen a significant rise in P2L3 when compared to the 100 mM NaCl control ($p < 0.05$) (Table 10). Additionally, a 62.2% and 76.2% significant decrease in Mg and K content was observed for P2L3 when compared to the control (100 mM NaCl) ($p < 0.05$) (Table 10). In the P5L3 endophytic inoculant group, a significant increase in Zn and Mn content was observed in the root tissue of *P. vulgaris* cv. Star 2000, with a rise of 50.8% and 21.8% each ($p < 0.05$) (Table 10). Whereas a notable decrease in Ca, Mg, K, P and Na was found for the P5L3 group when compared to the saline control, with reductions of 75.8%, 80.5%, 72.8%, 68.3% and 4.80% respectively ($p < 0.05$) (Table 10). Similar results were seen in the P3RB groups, where significant reductions in Ca, Mg, K, P and Na were also found, with a 71.0%, 75.4%, 72.1%, 69.0% and 3.59% decrease each, while a notable 50.8% increase was observed for Zn ($p < 0.05$) (Table 10). And lastly, a significant 75.9% and 60.8% increase in Zn and Mn content was displayed in the P3RC group when compared to the saline control, whereas a reduced nutrient content was observed for Ca, Mg, K and Na for this endophytic group, with a decrease in root nutrient content of 59.5%, 66.1%, 71.2% and 17.5%, respectively ($p < 0.05$) (Table 10).

4.3 Discussion

One-third of the world's crops are grown on salinity affected soils (Rani et al., 2019) and owing to the sensitivity of most crop plants to salinity stress (Glenn et al., 1999; Isayenkov, 2012), it is imperative to discover alternate measures and systems as to reduce the impacts of saline soils on crop growth, development and yield. One such glycophytic species with great importance in low-income countries and sensitive to salt stress is *P. vulgaris* cv. Star 2000 (Gama et al., 2007). It has been established that bacteria isolated from highly stressed environments possess traits that allow them to tolerate said stress and other characteristics that are beneficial to plants (Tiwari et al., 2011). In chapter 3, we had demonstrated the plant growth promoting (PGP) properties of our endophytes isolated from *A. calendula*. These include *Erwinia persicina* NBRC 102418^T, *Bacillus marisflavi* JCM 11544^T, *Ochrobactrum rhizosphaerae* PR17^T, *Microbacterium gubbeenense* DSM 15944^T and *B. zhangzhouensis* DW5-4^T, all of which had demonstrated a number of PGP characteristics. The dry mass, cell death, superoxide content and nutrient content of *P. vulgaris* cv. Star 2000 inoculated with these endophytes and treated with 0 mM and 100 mM NaCl was assessed in the present study.

Important cellular processes in plants are interrupted under salt stress, resulting in reduced plant growth and biomass (Li et al., 2017). In this study, we demonstrated the negative effect of salt stress on plant leaf and root dry mass, where the leaf and root dry masses (mg) of *P. vulgaris* cv. Star 2000 were greater in the 0 mM NaCl treatment when compared to the 100 mM NaCl treatment. In the 0 mM NaCl treatment, the plants inoculated with the endophytes P5L3 (*O. rhizosphaerae* PR17^T) and P3RC (*B. zhangzhouensis* DW5-4^T) displayed greater leaf and root dry masses than the control which had no inoculant (Figure 5, Figure 6). Whereas in the 100 mM NaCl treatment, only P3RB (*M. gubbeenense* DSM 15944^T) inoculated plants demonstrated greater leaf dry mass than the control (Figure 5). Additionally, P3L1 (*E, persicina* NBRC 102418^T), P5L3 (*O. rhizosphaerae* PR17^T) and P3RC (*B. zhangzhouensis* DW5-4^T) had displayed greater root dry mass than the control (Figure 6). Increased plant dry mass had also been demonstrated in a study by Ali et al. (2014), where tomato plants inoculated with the ACC deaminase producing endophytes *P. fluorescens* YsS6 and *P. migulae* 8R6 had demonstrated increased plant growth and tolerance to saline stress. Owing to the growth promoting characteristics of the endophytes in this study, we can infer that those traits have increased plant growth, and in saying that, the dry mass of *P. vulgaris* cv. Star 2000 under non-saline and saline stress.

Additionally, an increase in programmed cell death (PCD), is often seen in plants undergoing abiotic stress, such as salinity (Pennell et al., 1997). PCD is a physiological process dealing with the particular removal of unwanted cells (Pennell et al., 1997). These cells are often damaged and unable to function correctly (Pennell et al., 1997). The present study displayed interesting results for the leaf and root material *of P. vulgaris* cv. Star 2000 under 0 mM and 100 mM NaCl. Significant decreases in leaf cell death when comparing the saline treatment to the non-saline treatment was only observed for *P. vulgaris* cv. Star 2000 plants inoculated P3L1 (*E. persicina* NBRC 102418^T), P2L3 (*B. marisflavi* JCM 11544^T) and P5L3 (*O. rhizosphaerae* PR17^T) (Figure 7). Additionally, in the roots, the only significant decreases in root cell death were observed in the plants inoculated with P3L1 (*E. persicina* NBRC 102418^T) and P3RB (*M. gubbeenense* DSM 15944^T) (Figure 8). In a study conducted by Jha et al. (2015) on *Oryza sativa* (exposed to 150 mM NaCl) inoculated with the root-associated bacteria, *Pseudomonas aeruginosa* and *B. megaterium*, it was reported that both microorganisms provided protection against salinity-induced cell death (Jha et al., 2015). A similar result was observed in Jha et al. (2014), where *P. pseudoalcaligenes* and *B. pumilus* had both decreased cell death in *O. sativa* exposed to 1.5% NaCl. Here, this study provides evidence for protection against NaCl-induced PCD in *P. vulgaris* cv. Star 2000 by the species *E. persicina*, *B. marisflavi*, *O. rhizosphaerae* and *M. gubbeenense*.

Furthermore, the response of salt-sensitive plants to salinity is often to enhance its production of reactive oxygen species (ROS) ([b]Zhu, 2001; Gupta et al., 2014; Kumar et al., 2020). ROS molecules include singlet oxygen, superoxide, hydroxyl radical and hydrogen peroxide (Gupta et al., 2014; Kumar et al., 2020). These molecules are produced in the chloroplasts and mitochondria (Gill et al., 2010; Kumar et al., 2020), however, they are able to cause oxidative damage to almost all cellular components (Gill et al., 2010; Gupta et al., 2014). The formation of NaCl-induced ROS can lead to the damage of proteins, lipids, DNA and interrupt important cellular functions in plants (Gill et al., 2010; Gupta et al., 2014). However, PGPB are able to produce antioxidants, such as superoxide dismutase (SOD), which scavenge ROS, helping plants tolerate high salinities (Numan et al., 2018). In the present study, the leaf material *of P. vulgaris* cv. Star 2000 plants inoculated with P3RB (*M. gubbeenense* DSM 15944^T) had reported lower superoxide concentrations under salinity stress when compared to non-saline conditions (Figure 9). Additionally, decreases in root superoxide concentrations were observed for *P. vulgaris* cv. Star 2000 plants inoculated with P5L3 (*O. rhizosphaerae* PR17^T) and P3RB (*M. gubbeenense* DSM 15944^T) under salinity stress in relation to the non-saline treatment

(Figure 10). Thus, this study presents evidence for the alleviation of salt stress owing to the ability of *O. rhizosphaerae* and *M. gubbeenense* to reduce superoxide concentration in *P. vulgaris* cv. Star 2000

Lastly, many plants experience nutrient (N, Ca, K, P, Fe, Zn) deficiencies when exposed to high soil salinities (Shrivastava et al., 2015). This is a result of the competitive interactions Na^+ and Cl^- have with other soil nutrient ions for ion membrane transporters and by affecting soil pH (Shin et al., 2016). The present study assessed the nutrient (Ca, Mg, K, P, Na, Cu, Zn, Mo, Mn and Fe) content of the roots and leaves of *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl and inoculated with salt-tolerant endophytes. When assessing the nutrient content of plants inoculated with the endophytes in comparison to the control plants (within the non-saline treatment), P3L1 (*E. persicina* NBRC 102418^T) had demonstrated no significant decreases in any nutrient that was assessed (Table 9 and 10). Additionally, when compared to the control group, it had displayed increased leaf P, Cu, Mo, Mn and Fe content (Table 9). As such, it had presented the greatest for biofertilization of all isolates in the non-saline treatment. In the previous chapter, nitrogen fixation, ammonia production, siderophore activity, IAA production, phosphate and zinc solubilization had all been reported for *E. persicina* NBRC 102418^T. *E. persicina* had been described as weak, opportunistic phytopathogen entering susceptible host plants through natural openings and wounds or when the plant is undergoing abiotic stress (Nechwatal et a., 2019; Zrelovs et al., 2020). It had even been implicated for leaf spot disease in common bean (Santos et al., 2009). However, no symptoms of disease had been seen for the *P. vulgaris* cv. Star 2000 plants inoculated with *E. persicina* NBRC 102418^T in this study for both the non-saline and saline treatments. In fact, *E. persicina* NBRC 102418^T had presented significant plant growth promoting characteristics rather than exhibiting phytopathogenic properties. This result is supported by a study conducted by Shen et al. (2012), which had reported improved tomato quality and yield when inoculated with *E. persicinus* (*E. persicina*) RA2, even when irrigated with simulated seawater.

In contrast, under saline conditions, P2L3 (*B. marisflavi* JCM 11544^T) had displayed significant increases in root Zn, Mn, Fe and Mo content and leaf Ca, Mg, K, P and Mo content (Table 9 and 10) when compared to the control for the saline treatment, thus outperforming all the other isolates. In chapter 3, we had demonstrated the nitrogen fixation, ammonia production, siderophore activity, phosphate and zinc solubilization ability of *B. marisflavi* JCM 11544^T. It is well established that many *Bacillus* species possess plant growth promoting properties (Mohamed et al., 2012; Singh et al., 2018) and often appear as plant endophytes

(Hassan, 2017). In a study conducted by Mohamed et al. (2012)., radish (*Raphanus sativus*) inoculated with *B. subtilis* had displayed increased phytohormones, namely IAA and GA_3, as well as increased N, P, K, Ca and Mg content and decreased Na^+ and Cl^- content. Additionally, those plants had presented enhanced fresh and dry mass of the roots and leaves, enhanced photosynthetic pigments, proline, total free amino acids as well as crude protein contents (Mohamed et al., 2012). Additionally, *B. firmus* SW5 had mitigated the negative effects of salinity stress on soybean (*Glycine max* L.), by improving plant growth, biomass yield, root architecture, chlorophyll levels, transpiration rate, photosynthetic rate, stomatal conductance, soluble proteins and sugar content as well as improved nutrient uptake of N and P (El-Esawi et al., 2018). As such, it is unsurprising that *B. marisflavi* JCM 11544^T had been able to improve nutrient uptake under 100 mM NaCl, however, it remains a positive result.

4.4 Conclusion

The dry mass, cell death, superoxide concentration and nutrient content of common bean (*P. vulgaris* cv. Star 2000) were notably influenced by NaCl stress. However, inoculation of *P. vulgaris* cv. Star 2000 with plant growth promoting bacteria (*E. persicina* NBRC102418^T, *B. marisflavi* JCM 11544^T, *O. rhizosphaerae* PR17^T, *M. gubbeenense* DSM 15844^T and *B. zhangzhouensis* DW5-4^T) significantly enhanced plant growth under salt stress, by mitigating the negative effects of salinity on programmed cell death, reducing superoxide concentration and improving plant nutrient relations.

5. CONCLUSION AND FUTURE WORK

Little to no research had been performed on many of the species in this study when assessing their plant growth promoting properties and as such, little comparison could be made to the literature for the specific bacterial species and strains. The endophytes isolated from *A. calendula* (*E. persicina* NBRC 102418^T, *B. marisflavi* JCM 11544^T, *O. rhizosphaerae* PR17^T, *M. gubbeenense* DSM 15944^T and *B. zhangzhouensis* DW5-4^T) had each demonstrated a number of plant growth promoting factors, be it nitrogen fixation, ammonia production, phosphate solubilization, zinc solubilization, siderophore production or IAA production. This is a positive result and provides evidence for the potential use of these isolates in plant biofertilizers.

As such, future work should be directed at assessing other plant growth promoting properties including the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, jasmonates, abscisic acid (ABA), cytokinins and gibberellins (GA_3) and other plant hormone simulants. Additionally, owing to the lack of literature involving the endophytes identified in this study, they should be included in other abiotic stress studies, such as heavy metal tolerance, drought, heat and cold stress. Also, future studies could focus on the virulence and pathogenesis of a wide range of *E. persicina* strains in order to understand which strains pose a threat to agriculture and crop health, and which are beneficial to crop growth and yield.

Furthermore, the plant growth promoting endophytes in the current study were able to alleviate the adverse effects of salinity on plant growth, programmed cell death, superoxide concentration and nutrient uptake of *P. vulgaris* cv. Star 2000. Again, this is an encouraging result, as it supports the idea that the above-mentioned isolates can in fact be used in biofertilization and the remediation of crop species grown on saline soils. However, many of the other mechanisms which are able to enhance plant growth under salinity have not been addressed. Therefore, future studies should assess the impact of *E. persicina* NBRC 102418^T, *B. marisflavi* JCM 11544^T, *O. rhizosphaerae* PR17^T, *M. gubbeenense* DSM 15944^T and *B. zhangzhouensis* DW5-4^T on the nutrient content of bean pods (by means of ICP-OES), rate of photosynthesis, rate of transpiration, root architecture, other ROS molecules (hydroxyl radicals, perhydroxyl radical, alkoxyl radicals, hydrogen peroxide and singlet oxygen) and antioxidant enzymatic activity (superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, dehydroascorbate reductase,

glutathione peroxidase, guaicol peroxidase and glutathione-S- transferase) involved in the scavenging of ROS molecules and protecting plants against them. Lastly, future studies could assess the growth and development (dry mass, root architecture, programmed cell death, superoxide content, nutrient content, photosynthetic rate, transpiration rate and antioxidant enzymatic activity) of *P. vulgaris* cv. Star 2000 under salinity stress making use of a mixed endophyte culture and comparing its results to that of the growth and development of plants in the single isolate inoculation growth trials. Research has demonstrated the positive effects of mixed culture inoculation on crop plant and development. Thus, the result would be an interesting one to uncover.

6. APPENDIX

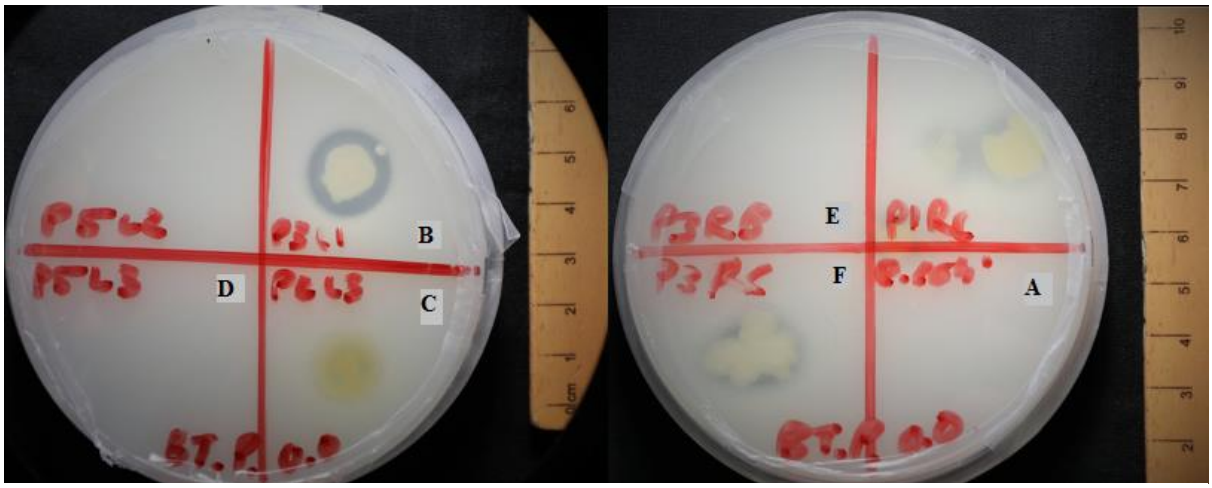

Figure 11. **Phosphate solubilization of endophytic isolates**. The isolates were observed for formation of zone of clearance in order to determine whether they possessed the ability to solubilize phosphate. A – *E. coli* KRX (control), B – P3L1, C – P2L3, D – P5L3, E – P3RB and F – P3RC.

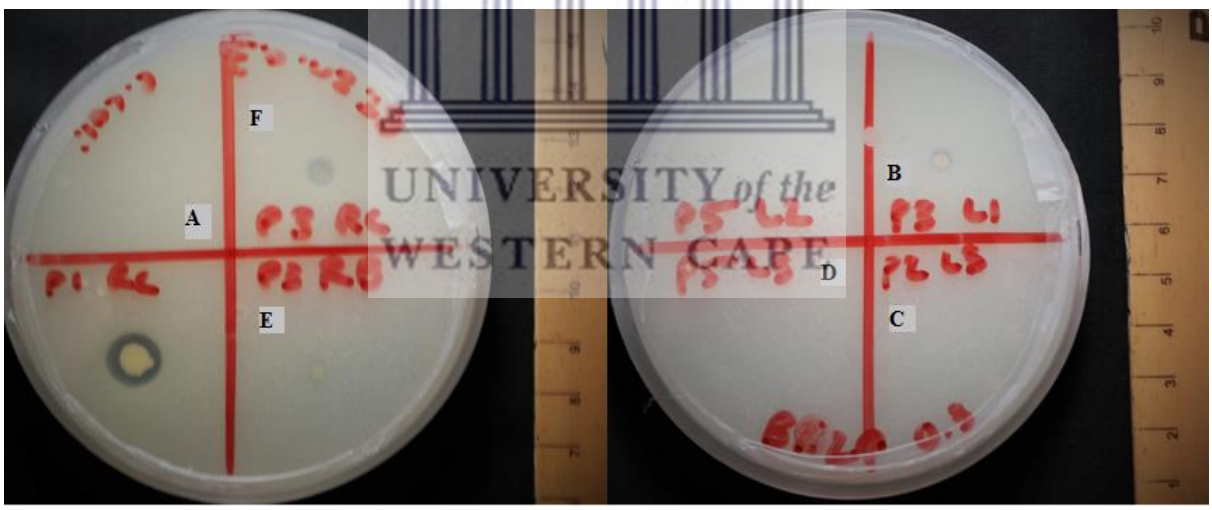

Figure 22. **Zinc solubilization of endophytic isolates**. The isolates were observed for formation of zone of clearance in order to determine whether they possessed the ability to solubilize zinc. A – *E. coli* KRX (control), B – P3L1, C – P2L3, D – P5L3, E – P3RB and F – P3RC.

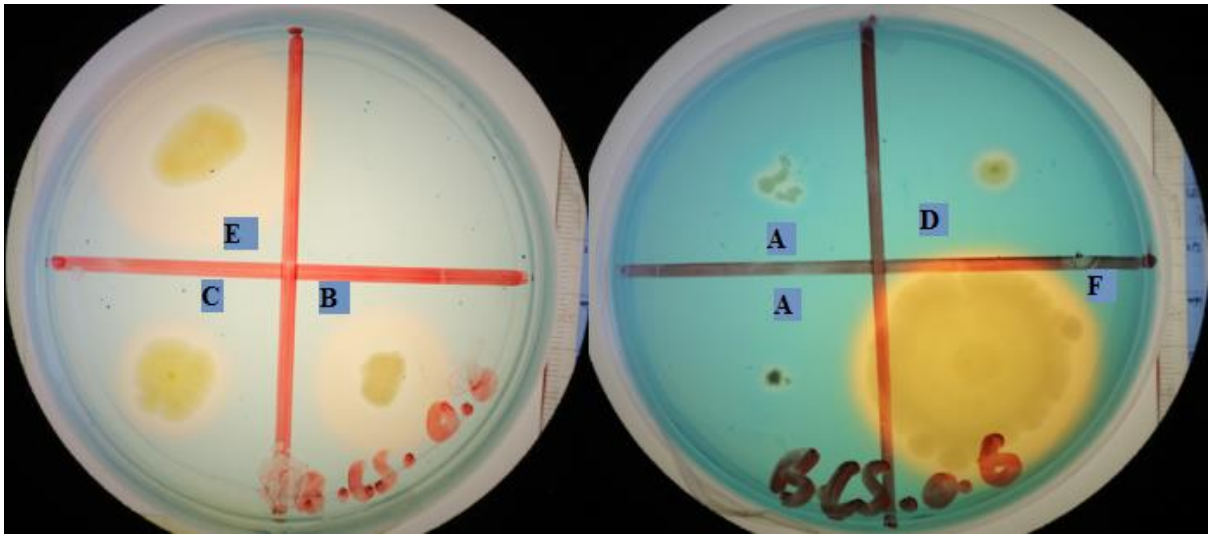

Figure 13. **Siderophore production of endophytic isolates**. The isolates were observed for formation of zone of clearance (orange halo) in order to determine whether they possessed the ability to produce siderophores. A – *E. coli* KRX (control), B – P3L1, C – P2L3, D – P5L3, E – P3RB and F – P3RC.

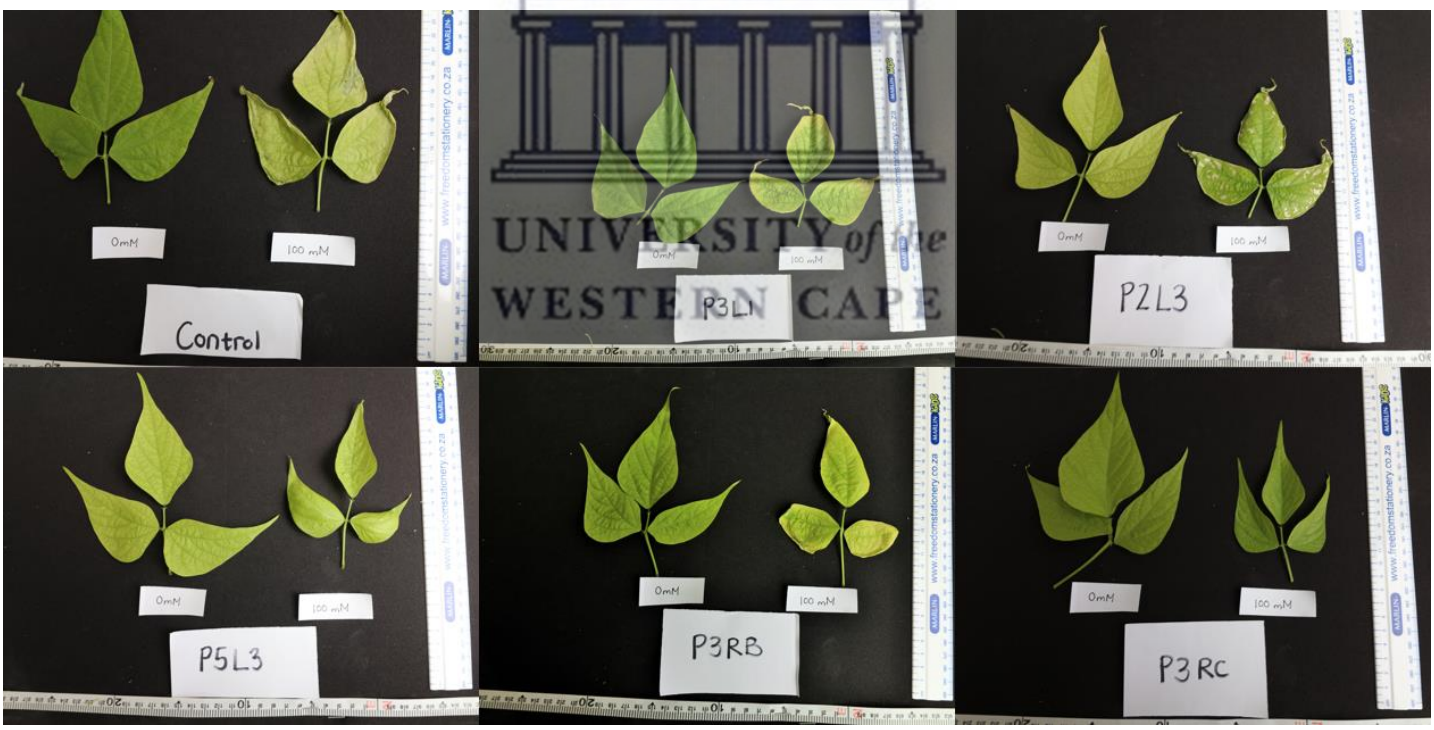

Figure 14. **The effect of salinity on leaf physiology**. Leaf trifoliates of *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl inoculated with the endophytes P3L1, P2L3, P5L3, P3RB and P3RC.

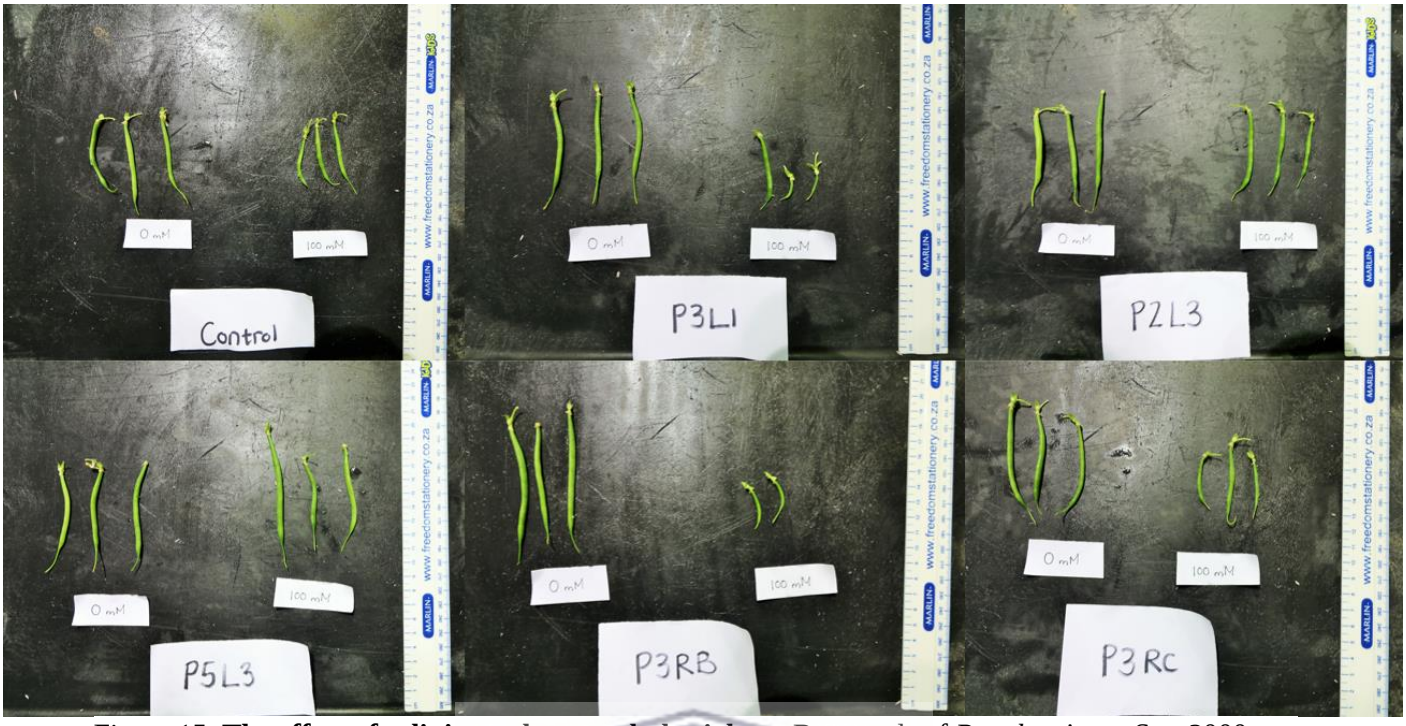

Figure 15. **The effect of salinity on bean pod physiology**. Bean pods of *P. vulgaris* cv. Star 2000 exposed to 0 mM and 100 mM NaCl inoculated with the endophytes P3L1, P2L3, P5L3, P3RB and P3RC.

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