Effect of salinity stress on growth, Exoproteome profiles and ability to enhance plant growth by members of a Commercial Microbial Consortium

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ABSTRACT

Plant growth promoting microorganisms (PGPM) such as *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H, and their derivatives, such as cell-free supernatants (CFS), enhance plant growth under stressed and ideal conditions. Salinity stress is a major global constraint affecting plants and their associated phytomicrobiome, posing a threat to the growth, survival, multiplication, and ability of PGPM to enhance plant growth. Identification of salt tolerant PGPM strains that can maintain their ability to enhance plant growth under saline conditions is important for crop production especially in salt affected areas.

The first study aimed at elucidating the effect of varying levels of NaCl on the growth of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H. The strains were cultured in M13 and De man, Rogosa and Sharpe (MRS) broth, respectively, supplemented with 0-1000 mM NaCl, at pH 7.0, and incubated at 30 and 37 °C, respectively, for 48 h, at 120 rpm. Both strains demonstrated a high tolerance to NaCl, up to 1000 mM. Growth rate and generation time varied across the different NaCl levels, for both strains.

The second study aimed at understanding changes in the exoproteome profiles of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, when exposed to salt stress. The strains were cultured in broth supplemented with 200 and 0 mM NaCl (control), at pH 7.0, and incubated at 30 and 37 °C, respectively, for 48 h, at 120 rpm. Microbial cultures were then centrifuged and filter sterilized, to obtain CFS whose proteome profiles were studied using LC-MS/MS analysis and quantified using scaffold. Treatment with 200 mM NaCl negatively affected the quantity of identified proteins in comparison to the control. Some proteins were upregulated while others were downregulated. Upregulated proteins were mostly those involved in cell wall metabolism, substrate transport, oxidative stress tolerance, gene expression and DNA replication and repair. Some of the upregulated proteins were reported to enhance plant growth under salinity stress and ideal conditions.

The third study aimed at understanding the ability of *B. amyloliquefaciens* EB2003A's CFS to enhance germination and root length of corn and soybean, under optimal and NaCl stressed growth conditions. Three NaCl levels (0, 50, and 75 mM) and four CFS concentrations (1.0, 0.2, 0.13, and 0.1% [v/v]) were used for the study. Treatment with CFS concentrations of 0.2%, 0.13%, and 0.1% significantly enhanced

root length of soybean grown at optimal conditions by 36.4, 39.70, and 39.91%. Treatment with CFS concentration of 1.0% significantly enhanced percentage germination of soybean exposed to 50 mM NaCl by 48.65%, at 24 h. CFS concentrations of 0.2% and 0.13% enhanced mean root length of corn exposed to 50 mM NaCl stress by 23.73 and 37.5%, respectively. Treatment with CFS concentrations of 0.2%, 0.13%, and 0.1% significantly enhanced percentage germination of corn exposed to 75 mM by 25.3% (in all 3), at 48 h.

The fourth study evaluated the effect of CFS obtained from *L. helveticus* EL2006H on its ability to enhance mean percentage germination and mean root length of corn and soybean, and growth variables of potato, using treatment formulations that consisted of 0.2 and 1.0% [v/v] *L. helveticus* EL2006H CFS concentrations and 100 and 150 mM NaCl levels. Results showed that treatment with CFS concentration of 0.2% enhanced percentage germination of soybean exposed to 100 mM NaCl stress by 44.37%, at 48 h. Treatment with CFS concentration of 1.0% significantly increased root length of corn by 23.04%. Treatment with CFS 0.2% significantly increased photosynthetic rate, leaf greenness and fresh weight of potato exposed to 100 mM NaCl.

In conclusion, based on findings of this project, *B. amyloliquefaciens* EB2003 and *L. helveticus* EL2006H are tolerant to high levels of NaCl stress. The two strains alter their exoproteome profile when exposed to salt stress, potentially upregulating proteins that enhance their tolerance to NaCl stress. The upregulated proteins could also be in part, potentially responsible for the bioactivity of the CFSs on corn, soybean, and potato. This project contributed to knowledge by being the first study, to show mechanisms employed by *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H to tolerate NaCl stress, at the exoproteome level, and relating it to the ability of their CFS to enhance plant growth. The project also elucidated that the two strains' CFS can be a promising approach for enhancing growth of crops at both ideal and NaCl stressed conditions.

RÉSUMÉ

Les micro-organismes favorisant la croissance des plantes tels que *Bacillus amyloliquefaciens* EB2003A et *Lactobacillus helveticus* EL2006H, et leurs dérivés, tels que les surnageants acellulaires, améliorent la croissance des plantes dans des conditions stressantes et idéales. Le stress de salinité est une contrainte mondiale majeure affectant les plantes et leur phytomicrobiome associé, constituant une menace pour la croissance, la survie, la multiplication et la capacité du PGPM à améliorer la croissance des plantes. L'identification de souches de PGPM tolérantes au sel qui peuvent maintenir leur capacité à améliorer la croissance des plantes dans des conditions salines est importante pour la production agricole, en particulier dans les zones touchées par le sel.

La première étude visait à élucider l'effet de différents niveaux de NaCl sur la croissance de *B. amyloliquefaciens* EB2003A et *L. helveticus* EL2006H. Les souches ont été cultivées dans des bouillons M13 et De man, Rogosa et Sharpe (MRS), respectivement, additionnés de 0-1000 mM de NaCl, à pH 7.0, et incubés à 30 et 37 °C, respectivement, pendant 48 h, à 120 rpm. Les deux souches ont démontré une tolérance élevée au NaCl, jusqu'à 1000 mM. Le taux de croissance et le temps de génération variaient selon les différents niveaux de NaCl, pour les deux souches.

La deuxième étude visait à comprendre les changements dans les profils d'exoprotéome de *B. amyloliquefaciens* EB2003A et *L. helveticus* EL2006H, lorsqu'ils sont exposés à un stress salin. Les souches ont été cultivées dans un bouillon additionné de NaCl 200 et 0 mM (témoin), à pH 7.0, et incubées à 30 et 37 °C, respectivement, pendant 48 h, à 120 rpm. Les cultures microbiennes ont ensuite été centrifugées et stérilisées sur filtre, pour obtenir le CFS dont les profils protéomiques ont été étudiés à l'aide d'une analyse LC-MS/MS et quantifiés à l'aide d'un échafaudage. Le traitement avec 200 mM de NaCl a affecté négativement la quantité de protéines identifiées par rapport au témoin. Certaines protéines ont été régulées à la hausse tandis que d'autres ont été régulées à la baisse. Les protéines régulées positivement étaient principalement celles impliquées dans le métabolisme de la paroi cellulaire, le transport du substrat, la tolérance au stress oxydatif, l'expression des gènes et la réplication et la réparation de l'ADN. Certaines des protéines régulées à la hausse ont également été signalées pour améliorer la croissance des plantes sous un stress de salinité et dans des conditions idéales.

La troisième étude visait à comprendre la capacité du CFS de *B. amyloliquefaciens* EB2003A à améliorer la germination et la longueur des racines du maïs et du soja, dans des conditions de croissance optimales et stressées par le NaCl. Trois niveaux de NaCl (0, 50 et 75 mM) et quatre concentrations de CFS (1.0, 0.2, 0.13 et 0.1 % v/v) ont été utilisés pour l'étude. Le traitement avec des concentrations de CFS de 0.2 %, 0.13 % et 0.1 % a significativement amélioré la longueur des racines du soja cultivé dans des conditions optimales de 36.4, 39.70 et 39.91 %. Le traitement avec une concentration de CFS de 1.0 % a significativement amélioré le pourcentage de germination du soja exposé à 50 mM de NaCl de 48.65 %, à 24 h. Des concentrations de CFS de 0.2 % et 0.13 % ont amélioré la longueur des racines du maïs exposé à un stress de NaCl de 50 mM de 23.73 et 37.5 %, respectivement. Le traitement avec des concentrations de CFS de 0.2 %, 0.13 % et 0.1 % a significativement amélioré le pourcentage de germination du maïs exposé à 75 mM de 25.3 % (dans les 3), à 48 h.

La quatrième étude a évalué l'effet du CFS obtenu à partir de *L. helveticus* EL2006H sur sa capacité à améliorer le pourcentage moyen de germination et la longueur des racines du maïs et du soja, ainsi que les variables de croissance de la pomme de terre, en utilisant des formulations de traitement composées de 0.2 et 1.0 % [v/ v] Concentrations de *L. helveticus* EL2006H CFS et niveaux de NaCl de 100 mM et de NaCl de 150 mM. Les résultats ont montré que le traitement avec une concentration de CFS de 0.2 % a amélioré le pourcentage de germination du soja exposé à un stress de 100 mM de NaCl de 44,37 %, à 48 h. Le traitement avec une concentration de CFS de 1.0 % a augmenté de manière significative la longueur des racines du maïs de 23.04 %. Le traitement avec CFS 0.2 % a significativement augmenté le taux de photosynthèse, la verdeur des feuilles et le poids frais de la pomme de terre exposée à 100 mM de NaCl.

En conclusion, sur la base des résultats de ce projet, *B. amyloliquefaciens* EB2003 et *L. helveticus* EL2006H sont tolérants à des niveaux élevés de stress NaCl. Les deux souches modifient leur profil d'exoprotéome lorsqu'elles sont exposées au stress salinité, régulant potentiellement à la hausse les protéines qui améliorent leur tolérance au stress NaCl. Les protéines régulées à la hausse pourraient également être en partie potentiellement responsables de la bioactivité des CFS sur le maïs, le soja et la pomme de terre. Ce projet a contribué à la connaissance en étant la première étude, pour montrer les mécanismes employés par *B. amyloliquefaciens* EB2003A et *L. helveticus* EL2006H pour tolérer le stress NaCl, au niveau de l'exoprotéome, et le relier

à la capacité de leur CFS à améliorer la croissance des plantes. Le projet a également élucidé que le CFS des deux souches peut être une approche prometteuse pour améliorer la croissance des cultures dans des conditions idéales et stressées par le NaCl.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Chapter 3

This is the first study to elucidate the effect of salt stress on EVL Inc consortium strains *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H. The study showed that the two strains were tolerant to varying concentrations, up to 1000 mM. It also showed that growth rate and generation time of the two strains varied across the different NaCl concentrations. Findings of the study could help the company to come up with new product combinations, especially those targeting salt affected areas.

Chapter 4

This is the first study to elucidate the effect of NaCl stress on the exoproteome profiles of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H. The study highlighted possible mechanisms through which the two strains tolerate salt stress at the exoproteome level. Some of the proteins upregulated have been reported to enhance growth of plants under salt stress and ideal conditions, which supplements results of chapters 5 and 6 of this thesis, as possibly being responsible, in part, for the ability of the two strains to enhance growth for corn, soybean and potato. Results of the study also support previous reports that when exposed to stress, microbes alter their exoproteome profiles. Findings of this study added to the knowledge regarding mechanisms through which *Bacillus spp* and *Lactobacillus spp* tolerate salt stress at protein level.

Chapter 5

This study is the first to show that cell-free supernatant of *Bacillus amyloliquefaciens* EB2003A exposed to 200 mM NaCl can enhance germination and root length of corn and soybean under both optimal and salt stressed conditions. The study showed the effect of plant species, NaCl level, and cell-free supernatant concentration, on the efficacy of *Bacillus amyloliquefaciens* EB2003A to enhance plant growth. The ability of the supernatant to enhance plant growth highlights the possibility that *Bacillus amyloliquefaciens* EB2003A produces a biologically active compound/s in its growth media, that is responsible for the effects observed on seed germination and root length. Therefore, findings of this study can be used as a baseline to isolate and identify any active compounds exuded by the strain in its growth media for possible use

on the market, as plant growth biostimulants. The findings also support previous reports that, the effect of plant growth promoting microorganisms and their derived compounds on plants varies between and within plant species and that, the effectiveness of microbial derived compounds at enhancing plant growth may be influenced by concentration of the compound, as well as the level of stress to which the plant is exposed.

Chapter 6

This study is the first to show that cell-free supernatant of *Lactobacillus helveticus* EL2006H exposed to 200 mM NaCl can enhance germination and root length of corn and soybean, and growth variables of potato, exposed to NaCl stress, under greenhouse conditions. The study showed the effect of plant species, NaCl level, and cell-free supernatant concentration, on the efficacy of *Lactobacillus helveticus* EL2006H to enhance plant growth. Findings of the study suggest a possible presence of bioactive substances in the cell-free supernatant, most probably exuded by the bacteria when exposed to 200 mM NaCl. Findings of this study could be used as a baseline for further studies that may involve isolation of the bioactive compounds. Findings of the study also support previous findings that microbial cell-free supernatant can enhance plant growth.

Chapter 1: General Introduction

The world population in 2023 is approximately 8 billion, greater than that of 2022 by 0.88%, and is expected to reach approximately 10 billion, by 2050, according to United Nations projections (Macrotrends, 2023). Therefore, there is an urgent need to meet food and nutrient demands for all people and for livestock animals, which necessitates increasing food production rates, especially, by increasing yield per unit area, since land is a static resource, and there is little scope left for converting marginal land into crop production areas (Barea, 2015). However, with the need to slow climate change and conserve the environment, optimising production requires use of environmentally friendly and sustainable approaches, such as the use of plant growth promoting microorganisms (PGPM) and their derivatives (Smith et al., 2015a; Egamberdieva & Lugtenberg, 2014; Zhou et al., 2016; Naamala & Smith, 2020).

The coexistence of plants and microbes is largely dependent on a cascade of chemicals produced by both partners, as a means of communication (signals), source of food/energy or simply as a survival mechanism, e.g., outcompeting competitors for niche space, and energy, as is the case for antibiotic and antifungal-compound producing microbes. The plant almost always regulates the composition of the population of microbes associated with it (the phytomicrobiome), especially in its rhizosphere, depending on development level and degree of stress, and conditions in its surrounding environment, mostly, through exudates, including signal compounds (Zhang et al., 2017). The plant may also benefit from microbial exudates as is the case of plants in association with plant growth promoting microorganisms.

Microbial exudates such as phytohormones, bacteriocins, exopolysaccharides, volatile organic compounds and microbe-to-plant signals have been reported to benefit plants in terms of enhancing growth and or mitigating effects of biotic and abiotic stressors (Souleimanov et al., 2002; Subramanian et al., 2016a, Subramanian et al., 2016b). However, the success of PGPM in a new environment is dependent on the inoculant strains' ability to survive in the rhizosphere while maintaining their ability to produce plant growth promoting substances (Martínez-Viveros et al., 2010). These two important aspects (survival and ability to promote plant growth) are affected by abiotic stresses such as salinity. Unfortunately, salinity is currently a major global constraint to crop production, with large areas of arable land already marginalised and more expected to follow suit (Li, 2008; Chakraborty et al., 2011; Xu et al., 2011;

Egamberdieva & Lugtenberg, 2014). In order to optimise crop production through PGPM technology, especially in saline environments, there is need to not only identify microbial strains tolerant to salinity stress and able to promote plant growth, but also complement and or supplement PGPM cells with other PGPM derivatives, such as PGPM cell free supernatant (CFS) containing PGPR derived compounds and PGPM derived compounds themselves; the combination of the two may address some of the inconsistencies associated with the sole use of PGPM cells, especially under field conditions (Schenk et al., 2012; Barea, 2015; Jha & Saraf, 2015; Naamala & Smith, 2020; Naamala & Smith, 2021a,b). Despite modern technology and equipment, a lot is yet to be uncovered about the phytomicrobiome of both domesticated and undomesticated plants (Lyu et al., 2020), partly due to their inability to grow/be cultured outside their natural environment. As a result, a lot is yet to be learned regarding microbial derived compounds.

1.1 Plant growth promoting microorganisms (PGPM)

Plant growth promoting microorganisms enhance plant growth, under stressful and optimal growth conditions (Hartmann et al., 2014). They are diverse and include groups such as bacteria, mycorrhizal fungi, protozoa, actinomycetes and algae (Schenk et al., 2012; Nadeem et al., 2014; Jha & Saraf, 2015; Ruzzi & Aroca, 2015; Ilangumaran & Smith, 2017; Ilangumaran et al., 2021). They occur naturally in the soil and are more prevalent in the plant rhizosphere. They can be symbiotic or free living, living on the outside or inside of plant tissues (Vessey, 2003; Gray & Smith, 2005; Hayat et al., 2010; Nadeem et al., 2016). As they associate with plants, they secrete substances such as volatile organic compounds, exopolysaccharides, and proteins, which may possess plant growth promoting characteristics (Burr et al., 1978; Barnawal et al., 2013; Kang et al., 2014b; Smith et al., 2015a; Zhou et al., 2016).

Research on PGPM has been on going for decades, which has yielded innovations such as PGPM based inoculants. PGPM based inoculants, which are packaged as single strains or consortia allow for application of PGPM on plants or around plants roots, depending on the plants' need, which makes the technology more effective, most times (Bashan et al., 2014; Barea et al., 2015; Gupta et al., 2015; Compant et al., 2019). Research on PGPM is currently dominated by plant growth promoting rhizobacteria (PGPR), often focused on diverse genera such as: *Bacillus*,

Pseudomonas, *Azospirillum*, *Burkholderia*, and *Rhizobium* (Gray & Smith, 2005; Khalid et al., 2009; Hayat et al., 2010; Nadeem et al., 2014; Ruzzi & Aroca, 2015). Mycorrhizal fungi and their relationship, especially with higher plants, have also been researched extensively (Marschner & Dell, 1994; Clark & Zeto, 1996b; Meding & Zasoski, 2008). The role PGPMs play in enhancing soil fertility, mitigating pathogens, mitigating effects of abiotic stress such as salinity, drought, extreme temperature, and extreme pH cannot be ignored and have been discussed in detail, in chapter two (Kang et al., 2015; Chen et al., 2016; Tiwari et al., 2016; Zhao et al., 2018). The use of PGPM technology is considered among the most successful sustainable and environmentally friendly approaches to enhancing plant growth, leading to enhanced uptake of the greenhouse gas CO_2, and so slowing development of climate change, and also making crop plants resilient to the stresses associated with climate change when they do develop (Naamala & Smith, 2020).

1.2 EVL coating® consortium

The consortium is made up of 5 microbial species from 4 genera, namely, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Pseudomonas putida*, *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. It is already on the market as a plant growth stimulant, currently being coated on inorganic fertiliser (NPK). Although members of the 4 genera have been individually investigated by other researchers, for their ability to promote plant growth (Hayat et al., 2010; Rahman et al., 2012; Sati et al., 2013; Nadeem et al., 2016; Punja et al., 2016; Moussa et al., 2017; Prasad et al., 2019), there is limited knowledge on the independent performance of the consortium's constituent strains, as plant growth stimulants, both under stressed and non-stressed environments, and it is generally known that efficacy often varies between and within species and strains. Furthermore, the effect of salinity stress on growth and ability of individual member strains to produce plant growth promoting substances is yet to be understood. This knowledge would be pivotal to EVL, in developing new products, especially those aimed at addressing salt stress mitigation. This study focused on *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H. Both strains are gram positive and widely used in the food processing sector (Woldemariam et al., 2020). *B. amyloliquefaciens* are rod shaped and form endospores when exposed to stress (Woldemariam et al., 2020), while *L. helveticus* produce lactic acid which enables them to acidify their growth environment. Both species and their derivatives, have also

been reported to enhance plant growth under stressed and non-stressed conditions (Duan et al., 2021; Kazerooni et al., 2021; Naamala et al., 2022a; Naamala et al., 2022b). The two strains were chosen for this project because there is not much published information on their performance or their derivatives as plant growth biostimulants, especially *L. helveticus* EL2006H, under saline conditions.

1.3 PGPM derivatives

1.3.1 Microbial cell-free Supernatant

Microbial cell-free supernatant (CFS), also referred to as spent medium, may refer to microbial medium which has been used to culture microbes for a given period of time, after which microbial cells are removed either through centrifugation and/or filtration (Naamala et al., 2022; Naamala et al., 2023; Monjezi et al., 2023; Msimbira et al., 2023). The CFS of various microbial species have been reported to enhance growth of varying plant species. For example, CFS of *B. amyloliquefaciens EB2003A* and *L. helveticus* EL2006H enhanced growth for corn, soybean, and potato, under optimal and NaCl stressed conditions (Naamala et al., 2022; Naamala et al., 2023). Cell-free supernatant of *Bacillus subtilis* cultured at varying pH levels enhanced growth of tomato, corn and potato grown under ideal and acidic conditions (Msimbira et al. 2022a; Msimbira et al., 2023). Cell-free supernatant of *Devosia* sp. enhanced germination of soybean and canola seeds under ideal and NaCl stress conditions (Shah et al., 2022; Monjezi et al., 2023). The bioactivity of CFS could be attributed to the fact that during microbial culturing, microbes produce substances such as metabolites and proteins of varying quality and quantity, into their growth medium, some of which have been reported to enhance plant growth (Subramanian et al., 2021; Nazari et al., 2022).

1.3.2 Microbe-derived compounds

Invention of technologies, such as high-pressure liquid chromatography, tandem mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy, has allowed the isolation and identification of substances secreted into spent media (Armengaud, 2013; Kucharova and Wiker, 2014; Otto et al., 2014; Subramanian et al., 2021). Microbe-derived compounds are mostly secondary metabolites that are excreted by microorganisms, into their growth medium, in response to stimuli (Schulz & Dickschat, 2007; Piechulla et al., 2017; Schmidt et al., 2015; Schulz Bohm et al., 2017).

They include hormones, volatile organic compounds (VOCs), enzymes, antimicrobials and siderophores (Crowley et al., 1988; Bais et al., 2006; Dimkpa et al., 2009; Lemfack et al., 2014, 2018). A number of microbially derived compounds, such as thuricin17 and lipo-chitooligosaccharide have been reported to enhance plant growth under stressed and non-stressed conditions (Souleimanov et al., 2002; Subramanian et al., 2016a, b; Nazari et al., 2022), which is a potentially key finding for sustainable agriculture.

1.4 The Microbial Proteome

The microbial proteome loosely translates to all proteins associated with a given microbe, grouped into endoproteome and exoproteome proteins (Fuchu et al., 2016). Microbial exoproteome refers to proteins found in the immediate extracellular milieu of a microbe, arising from active cellular secretion, passive excretion and or cell lysis (Desvaux et al., 2010; Armengaud et al., 2012; Rubiano-Labrador et al., 2015; Schoof et al., 2022). The microbial endoproteome refers to proteins on the inside of the microbe, that can be accessed after microbial cell lysis. Both exoproteome and endoproteome compositions reflect a microbe's physiological state at a given time and can provide insight into a microbe's interactions with its surroundings (Armengaud et al., 2012). Abiotic stresses such as salinity, acidity and alkalinity affect the quantity and quality of the microbial proteome (Singleton et al., 1982a; Soussi et al., 2001; Msimbira et al., 2022).

1.5 Soil salinity

Soil salinity is a constraint for crop production and productivity at locations around the world, especially in arid and semi-arid areas (Zahran, 1997, 1999; Egamberdieva & Lugtenberg, 2014; Shrivastava & Kumar, 2015). In the agricultural context, soil salinization refers to the accumulation of water-soluble salts ions, such as Na^{+}, K^{+}, Mg^{+2} and Ca^{+2} and anions such as Cl^{-}, SO_2^{-4}, HCO_3^{-}, NO_3^{-} and CO_2^{-3}, in the root zone, to a level that negatively affects plant growth (Tanji, 2002; Rengasamy, 2006; Bui, 2013; Arora et al., 2021). Generally, when the electrical conductivity of a saturated soil paste extract (ECe) is greater or equal to 4 dS m^{-1}, equivalent to 40mM NaCl, the soil is classified as saline (US salinity laboratory staff, 1954; Shrivastava & Kumar, 2015; Forni et al., 2017; Arora et al., 2021). Soil salinity is caused by both

natural and artificial factors such as weathering of rocks, application of fertilisers, deforestation, and irrigation (Ghassemi et al., 1995; Tanji, 2002; Tank & Saraf, 2010; Rousk et al., 2011; Egamberdieva and Lugtenberg, 2014; Shrivastava & Kumar, 2015; Yan et al., 2015).

It has been reported that more than 100 countries worldwide are affected by either primary or secondary salinity, or both (Tanji, 2002; Rengasamy, 2006). Approximately 1 billion ha of land worldwide, that is approximately 7% of the planet's surface area is affected by salinity (Metternicht & Zinck, 2003; Yensen, 2008). The economic effects of salinity worldwide are estimated to be about US$ 12 billion (FAO, 2020). Unfortunately, soil salinization is expected to expand at a high rate, of about 10% annually, in part due to the expected expansion of crop production into marginal areas, which will require irrigation (Patel et al., 2011; Shrivastava & Kumar, 2015).

1.5.1 Effect of salinity stress on Plant Growth Promoting Microorganisms

While a lot of research has reported the effect of salinity stress on plants, its effect on PGPM is not as widely documented. However, salinity affects growth, survival, and diversity of soil microbial communities (Singleton et al., 1982a; Vriezen et al., 2007; Yan & Marschner, 2012; Yan & Marschner, 2013; Miransari et al., 2013; Egamberdieva et al., 2017). Because salinity can potentially affect general morphology of a microbe, as well as the nature and quantity of substances it exudes, in turn, it can affect the efficacy of the microbe in as far as plant growth promotion is concerned (Zahran, 1997; Soussi et al., 2001; Nadeem et al., 2016). In rhizobia for example, salinity can affect their ability to fix nitrogen by affecting rhizobial root colonisation and infection as well as affecting the nitrogenase enzyme itself (Singleton et al., 1982a; Zahran, 1997; Zahran, 1999). There is variation in the effect of salinity on microbes at strain, species, and genus levels (Singleton et al., 1982a; Zahran, 1997; Nadeem et al., 2016; Ilangumaran & Smith, 2017). When exposed to salt levels beyond their tolerance levels, microbes cease growth and can die.

1.6 Potato, corn, and soybean

1.6.1 Potato

Potato (*Solanum tuberosum*), from the plant family *Solanaceae*, is the world's second most widely distributed crop after corn, and 4th most important food crop after

rice, wheat, and corn (Beukema & van der Zaag, 1990; Jaarsma et al., 2013). Although it was first domesticated in the Andes (present day southern Peru and northwestern Bolivia), more than 7000 years ago, potato is currently produced in more than 100 countries, and a staple in many regions worldwide (Beukema & van der Zaag, 1990; Levy & Veilleux, 2007). Potato is the most valuable vegetable to the Canadian population, constituting more than half the total of fresh vegetables consumed in the country (Daniels-Lake, 2017). Potato also contributes to the trade revenue of Canada, through exports of potato products worth $1 billion annually (Daniels-lake, 2017). The provinces of Prince Edward Island, Manitoba, New Brunswick, and Alberta are, respectively, the leading potato producers in Canada (Daniels-lake, 2017). Although potato is a very important food and cash crop, its production is constrained by salinity stress, among other biotic and abiotic factors. Cultivated potatoes (*Solanum tuberosum* L.) are moderately sensitive to salinity, with most cultivars' growth negatively affected by salt levels as low as 15-30 mM NaCl (Shaterian et al., 2005; Levy & Veilleux, 2007; Jaarsma & de Boer, 2018). However, salinity tolerance in potato varies between and within varieties (Shaterian et al., 2005), with some cultivars able to grow at as much as 150 mM NaCl.

1.6.2 Corn (Maize)

Corn (*Zea mays* L.) is a monocotyledonous, annual C4 plant from the grass family *Poaceae*, and is the world's third most important cereal crop, after rice, and wheat (Farooq et al., 2015). It was domesticated in central Mexico about 7000 years ago (McCann, 2009; Adams, 2015). The primary use of corn varies across regions, being predominantly grown for human consumption (95%) in Africa while in Europe, East Asia and North America, corn is largely used as animal feed (fodder), raw material for biofuel, and in paint and plastic production (McCann, 2009; Adams, 2015; Guyader et al., 2017). In Canada, corn is an important feed and industrial crop, largely used in the production of animal feeds, and biofuel (Guyader et al., 2017). Corn is very important to the economy of Canada with an estimated $2 billion annual farm gate value. Each year, the crop occupies more than 1,400,000 ha of the country's arable land with an estimated production of about 10,700,000 tonnes (Statistics Canada, 2018). The provinces of Quebec and Ontario are the leading corn producers, in Canada, contributing approximately 90% to Canada's total corn production. Corn is considered

moderately sensitive to salinity, although variation occurs among cultivars (Farooq et al., 2015). Salinity stress affects all stages of corn development, from germination to maturity. As little as 0.1 M NaCl causes visible reductions in plant height, compared to corn grown under optimal conditions (Farooq et al., 2015).

1.6.3 Soybean

Soybean *(Glycine max* [L.] Merrill*)* is an annual grain crop, belonging to the family *Fabaceae* and sub-family *Papilionoideae* (Werner & Newton, 2005; Herridge *et al*., 2008). The crop was first domesticated in China, around 1100 BC (BASF USA, 2015), and subsequently spread to the rest of the world, reaching Canada in the mid-1800s (Dorff, 2007). The United States is the leading producer of soybean, while Canada ranks 17th, contributing 1.3 % to global soybean production. In the past decade, soybean production in Canada increased by 103%, making it the 3rd most important field crop in the country, after canola and wheat. The provinces of Ontario, Manitoba and Quebec are the leading soybean producers in Canada, respectively (Dorff, 2007; Soy Canada, 2019). Canada earns approximately $ 2.7 billion from the export of food grade soybean to other countries (Soy Canada, 2019). Besides its economical and nutritional value, as well as its nutraceutical properties, soybean also plays significant roles in sustainable agriculture, through biological nitrogen fixation (BNF), when in symbiotic association with rhizobia (Miransari et al., 2013). Given its enormous benefits, the demand for high quality soybean is substantial yet, over time, climatic conditions are deteriorating, to levels that may compromise yield and quality. Soybean is sensitive or moderately tolerant to salinity stress, depending on cultivar and growth stage (Kondetti et al., 2012). High salt concentrations affect germination (Kondetti et al., 2012), early plant growth, and the nitrogen fixation process (Zahran, 1997, 1999; Egamberdieva & Lugtenberg, 2014). Therefore, sustainable approaches for enhancing soybean production, in deteriorating climatic conditions, are priority (Soy Canada, 2019)

1.7 Hypotheses

1. *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H can grow under salinity stress conditions.
2. Salinity stress alters the exoproteome composition of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H.

3. *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H cell-free supernatants enhance growth of corn, soybean, and potato, in stressed and non-stressed environments.

1.8 Objectives

1. To determine the ability of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H strains to grow under salinity stress.
2. To understand the effect of salinity stress on the exoproteome composition of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H.
3. To understand effect of salinity stress on the ability of the cell-free supernatants of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H to promote growth of corn, soybean, and potato, under controlled conditions.

Chapter 2: Literature Review
Relevance of Plant Growth Promoting Microorganisms and Their Derived Compounds, in the Face of Climate Change

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2.1 Abstract

Climate change has already affected food security in many parts of the world, and this situation will worsen if nothing is done to combat it. Unfortunately, agriculture is a meaningful driver of climate change, through greenhouse gas emissions from nitrogen-based fertilizer, methane from animals and animal manure, as well as deforestation to obtain more land for agriculture. Therefore, the global agricultural sector should minimize greenhouse gas emissions in order to slow climate change. The objective of this review is to point out the various ways plant growth promoting microorganisms (PGPM) can be used to enhance crop production amidst climate change challenges, and effects of climate change on more conventional challenges, such as: weeds, pests, pathogens, salinity, drought, etc. Current knowledge regarding microbial inoculant technology is discussed. Pros and cons of single inoculants, microbial consortia and microbial compounds are discussed. A range of microbes and microbe derived compounds that have been reported to enhance plant growth amidst a range of biotic and abiotic stresses, and microbe-based products that are already on the market as agro-inputs, are a focus. This review will provide the reader with a clearer understanding of current trends in microbial inoculants and how they can be used to enhance crop production amidst climate change challenges.

2.2 Introduction

The world is at a point where we can no longer prevent all of the effects of climate change (because some of it is already here) but can only slow its further progress. The purpose of this paper is therefore to give the reader an understanding of why plant growth promoting microorganisms, or their products, are relevant, amidst climate change challenges, by showing how they can be used to mitigate the effects of climate change on crop production. The paper also highlights the various ways in which this approach can be used, and the role that inoculant formulation plays in maintaining the efficacy, durability, and handling of microbial inoculants. The major drivers of climate change are human driven (Lott et al., 2011; Rossi et al., 2015; Bradley et al., 2016). Burning of fossil fuels for energy, agriculture and industrialisation all contribute to emission of greenhouse gases (GHGs), such as: methane, carbon dioxide and nitrous oxide (N_2O). Agriculture is a major contributor to greenhouse gas emissions (Richards et al., 2018; Loboguerrero et al., 2019), especially with the use of N based fertilizers, methane emissions from animals and animal manure, deforestation to acquire more land for crop production, etc. According to the intergovernmental panel on climate change (IPCC) report on GHG emissions, energy consumption contributes about 35%, agriculture, forestry, and related land use 24%, industry 21% and transport 14% (IPCC, 2014). The greenhouse gases then trap heat radiating from the earth's surface, causing global warming. Unfortunately, climate change also adversely affects agriculture (IPCC, 2014; Porter et al., 2014), especially because, along with increases in global temperature, comes the increased prevalence of biotic and abiotic stresses that are detrimental to agriculture production, such as: pests, pathogens, nutrient deficiencies, salinity and weather extremes (Bouwer et al., 2010; Mirza, 2011; Lott et al., 2013; Dawson et al., 2016), some of which may encourage the further use of chemicals to correct, while there is little that can be done about others such as high temperatures and floods. Unmanaged, such factors affect plant growth and render arable land unproductive. This puts us in a challenging situation, especially because the world population is growing so that there is a need to increase food production (Loboguerrero et al., 2019), both through increasing yield per unit area and reclaiming more land for crop production (Nam et al., 2015). Therefore, while we strive hard to hold greenhouse gas emissions to 'bearable' levels, there is also a need for sustainable approaches that will ensure increased food production in the face of climate change. The use of agrochemicals has boosted crop productivity and contributed to food security,

especially in developed countries. However, shortcomings related to their improper and continuous use, such as: increased greenhouse gas emissions (which is a major contributor to global warming), surface and ground water contamination, residual contamination of crop harvest, which poses health concerns to both humans and animals, as well as high costs related to their use. These circumstances have created a need for a more ecofriendly and sustainable approach for enhancing crop productivity in the face of climate change (Barea, 2015; Gupta et al., 2015; Nam et al., 2015).

Several approaches have been suggested; the use of plant growth promoting microorganisms and compounds that they produce is perhaps the most promising (Bender et al., 2014). The holobiont refers to plants and their associated microbes, which probably coexisted since the colonization of land by the first terrestrial plants (Babalola & Glick, 2012; Smith et al., 2015; Smith et al., 2017; Backer et al., 2018). This association is dynamic, with the plant asserting a great influence on the nature of phytomicrobiome, especially in its rhizosphere (Hartmann et al., 2014), which is mainly attributed to the composition of their root exudates. The rhizosphere, endosphere and phyllosphere may be comprised of pathogenic, neutral, and beneficial microbes, in relation to the plant (Sánchez-Cañizares et al., 2017; Backer et al., 2018). Microbes that exert beneficial effects on the plant are termed plant growth promoting microorganisms (PGPM). These microbes may inhabit the rhizosphere, rhizoplane, phyllosphere, endosphere, and other parts of the plant. (Hartmann et al., 2014). For decades, PGPM such as rhizobia, mycorrhizae and plant growth promoting bacteria (PGPR, first defined by Kloepper and Schroth, in 1978) have been reported to enhance plant growth under stressed and non-stressed conditions. The use of microbial inoculants is an old practice (Compant et al., 2019) that has recently gained more prominence during the last three decades. Much research has been done on rhizobia, and currently, a lot is being done on plant growth promoting rhizobacteria and PGPR derived compounds. The ability of microbes to suppress plant pathogens, as well as mitigate the effect of abiotic stress on plants, has been investigated by many researchers, and the findings are promising.

Although they occur naturally in the rhizosphere, and plant tissue, PGPM populations are often insufficient to induce desired effects, hence, it is recommendable to isolate them from their natural environments and multiply their populations before reintroduction into the soil or onto the plant as microbial inoculants (Bender et al., 2014). Products in the form of microbe-produced compounds are currently gaining popularity among researchers, although they are less known among farmers, in

comparison to microbial cell inoculants, packaged as either single microbial strains or consortia, which have been commercialised for quite some time (Berg & Koskella, 2018; Compant et al., 2019). Microbe based inoculants are generally from the bacteria (such as *Bacillus* and *Rhizobia*) and fungi (especially *Trichoderma*) subgroups (Bashan et al, 2014; Hartmann et al., 2014; Berg & Koskella, 2018), although some groups of archaea have also been reported to enhance plant growth. Microbially produced compounds, such as lipo-chitooligosaccharides (LCO), as plant growth enhancers, on the other hand, are only gaining attention recently, which may explain their lesser availability on the agro-input market. Figure 1 below summarizes some of the mechanisms PGPM employ to mitigate the effects of biotic and abiotic stress on plants, which are later discussed in detail.

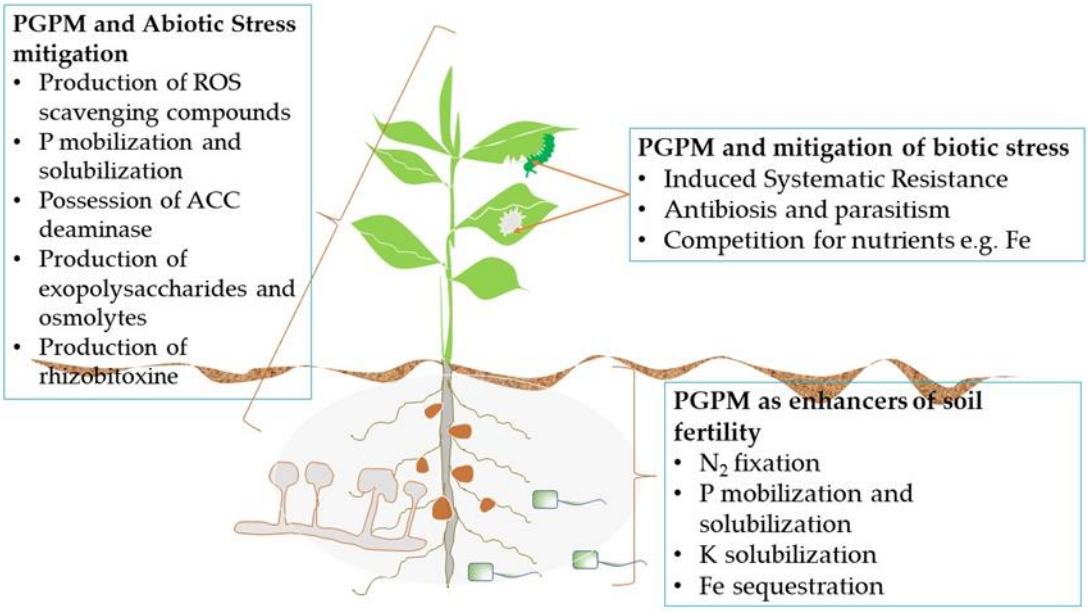

Figure 2.1: Mechanisms employed by plant growth promoting microorganisms (PGPM) to mitigate effects of biotic and abiotic stress on plants.

2.3 PGPM as Enhancers of Soil Fertility

For proper growth and development, plants need enough supply of essential macro (Nitrogen [N], Phosphorus[P], Potassium [K], Magnesium [Mg], Calcium [Ca], etc.) and micro (iron [Fe] , manganese [Mn], boron [B], zinc[Zn], molybdenum [Mo], copper[Cu]) nutrients. Nitrogen, P and K are the most limiting as far as plant growth is concerned. Unfortunately, with climate change comes abiotic stresses like high

temperature, drought, and salinity, which influence the biogeochemical transformation of nutrients like P, K, and N, making them either available or less available for plant uptake (Silva et al., 2011; Malusa et al., 2012; Alori et al., 2017). While the lack of bioavailable macro and microelements is natural in the soil, it could be worsened by climate change. Nitrogen, P, and K as the most plant growth limiting elements and their biogeochemical cycle, are affected by temperature and rainfall amongst other abiotic factors, which happen to be affected by climate change. Processes like decomposition, mineralisation, immobilisation, etc. are largely influenced by temperature and rainfall. Processes like soil erosion should also be noted, which is majorly due to run off and wind affect soil fertility as the nutrient rich topsoil is washed away.

Alkalinity affects the availability of Fe, Cu, Zn and Mn, while very low pH is associated with Aluminium toxicity. Processes such as mineralization and nitrogen fixation are affected by moisture, temperature, and pH, because they are driven by soil microorganisms like rhizobia, nitrifying bacteria, etc., and enzymes (Silva et al., 2011; Alori et al., 2017), which are also affected by abiotic stress. A study by DaMatta et al. (2002) showed a decrease in leaf N content of *Coffea canephora* due to water stress. For PGPM technology to be relevant, amidst climate change, it is paramount that stress tolerant strains are identified and used. At the same time, the availability of these nutrients is essential, because they play a key role in minimizing the effects of other abiotic stresses like drought, salinity, and high temperature on crops. The roles N, P, K, Ca, Mg and Fe play in the mitigation of abiotic stress have been reported by many researchers (Tietema et al., 1992; DaMatta et al., 2002; Waraich et al., 2011; Waraich et al., 2012; Wang et al., 2013; Karmakar et al., 2016; Triphati et al., 2018). For instance, N and P have been reported to minimize the effects of drought stress (Faye et al., 2006; Wu et al., 2008; Silva et al., 2011; Malusa et al., 2012; Alori et al., 2017). Potassium plays a major role in drought stress as well since it is involved in the opening and closing of the stomata. Agricultural soils have been degraded due to continuous and intense cropping. Agricultural practices like continuous cropping, especially monocropping of non-leguminous crops, without application of fertiliser, is one way of depleting soils of nutrients (Alori & Fawole, 2018). This is a common practice of many smallholder farmers, especially in sub-Saharan Africa, due to the inaccessibility and cost of fertiliser (Lal., 2015). Climate change is only further degrading the situation because factors such as high temperatures, drought, flooding, salinity, extreme pH, etc. may cause changes in the physiochemical properties of essential soil nutrients such as

N, Fe, P and K, thereby limiting their mobility and/or affect their availability for plant uptake, while enhancing the accumulation of toxic elements such as aluminium (Al^{3+}). The role of stress tolerant beneficial microbes in maintaining/increasing crop production amidst climate change challenges cannot be ignored. In order to reclaim land that has been abandoned due to inadequate nutrients for crop growth, considering the financial and environmental costs related to synthetic fertilisers, stress tolerant plant growth promoting organisms can be a cheaper and sustainable approach. With the need to reclaim more land for crop production, emphasis on enhancing soil fertility is inevitable, because nutrients can enhance plant tolerance to abiotic stress. Therefore, there is a need to address the issue using more sustainable approaches. With limited alternatives, and research output so far, microbial inoculants are a promising approach to enhance soil fertility, particularly in conjunction with the various challenges associated with climate change. Microbial inoculants may be defined as formulations comprised of microorganisms, such as bacteria and fungi, as the active ingredients, which once applied on plants, can enhance their growth (Hartmann et al., 2014; Alori & Babalola, 2018; Berg & Koskella, 2018). They may also enhance plant quality through the increased concentration of essential nutrients such as proteins (Bender et al., 2014), and valuable metabolites such as flavonoids, phenolics, alkaloids and carotenoids (Bashan et al, 2014). Microbial inoculants may also enhance soil biodiversity and properties such as soil structure (Berg & Koskella, 2018). As biofertilizers, microbial inoculants enhance the availability and uptake of essential plant nutrients, such as: N, P, Fe, Zn, and K (Barea, 2015; Gupta et al., 2015; Nam et al., 2015), which, if lacking or available in inadequate quantities, could limit plant growth.

2.3.1 Nitrogen Fixation

Some free-living and symbiotic bacteria fix atmospheric dinitrogen into plant usable forms, initially ammonium, through biological nitrogen fixation. Symbionts such as *Rhizobia*, *Bradyrhizobium*, *Sinorhizobium*, *Frankia*, *Actinobacteria* and *Burkholderia* form specialized structures called nodules on their host plants, where they obtain nourishment and shelter, and in turn, fix nitrogen (Naamala et al., 2016; Alori & Babalola, 2018). The process is referred to as symbiotic nitrogen fixation and it occurs in both legumes and non-leguminous plants, although that of legumes is the most studied. Communication in the form of molecular signals from both the microbe and host plant, as well as a complex of enzymes (e.g., nitrogenase) and genes (*nif* and/or

symbiotic genes), are involved in the process of nitrogen fixation. On the other hand, free-living nitrogen fixing bacteria such as *Azotobacter* do not need to occupy plant tissue to fix nitrogen. Because of the high energy requirement for BNF, plants tend to prefer applied N fertilizer to the former, hence, for effectiveness, synthetic N should not be used along with biological nitrogen fixing organisms, because the plant may suppress the nitrogen fixing symbiosis. Where a starter dose of synthetic N is necessary, it should be applied cautiously, because high N supply can have an inhibitory effect on nodulation (nodule dry weight and number of nodules) and nitrogenase activity (Graham et al., 1981; Sprent et al., 1988; Alori et al., 2017). Arbuscular mycorrhizal fungi, through their hyphae, can enhance the acquisition of soil N by the plant (Sprent et al., 1988; Marschner & Dell, 1994), although there are wide variabilities as to the degree of this, whose causes are not yet known (Berg & Koskella, 2018). The efficiency and effectiveness of nitrogen fixing bacteria varies among and within plant species, and, in the agricultural context, are largely limited to members of the *fabaceae* family. Other crops can benefit from the symbiosis by including legumes in crop rotation regimes. There is also a need for more research on how to extend such modifications to non-leguminous plants. Approaches such as genetic engineering to enable non-legume nitrogen fixation and enhance effective communication with N fixing microorganisms can be further researched. Although genetic engineering is questionable, especially its ecological impact, some of the questions are likely from a lack of adequate information on the technology. Extensive research to address most of the questions can be very helpful.

2.3.2 Phosphate Mobilisation and Solubilisation

Although phosphorus is an abundant element in most soils, it frequently occurs in forms unavailable for plant use. The application of external sources of P fertiliser, such as single super phosphate, diammonium phosphate, etc., can help meet plants' P requirements, but this too may be immobilised shortly after application, making it largely unavailable for plant uptake (Glick, 2012). Mobilization (chemical solubilization and mineralization), which results in plant available forms of the respective nutrients and solubilization, which is a more general term and does not necessarily result in readily plant available forms. For instance, the solubilization of organic P does not necessarily mean that the P is already plant available, as it may still

be bound in unavailable organic forms (e.g., phytates). PGPM may enhance soil phosphorus availability for plant uptake through solubilisation and/or mobilisation of inorganic phosphorus. A PGPM may possess both or either mechanism. The terms, phosphorus solubilisation and phosphorus mobilisation are often used synonymously by many researchers, although they are not necessarily the same thing. P solubilisation is the broader term, which may entail P mobilisation. Goldstein and Krishnaraj (2007) described phosphate solubilising microorganisms as those that convert sparingly soluble organic or mineral P, into soluble orthophosphate, in a way that significantly increases P availability to a specific plant or plant population within the microorganism's native soil ecosystem. The same author defined phosphate mobilising microorganisms as those that convert sparingly soluble organic or mineral P, into soluble orthophosphate P, in a way that significantly contributes to pool of available orthophosphate (Pi) in the native soil ecosystem. Phosphorus solubilising bacteria, such as: *Pseudomonas*, *Bacillus*, *Burkholderia* and *Rhizobium*, and some fungal species solubilise inorganic phosphates from sparingly soluble forms such as: tricalcium phosphate, dicalcium phosphate and aluminum phosphate, to forms such as hydrogen phosphate (HPO_4^{-2}), or dihydrogen phosphate ($H_2PO_4^{-1}$), which plants can utilise (Sprent et al., 1988; Marschner & Dell, 1994; Goldstein & Krishnaraj, 2007; Meding & Zasoski, 2008; Hayat et al., 2010; Glick, 2012; Lal, 2015) through the production of low molecular weight organic acid anions, such as gluconate, lactate, glycolate and oxalate. Phosphorus mobilisers, on the other hand, produce enzymes (such as phosphatase, phytase and phosphonoacetate hydrolase) that chelate cations, bind phosphates, and dephosphorylate organic phosphates (Alori et al., 2017; Berg & Koskella, 2018). Dephosphorylation is catalyzed by hydrolase enzymes such as phosphonoacetate, which some PGPM can produce. For instance, ectomycorrhiza and ericoid mycorrhizal fungi produce extracellular acid phosphatases and phytases, which catalyse the mineralisation of P from organic complexes in the soil (Straker & Mitchell, 1986; Sprent et al., 1988; Marschner & Dell, 1994). Other fungal species, such as *Aspergillus niger*, also produce organic acids which aid the process of P solubilisation (Khan et al., 2010; Elias et al., 2016). Through the possession of hyphae, some mycorrhizae such as arbuscular mycorrhizae can deliver up to 80% of the phosphorus taken up by the host plant (Graham et al., 1981; DaMatta et al., 2002).

2.3.3 Sequestering of Iron

Some PGPM, like *Pseudomonas fluorescens* and *Rhizobia meliloti*, sequester iron through the production of siderophores, which can be grouped into four, namely: hydroxamates, catecholates, carboxylates and pyoverdines (Daly et al., 2017). Currently, about 500 siderophores have been reported by researchers. Although plants cannot absorb Fe^{3+}, siderophores have a high affinity for Fe^{3+}, which results in an iron-siderophore complex that is then absorbed by plants (Khan et al., 2019), into their tissues, hence, aiding plants in meeting their iron requirements (Bender et al., 2014; Smith et al., 2017). In 2013, study findings of Radzki et al. (2013) showed an increase in iron content at 12 weeks for iron deficient tomato plants, following inoculation of siderophore producing bacteria, evidence that microbial siderophores can be a source of iron for plants. A study by Sharma and Johri (2003) also showed an increase in maize plant growth following inoculation with siderophore producing PGPR. The uptake of Fe-microbial siderophore complexes by strategy II plants, via ligand exchange, between ferrated microbes and a phyto siderophore, was also reported by Yehuda et al. (1996). It should also be noted that some plant species can also produce siderophores which bind Fe^{3+}, to form a complex that can be taken up by the plant with the aid of ligands. Production of siderophores is also a benefit in the context of biocontrol in a sense that potential plant pathogens, especially fungal pathogens, are outcompeted for iron sources, which may lead to their death, or ineffectiveness.

2.3.4 Potassium solubilisation

Microbes such as: *Arthrobacter sp*., *Bacillus edaphicus, Bacillus circulans* and *Bacillus mucilaginosus* convert sparingly soluble and mineral potassium to soluble forms available for plant use (Nadeem et al., 2010). Through the release of H^+ and organic anions, such as citrate, malate and oxalate, arbuscular mycorrhiza can also increase the solubility of mineral K (Meena et al., 2014), thereby increasing the availability of potassium anions for plant uptake, although the increase in K^+ availability is sometimes related to the increase in phosphorus availability (Cardoso & kuyper, 2006; Berg & Koskella, 2018). Some PGPR can also directly influence plant growth through the production of phytohormones such as auxins and gibberellins, which enhance plant growth when plant phytohormones are at suboptimal concentrations (Lal, 2015). They may also produce enzymes which regulate hormone

concentration in plant tissue. For instance, some plant growth promoting microorganisms can produce an enzyme, *ACC deaminase*, which breaks down ACC, a precursor of ethylene, into an alpha keto butyrate and ammonium, hence lowering ethylene concentration in plant tissues (Nadeem et al., 2010; Jalili et al., 2008; Ali et al., 2014; Pérez-Montano et al., 2014; Jha & Saraf, 2015). With more research and proper manipulation, PGPM, with the ability to enhance plant growth, may not necessarily fully replace chemical fertilizer, but lower their use, directly and indirectly, through increasing the plants' nutrient uptake efficiency from applied chemical fertilizers (Dodd & Ruiz-Lozano, 2012). Manipulations such as developing an effective consortium of microbes that can make available key elements in the soil would greatly reduce the need to use chemical fertilizers. For instance, rhizobial species require iron for good growth, and in their nitrogenase complex, hence co-inoculation of rhizobia and siderophore producing PGPM could enhance nodulation and nitrogen fixation (Hassen et al., 2016). Use of biofertilizers can lower the need to burn fossil fuels for fertilizer production, and the associated contribution to greenhouse gas emissions.

2.4 PGPM and Control of Plant Pests and Diseases

With global warming comes new species of pests, weeds, and pathogens currently prevalent in warmer environments. The use of chemicals to suppress such plant growth inhibitors is effective but with negative outcomes related to improper use, cost, and increasing evolution of tolerance to the chemical. The antagonist properties of biocontrols against such plant growth suppressors have been reported by many researchers, and the results are promising. A diversity of PGPM with biocontrol properties has been identified by researchers, conferring benefits to a variety of crop species (Recep et al., 2009; Moussa et al., 2013; Diaz et al., 2013; Vanitha & Ramjegathesh, 2014; Dixit et al., 2016; Li et al., 2017; Wu et al., 2017; Zhao et al., 2018; de Vrieze et al., 2018). Berendsen et al. (2018) showed that plants, when exposed to pathogen attack, can recruit specific plant growth promoting microbes with biocontrol activity, against the pathogen in question. It is believed that manipulating plant recruited PGPM for inoculant production could be more effective in controlling targeted pathogens, than PGPM isolated from places with no pathogen attack. Biocontrols have the potential to minimise the use of industrially manufactured chemicals in agricultural production. This would mean a decline in burning of fossil

fuels, and hence a reduction in greenhouse gas emissions. This is because some pesticides are synthesized in laboratories using hydrocarbons like petroleum, which is a fossil fuel. Reduction in their use can mean a reduction in burning fossil fuels, hence less CO_2 emission to the atmosphere. It would also reduce effects on non-targeted members in the ecosystems, which are sometimes affected by chemical use.

Biocontrols may act directly to inhibit growth of biotic agents through hyper parasitism and production of bioactive substances, such as: antibiotics, hydrogen cyanide and phenazines (de Vrieze et al., 2018), or indirectly through competition for nutrients and active sites on plants, as well as inducing the plant's systemic resistance against the harmful biotic factor (Alori & Babalola, 2018; Berg & Koskella, 2018). Siderophore producing PGPM tend to outcompete other microorganisms for iron sources, which causes inefficiencies in terms of pathogen activities, especially for fungal pathogens, which eventually leads to their death (Hassen et al., 2016) Induced systemic resistance is triggered by microbe associated molecular patterns (MAMPS), such as lipopolysaccharides, that plants recognise and respond to by turning on their defence systems (Gadhave et al., 2016). Since MAMPS differ among PGPM, it is believed that microbial consortia made up of more than one microbe may induce stronger systemic resistance than single strains (Zhao et al., 2018), although further research needs to be done for a clearer understanding of this potential. PGPM can not only mitigate crop pathogens, but also suppress crop pests, such as spidermites (Schausberger et al., 2012), moths (Pangesti et al., 2015), aphids (Herman et al., 2008), nematodes (Diaz et al., 2013; Velivelli et al., 2014), leaffolder pest (Karthiba et al., 2010) and cutworms (Bano et al., 2017), which greatly contribute to losses incurred in crop production, right from planting to harvesting and storage, if not managed well.

PGPR control pests through mechanisms such as production of volatile compounds, such as β-ocimene and β-caryophyllene (Schausberger et al., 2012), that attract natural enemies of the pest in question. For example, a study by Pangesti et al. (2015) showed an increase in the concentration of parasitoid *Microplitis mediator*, a natural predator of *Mamestra brassicae* following the inoculation of *Arabidopsis thaliana* roots with the rhizobacterium *Pseudomonas fluorescens* WCS417r. Other mechanisms through which PGPM mitigate the effects of pests include increased activity of antioxidant enzymes and increased content of proteins and phenolics in plants, etc. In other cases, the biocontrol agent may not influence the biotic antagonist but will enhance plant yield in the presence of the antagonist (Herman et al., 2008).

This strategy seems very useful, especially in cases where biotic stress factors such as weeds, and pests become resistant or unresponsive to other control strategies. It may also enhance/preserve species diversity, hence maintaining ecosystem functionality.

Some PGPM are efficient against pathogens as single strains, while others perform better as a consortium. Details of single strains vs. consortia are discussed later in this review. Table 1 lists PGPM with potential biocontrol activities against the pathogens of various crop species. With the increasing campaign against the use of chemicals, as a means of combating climate change, such strains are a promising substitute for chemicals that are currently prevalent in agricultural production. Currently, the global biocontrol market is approximately 2 billion USD (Velivelli et al., 2014) and is expected to grow further. More research on existing microbial species or microbe-produced compounds with biocontrol properties is still desirable, as is the identification of new ones.

Table 2.1: Biocontrol species against biotic stressors of different crop species

PGPM	Biotic Stress	Host Plant	Reference
Bacillus amyloliquefaciens LY-1	*Peronophythora litchii*	Litchi (*Litchi chinensis Sonn.*)	(Wu et al., 2017)
Burkholderia cepacia	*Fusarium oxysporum*	Potato (*Solanum tuberosum*)	(Recep et al., 2009)
Pseudomonas fluorescens	*Fusarium graminearum*	Wheat (*Triticum aestivum* cv. Tabuki)	(Moussa et al., 2013)
Pseudomonas fluorescens CHAO	*Gaeumannomyces graminis* var. tritici	Wheat (*Triticum sp*)	(Hassen et al., 2016)
Pseudomonas fluorescens CHAO	*Thielaviopsis basicola*	Tobacco (*Nicotiana tabacum*)	(Hassen et al., 2016)
Bacillus spp.	*Heterodera glycines*	Soybean (*Glycine max.*)	(Xiang et al., 2017)

Serratia proteamaculans	*Meloidogyne incognita*	Tomato (*Solanum lycopersicum L.*)	(Zhao et al., 2018)
Bacillus aryabhattai A08	*Meloidogyne incognita*	Tomato (*Solanum lycopersicum L.*)	(Viljoen et al., 2019)
Serratia plymuthica HRO-C48	*Botrytis cinerea*	_	(Frankowski et al., 2001)
Serratia plymuthica strain C-1, *Chromobacterium sp.* strain C-61 and *Lysobacter enzymogenes* strain C-3 consortium	*Phytophthora capsici*	Pepper (*Cupsicum spp*)	(Kim et al., 2008)
Paenibacillus sp. 300 + *Streptomyces sp.* 385	*Oxysporum f. sp. Cucumerinum*	Cucumber (*Cucumis sativus*)	(Singh et al., 1999)
Pseudomonas fluorescens WCS 358	*Fusarium oxysporum* f sp. Raphani	Radish (*Raphanus sativus*)	(Leeman et al., 1996)
Pseudomonas fluorescens	*Macrophomina phseolina*	*Coleus forskohlii* Briq.	(Vanitha & Ramjegathesh, 2014)
Pseudomonas aeruginosa 7NSK2	*Pythium splendens*	Tomato (*Lycopersicon esculentum*)	(Buysens et al., 1996)
Pseudomonas fluorescens	*Pythium spp*	Wheat (*Triticum sp*)	(Hassen et al., 2016)

Pseudomonas fluorescens	*Pythium ultimum*	Cotton (*Gossypium sp.*)	(Hassen et al., 2016)
Bradyrhizobium japonicum NCIM 26	*Rhizopus sp.* and, *Fusarium sp*	Soybean (*Glycine max L.*)	(Khandelwal et al., 2002)
Paenibacillus lentimorbus B30488	*Scelerotium rolfsii*	Tomato (*Solunum lycopersicum* L.)	(Dixit et al., 2016)
Pseudomonas putida UW4	*Agrobacterium tumefaciens*	Tomato (*Solanum lycopersicum* L.)	(Toklikishvili et al., 2010)
Burkholderia phytofirmans PsJN	*Agrobacterium tumefaciens*	Tomato (*Solanum lycopersicum* L.)	(Toklikishvili et al., 2010)
Bacillus cereus PX35, *Bacillus subtilis* SM21 and *Serrati asp* XY2	*Meloidogyne incognito*	Tomato (*Solanum. Lycopersicum* L.)	(Niu et al., 2016)
Pseudomonas fluorescens strain S35	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(de Vrieze et al., 2018)
Pseudomonas frederiksbergensis strain 49 and *Pseudomonas fluorescens* strain 19 consortium	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(de Vrieze et al., 2018)
Pseudomonas putida strain R32	*Phytophthora infestans*	*Solanum tuberosum*	(de Vrieze et al., 2018)

Pseudomonas chlororaphis spp strain R47	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(de Vrieze et al., 2018)
Pseudomonas spp strain S49	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(de Vrieze et al., 2018)
Bacillus and *Pseudomonas spp* consortium	*Fusarium oxysporum U3 and Alternaria* sp U10	Coyote tobacco (*Nicotiana attenuata*)	(Santhanam et al., 2015)
Chaetomium sp. C72 and *Oidodendron sp.* Oi3 consortium	*Fusarium oxysporum U3 and Alternaria sp* U10	Coyote tobacco (*Nicotiana attenuata*)	(Santhanam et al., 2015)
Pseudomonas chlororaphis R47	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(Dixit et al., 2016; Hunziker et al., 2015)
Pseudomonas fluorescens strain LBUM 636	*Phytophthora infestans*	Potato (*Solanum tuberosum*)	(Guyer et al., 2015)
Agrobacterium radiobacter var radiobacter	*Crown gall*	Tomato (*Solunum lycopersicon* L.)	(New & Kerr, 1972)
Tricoderma koningiopsis Th003 WP	*Fusarium oxysporum*	Cape gooseberry (*Physalis peruviana*)	(Diaz et al., 2013)
Trichoderma harzianum Tr6 + *Pseudomonas sp.* Ps14	*Fusarium oxysporum f. sp. radicis cucumerinum*	Cucumber (*Cucumis sativus*)	(Allizadeh et al., 2013)
Pseudomonas sp. Ps14	*Botrytis cinerea*	Arabidopsis (*Arabidopsis thaliana*)	(Allizadeh et al., 2013)

Trichoderma harzianum Tr6	*Botrytis cinerea*	Arabidopsis (*Arabidopsis thaliana*)	(Allizadeh et al., 2013)
Pseudomonas putida	*Spodoptera litura*	Tomato (*Solanum lycopersicum L.*)	(Allizadeh et al., 2013)
Pseudomonas fluorescence Pf1, *Bacillus subtilis Bs and Trichoderma viridae* Tv consortium	*Lasiodiplodia theobromae*	Tuberose (*Polianthes tuberosa L.*	(Durgadevi et al., 2018)
Pseudomonas sp. 23S	*Clavibacter michiganensis*	Tomato (*Solanum lycopersicum L.*)	(Takishita et al., 2018)
Peanibacillus lentimorbus B-304	*cucumber mosaic virus*	Tobacco (*Nicotiana tabacum* cv White burley)	(Kumar et al., 2016)
Serratia liquefaciens MG1	*Alternaria alternate*	Tomato (*Solanum lycopersicum* L.)	(Schuhegger et al., 2006)
Xanthomonas sp. WCS2014-23, *Stenotrophomonas sp.* WCS2014-113 and *Microbacterium sp.* WCS2014-259	*Hyaloperonospora arabidopsidis*	Arabidopsis (*Arabidopsis thaliana*)	(Berendsen et al., 2018)
Lactobacillus plantarum SLG17 and *Bacillus*	*Fusarium spp*	Durum wheat (*Triticum durum*)	(Baffoni et al., 2015)

amyloliquefaciens FLN13			
Fusarium oxysporum strain Fo162	*Aphis gossypii Glover*	Zucchini (*Cucurbita pepo*)	(Martinuz et al., 2012)
Rhizobium etli strain G12	*Aphis gossypii Glover*	Zucchini (*Cucurbita pepo*)	(Martinuz et al., 2012)
Bacillus subtilis strain BEB-DN	*Bemisia tabaci*	Tomato (*Solanum lycopersicum* L.)	(Valenzuela-Soto et al., 2010)
Bacillus amyloliquefaciens (SN13)	*Rhizoctonia solani*	Rice (*Oryza sativa*)	(Shrivasta et al., 2016)
Pseudomonas fluorescens Migula strains Pf1 and AH1	*Desmia funeralis*	Rice (*Oryza sativa*)	(Karthiba et al., 2010)
Pseudomonas putida and *Rothia sp.*	*Spodoptera litura*	Tomato (*Solanum lycopersicum*) L.)	(Bano et al., 2017)

2.5 PGPM and Abiotic Stress

With climate change, the occurrence of extreme abiotic stresses, such as floods, salinity, high temperature and drought are expected to increase (Bouwer et al., 2010; Mirza, 2011; Collins et al., 2013; Bradley et al., 2016; Dawson et al., 2016). In fact, much of this is already being experienced in some parts of the world. Winters are becoming warmer in some regions; rainfall is becoming scarcer and more erratic, causing droughts and desertification (Lott et al., 2013; Rossi et al., 2015; Shrivasta et al., 2016) in other regions. With less rainfall, salinity is more likely to occur, either through irrigation or natural causes (Tank & Saraf, 2010; Xu et al., 2010; Rousk et al., 2011; Egamberdieva et al., 2014; Shrivasta & Kumar, 2015; Yan et al., 2015). All these

factors affect crop production, and their management inputs are sufficiently costly that many farmers may not be able to afford them. Factors such as high temperatures can generally not be managed under field conditions. Therefore, there is the need for a strategy that is ecofriendly and manageable by most crop producers. PGPM have been reported to mitigate effects of abiotic stress on plants, hence, allowing the plant to grow and yield relatively well under stress conditions (Subramanian et al., 2015; Chen et a., 2016; Tiwari et al., 2016; Wang et al., 2016). Various researchers have reported the ability of a wide range of PGPM to enhance plant growth, in the presence of abiotic stressors, such as salinity (Bhartirt et al., 2016; Subramanian et al., 2016a,b); drought (Rolli et al., 2015; Tiwari et al., 2016; Molina-Romero et al., 2017), heavy metals and acidity. In fact, the ability of some PGPMs to enhance plant growth is only triggered in the presence of stress (Rolli et al., 2015). They employ mechanisms such as the production of ROS scavenging compounds, possession of *ACC deaminase* (an enzyme that lowers ethylene concentration in plants exposed to stress), and the production of exopolysaccharides and osmolytes. For example, Akhtar et al. (2020) observed an increase in the antioxidant activity of catalase (CAT) in the roots of drought stressed maize plants treated with *Bacillus licheniformis* (FMCH001). Treated plants also exhibited a higher dry weight and higher water use efficiency. Yang et al. (2020) also reported the increased activity of catalase and dehydroascobate reductase enzymes in salinity stressed Quinoa plants treated with an endophytic bacterium known as *Burkholderia Phytofirmans* PsJN, compared to the untreated plants. The former also exhibited a higher shoot biomass, grain weight and grain yield compared to the latter. Some *Rhizobia spp*. produce the compound rhizobitoxine, which inhibits the activity of *ACC synthetase,* hence lowering ethylene activity that would otherwise inhibit nitrogen fixation. A PGPM may possess one or more of these mechanisms, all of which act to help a plant thrive under stress conditions. Like plants, PGPM can also be affected by abiotic stress, such as salinity, high temperature and drought, which can lower their efficacy in promoting plant growth, or even death of the microbe, in cases of prolonged exposure to extremes of such conditions (Zahran, 1999). Therefore, it is essential that the strains chosen for use are tolerant to the abiotic stress, whose effect in plants they tend to mitigate. Strains isolated from areas affected by abiotic stress may have an edge over those isolated under normal conditions, although this may not always be the case. The use of microbial consortia may be helpful, especially in areas where more than one factor inhibits crop growth (which is almost always the case under field conditions).

However, more research needs to be conducted, for the better deployment of PGPM technology. The exploitation of such microbes has a definite potential to maintain crop production amidst increasing abiotic stresses that are rendering some currently arable land unfit for crop production. Table 2, below, shows some PGPM strains that have been discovered and characterized by researchers, with the potential to mitigate the effects of abiotic stress on a range of plant species.

Table 2.2: Examples of PGPM that enable plants to withstand abiotic stress.

PGPM	**Abiotic Stress**	**Host Plant**	**Reference**
Pseudomonas putida MTCC5279	Drought	chickpea *(Cicer arietinum)*	(Tiwari et al., 2016)
Pseudomonas fluorescens REN1	Flooding	Rice *(Oryza sativa)*	(Etesami et al., 2014)
Variovorax paradoxus 5C-2,	Salinity	Peas (*Pisum sativum*)	(Wang et al., 2016)
Bacillus amyloliquefaciens SQR9	salinity	Maize (*Zea mays*)	(Chen et a., 2016)
Dietzia natronolimnaea	Salinity	Wheat (*Triticum aestivum*)	(Bhartirt et al., 2016)
Serratia nematodiphila	Low temperature	pepper (*Capsicum annum*)	(Kang et al., 2015)
Burkholderia phytofirmans PsJN	Low temperature	grapevine (*Vitis vinifera*)	(Fernandez et al., 2012)
Pseudomonas vancouverensis	Low temperature	Tomato (*Solanum lycopersicum*)	(Subramanian et al., 2015)
Pseudomonas sp S1	drought	Pepper (*Capsicum annum*)	(Rolli et al., 2015)
Pseudomonas sp S1	drought	Grape (*Vitis vinifera*)	(Rolli et al., 2015)

Achromobacter xylosoxidans	Flooding stress	Basil (*Ocimum sanctum*)	(Barnawal et al., 2012)
Pseudomonas sp. 54RB + *Rhizobium* sp. Thal-8	Salinity	Maize (*Zea mays cv. Agaiti 2002*)	(Bano & Fatima, 2009)
Pseudomonas putida KT2440, *Sphingomonas sp*. OF178, *Azospirillum brasilense* Sp7 and *Acinetobacter* sp. EMM02) consortium	drought	Maize (*Zea mays*)	(Molina-Romero et al., 2017)
Achromobacter xylosoxidans	salinity	Periwinkle (*Catharanthus roseus*)	(Karthikeyan et al., 2012)
Burkholderia cepacia SE4	salinity	Cucumber (*Cucumis sativus* L.)	(Kang et al., 2015)
Pseudomonas putida (W2)	salinity	Wheat (*Triticum aestivum* L.)	(Nadeem et al., 2010)
Pseudomonas fluorescens (W17)	salinity	Wheat (*Triticum aestivum* L.)	(Nadeem et al., 2010)
Kocuria flava AB402	*Arsenic toxicity*	Rice (*Oryza sativum*)	(Mallick et al., 2018)
Bacillus vietnamensis AB403	*Arsenic toxicity*	Rice (*Oryza sativum*)	(Mallick et al., 2018)
Trichoderma spp strain, M-35	*Arsenic toxicity*	Chickpea (*Cicer arietinum*)	(Tripathi et al., 2017)
Burkholderia cepacia and Penicillium chrysogenum consortium	*waste motor oil toxicity*	Sorghum (*Sorghum bicolor*)	(Sánchez-Yáñez et al., 2015)
Bacillus safensis	High temperature	Wheat (*Triticum aestivum L.*)	(Sarkar et al., 2018)

Pseudomonas aeruginosa	*Zn-induced oxidative stress*	Wheat (*Triticum aestivum L.*)	(Islam et al., 2018)
Bacillus licheniformis (FMCH001)	*oxidative stress Drought*	Maize (*Zea mays L. cv. Ronaldinho*)	(Akhtar et al., 2020)
Burkholderia phytofirmans PsJN	*Salinity*	Quinoa (*Chenopodium quinoa* Wild)	(Yang et al., 2020)

2.6 Commercialisation of Microbial Inoculants

Making PGPM technology available for farmers is key to ensuring their adaptation as agricultural inputs. Commercialisation of promising strains is one way of making promising strains accessible by farmers. Although various strains that possess desirable properties under laboratory and greenhouse conditions may be isolated, developing a commercial product, effective under field conditions, is not an easy task, especially because numerous factors determine the efficiency of introduced species. Characteristics such as: possession of multiple mechanisms of enhancing plant growth, ability to compete favorably and establish populations in the rhizosphere, persistence in the rhizosphere over seasons, and ability to be cultured in artificial environments (Khandelwal et al., 2002; Babalola & Glick, 2012; Pérez-Montano et al., 2014) are desired for potential PGPM strains. However, many plant and soil factors, such as plant species, soil temperature, composition and prevalence of native microbes, soil pH, etc., may work together against a strain which is otherwise excellent under controlled environment conditions. Even before introduction into the field, factors such as formulation play a major part concerning a product's efficacy. For instance, solid inoculant formulations are desired for their longer shelf life, however, the process of drying microbes often results in lower microbial cell counts, hence lowering their competitiveness, since number contributes greatly to their ability to compete with native microbes (Berninger et al., 2018). Exposing a potential PGPM to some level of stress before formulation may increase its survival rates during formulation and after field application (Berninger et al., 2018). Before introducing a potential PGPM

inoculant into the market, a series of events, such as greenhouse and field trials, characterization, toxicology profiling, etc. occur, most of which are intended to increase strain survival and efficacy in the field.

2.6.1 Formulation of Microbial Inoculants for Commercial Purposes and Their Mode of Application

Microbial inoculants are usually a combination of microbial cells and/or their parts/compounds and a nonliving carrier that may be in form of a liquid or solid material (Babalola & Glick, 2012; Alori et al., 2017; Alori & Babalola, 2018). Microbial cells may be either active or dormant; in the latter case, they must be activated before or after inoculation (Babalola & Glick, 2012). They may also be pure cultures (single strains) or a combination of microbial strains (microbial consortia) (Babalola & Glick, 2012; Alori & Babalola, 2018). Formulation is a major contributor to the variation in performance of inoculants observed in farmers' fields and at research stations. Formulation can shield the microbe from adverse environmental conditions, increase their shelf life and supply their nutritional requirements, hence enhancing their chances of survival in the field (Bashan et al, 2014; Berninger et al., 2018). Normally, a group of microbes are isolated from their natural habitat (soil or plant tissue), tested for their ability to promote plant growth under a range of conditions, and the superior strains are selected for commercialisation purposes. The strains are multiplied and formulated under controlled environment conditions, after which the efficiency of the inoculant is evaluated under field conditions (Bashan et al, 2014). The method of formulation ought to consider the target crop, target market and mode of application, the latter because the type of formulation often dictates the mode of application of the inoculant. For instance, solid formulations are mainly applied through seed dressing, or broadcasting onto the field, while liquid formulations have a wide range of application methods (Babalola & Glick, 2012; Alori et al., 2017; Alori & Babalola, 2018). Liquid carriers are mostly water and/or organic solvents (other than microbial media), such as glycerol and carboxymethyl cellulose that are added to increase properties such as stickiness and dispersal abilities (Bashan et al, 2014). There are several types of solid carriers, such as clay, vermiculite, peat, and charcoal (Babalola & Glick, 2012). Care should be taken, when selecting microbial carriers, to ensure they have no negative impact on the environment or the microbe itself (Babalola & Glick, 2012; Alori & Babalola, 2018).

Although they are easy to handle and work with, liquid carriers may require specialised storage conditions (cool conditions that necessitate a cooling mechanism) for a long shelf life (Bashan et al, 2014), which makes their marketing and use in developing countries difficult, due to limited and unstable power supply on most farms. Solid formulations, on the other hand, are bulky and may require larger storage facilities, when compared to liquids. However, materials such as peat have an outstanding reputation as inoculant carriers and are successfully used in both North and South America (Bashan et al, 2014). The formulation method opted for should ensure the affordability of the final product by the target market, since a very expensive product is likely to meaningfully increase production costs, which is undesirable. For instance, sterile carriers are preferred over nonsterile carriers (Bashan et al, 2014), however the former are costlier than the latter, which may make them unaffordable to many farmers across the globe. The formulation method should also ensure the compatibility of the inoculant with agronomic practices, such as weed control methods, irrigation, etc.

Once a formulated product exhibits positive responses, in field and greenhouse trials, it is put on the market for accessibility by farmers. While the isolation and characterisation of microbial strains from their natural habitats is largely done by academic research institutions, the production of microbial inoculants for commercial purposes is dominated by registered companies, which obtain patents and rights over specific inoculants. Table 3 below shows such microbial based products on the market as plant growth stimulants.

Table 2.2: Examples of microbial inoculants currently available on the market, and their producing companies.

Inoculant	Country	Producer	Use	Reference
Bacillus megaterium	Sri Lanka	BioPowerLanka	Phosphorus solubilisation	(Mehnaz, 2016)
Pseudomonas striata, *B. Polymyxa* and *B.megaterium* consortium	India	AgriLife	Phosphorus solubilisation	(Mehnaz, 2016)
Acidithiobacillus ferrooxidans	India	AgriLife	Iron mobilization	(Mehnaz, 2016)

Trichoderma and *Bradyrhizobium Spp* (Excalibre-SA) consortium	USA	ABM®	N fixation Growth stimulation	(Backer et al., 2018)
BIODOZ® *(B. japonicum)*	Denmark	Novozymes	Nitrogen fixation	(Berninger et al., 2018)
Cell-Tech® *(B.japonicum)*	Belgium	Monsanto (Bayer)	Nitrogen fixation	(Berninger et al., 2018)
Nitragin® *S. meliloti*	Belgium	Monsanto BioAg™ (Bayer)	Nitrogen fixation	(Berninger et al., 2018)
Cedomon® *Pseudomonas chlororaphis*	Sweden	BioAgriAB	Biopesticide	(Berninger et al., 2018)
Sheathguard™ *Pseudomonas fluorescens*	India	AgriLife	Biopesticide	(Berninger et al., 2018)
Galltrol® -A *Agrobacterium radiobacter*	USA	AgBioChem	Biopesticide	(Berninger et al., 2018)
HISTICK® *Bradyrhizobium japonicum*	Germany	BASF SE	Nitrogen fixation	(Mehnaz, 2016)
Bacillus +Pseudomonas+ Lactobacillus+ Saccharomyces spp	Canada	EVL Inc	Biostimulant	
Xen Tari *(Bacillus thuringiensis)*	USA	Valent USA	Biopesticide	(Arthur & Dara, 2018)
VOTIVO FS seed treatment *(Bacillus firmus)*	USA	Bayer	Biopesticide	(Arthur & Dara, 2018)

VectoLex *FG* *(Bacillus sphaericus)*	USA	Valent Biosciences	Biopesticide	(Arthur & Dara, 2018)
Venerate XC *(Burkholderia rinojensis)*	USA	Marrone Bio Innovations	Biopesticide	(Arthur & Dara, 2018)
Zequanox *(Pseudomonas fluorescens)*	USA	Marrone Bio Innovations	Biopesticide	(Arthur & Dara, 2018)
BotaniGard ES/WP, Mycotrol *(Beauveria bassiana)*	USA	Lam International	Biopesticide	(Arthur & Dara, 2018)
Naturalis L *(Beauveria bassiana)*	USA	Troy BioSciences	Biopesticide	(Arthur & Dara, 2018)
BioCeres WP (*Beauveria bassiana*)	USA	BioSafe	Biopesticide	(Arthur & Dara, 2018)
Met-52 EC and Met-52 G (*Metarhizium brunneum* (*anisopliae s.L.*)	USA	Novozymes	Biopesticide	(Arthur & Dara, 2018)
MeloCon WG (*Purpureocillium lilacinum*)	USA	Bayer	Biopesticide	(Arthur & Dara, 2018)
Cyd-X, Cyd-X HP (*Cydia pomonella* (CpGV)	USA	Certis USA	Biopesticide	(Arthur & Dara, 2018)
FruitGuard (*Plodia interpunctella GV*)	USA	Agrivir	Biopesticide	(Arthur & Dara, 2018)
Serenade (*Bacillus subtilis* QST 713)		Agraquest	Biocontrol	(Velivelli et al., 2014)
Bacillus firmus I-1582 WP5 (*B. firmus* I-1582)		Bayer Crop Science	Biocontrol	(Velivelli et al., 2014)

Cedomon (*Pseudomonas chlororaphis* MA342)		Bioagri		(Velivelli et al., 2014)
Proradix (*Pseudomonas sp.* DSMZ 13134)		Sourcon–Padena Germany, Itary	Biocontrol	(Velivelli et al., 2014)
Novodor (*B. thuringiensis ssp.* tenebrionis NB 176)	USA	Valent Bioscience	Biocontrol	(Velivelli et al., 2014)

2.6.2 Limitations to Global Use of Microbial Inoculants

Although microbial inoculants are viewed as the most viable hope, with regard to sustainable agriculture in the face of climate change, their use and adoption globally are still wanting, due to a range of reasons, that vary between developed and developing countries. Adaptation to use of microbial inoculants is developing at a relatively faster pace (Alori et al., 2017) in the developed world than in developing areas, such as Africa, where their use is restricted by limited availability of resources and knowledge, among other factors. In the developed world, microbial use is slowed largely by inconsistencies in enhancing plant growth, in which case crop producers opt for chemicals, which generally provide stable results. There are many cases where the excellent performance of an inoculant observed during pre-commercialisation trials does not translate to efficiency on farmers' fields. Even when it does, sometimes the results are not consistent, which frustrates the farmers. Some of these inconsistences may be attributed to biotic and abiotic soil factors and plant factors which directly or indirectly affect the introduced microorganism(s) (Bashan et al, 2014). For instance, some inoculants are cultivar and species specific, in that applying them outside the target species will yield no results. Soil factors such as salinity and temperature are dynamic and affect the survival and effectiveness of the applied microbial strains. This implies that soil conditions should always be favorable for the introduced microbe, otherwise inconsistencies are bound to prevail. Therefore, there is a need to sensitise farmers regarding the proper use of microbial products to minimise such inconsistencies. Unless sensitisation is properly conducted, we cannot rule out inappropriate practices such as farmers applying rhizobial inoculants together with high doses of nitrogen fertilizer,

expecting better results than the inoculant or fertilizer used alone. In fact, nitrogen fertilizer will inhibit biological nitrogen fixation. Similarly, applying a biocontrol to a soil or plant that lacks the pathogen it can antagonise/suppress may not yield results. It is also important to understand the status of the soil/plant as the application of microbial inoculants may inhibit plant growth where the soil/plant already contains optimal concentrations of the compound that the microbe produces to enhance plant growth. For instance, application of IAA producing PGPM on plants with an already optimal concentration of IAA may yield negative effects on the plant, due to excess IAA (Glick, 2012). Understanding soil conditions will also guide the farmer regarding how often to apply the inoculant. Some require seasonal, annual, or even twice in a season application, while after some time, application may not be necessary, especially where the microbe establishes reasonable populations in the soil. Successful microbial inoculants employ mechanisms that give them a competitive advantage over the native strains. For instance, rhizobia and mycorrhizal fungi have a signaling system with their host plants, which gives them an advantage over their competitors. Introduced microbes may also outcompete native microbes through the production of antimicrobials, which may kill or deter other microbes, as well as the production of siderophores that give them a competitive advantage over other microbes for iron resources in the soil, hence proliferating better, especially in iron limited soils (Bender et al., 2016). Nevertheless, it is important to increase the competitive advantage of introduced microbes, by ensuring high microbial concentrations in the inoculant and use of adequate formulations (Backer et al., 2018). With approaches such as metagenomics, the microbial population of the target environment can be studied, and potential PGPM studied for their ability to out compete the latter in field, greenhouse, and laboratory conditions. However, this may not be an easy task, given that microbial populations in crop production fields may differ meaningfully due to a wide range of factors. Location and plant specific nature of some phytomicrobiome elements for inoculant production should also be prioritised, since such microbes, to a great extent, are more adapted to the environment and/or plant conditions, which may increase their chances of survival and persistence in the soil. The idea of using microbial consortia may also work to our benefit, as will be discussed below. This does not, however, disqualify single strain inoculants; their advantages are also discussed below.

In less developed countries, especially in sub-Saharan Africa, reasons for low adoption also vary between large- and small-scale farmers. For large-scale farmers,

such as those in Zimbabwe, South Africa and Kenya, the ineffectiveness of many microbe-based products in the field contributes meaningfully to the low adoption of microbial inoculants (Aremu et al., 2017; Babalola & Glick, 2017). For small-scale farmers, costs and inadequate knowledge of such products are the major drivers. These two factors, especially costs, also limit the use of other agricultural inputs, such as high-quality seed. Exceptions can be made for a smaller group of small scale farmers, whose farms' researchers run experiments/field trials, because then, they can obtain access to the inputs from researchers largely free of charge, otherwise, they mostly depend on crop rotations (which are sometimes not properly done) and animal manures, while others just grow their preferred crops year in year out. The lack of knowledge can be attributed to the large gap between research and extension. Researchers achieve good findings, but due to poor funding and poor dissemination techniques, this knowledge never reaches the farmer (Aremu et al., 2017). Publications do not help much, because many small-scale farmers are illiterate, and even those who can read have limited access to technologies such as smart phones, computers, and the internet. It should be noted that many small-scale farmers are also low-income earners, who struggle to meet their basic needs. In countries where governments are not directly involved in the distribution of agricultural inputs, dealers may not be willing to extend products to people who they well know cannot afford them, which leads to unavailability of and/or inaccessibility of the products by the farmers. In such cases, intervention strategies should be at least a bit different and more vigorous. First and foremost, the knowledge of existence of PGPM technology needs to be spread to these largely small-scale farmers. Projects like N2 Africa have done a good job in trying to spread the BNF technology, although more effort is still needed. Extension officers should be updated on new findings and products and be properly facilitated to extend this knowledge to the farmers. Governments may consider subsidizing products and getting directly involved in their distribution to the farmers. Promiscuous soybean varieties are already a good strategy of eliminating the need for inoculation. It would be better to develop strategies that enable the use of farm-based PGPM inoculants, as many farmers have limited access to agro-input markets, in part due to poor transport networks. Locally made cooling facilities such as charcoal based refrigerators and unglazed clay pots may also be helpful. However, the former would be a contradictory measure, given that it would encourage deforestation. The whole sensitisation process should involve all stakeholders, such as governments, extension officers, agricultural schools, and private companies that contract small scale

farmers to grow crops for them for use as raw materials. The latter, especially, provide the farmer with chemicals such as pesticides and fertilizers; therefore, their involvement cannot be ignored.

2.6.3 Microbial Consortia

In order to address issues associated with the use of single strains as inoculants, microbial consortia have gained popularity. This may be relevant, especially now that the prevalence of both biotic and abiotic stresses due to climate change are likely to increase. Microbial consortia technology involves the use of more than one microbial species in a single inoculant product. The microbes may have the same or different modes of action (Khandelwal et al., 2002; Li et al., 2017; Backer et al.,2018), and may be from different phyla, genera, or even other groupings, for example, a combination of bacterial and fungal strains. Microbial consortia may have an advantage over single strains when the species synergistically interact and confer benefits to each other (Khandelwal et al., 2002; Subramanian et al., 2016a; Li et al., 2017; Wu et al., 2017). For instance, one strain may breakdown a substrate, unavailable to other species, converting it into forms that the other members of the consortium can utilise as a source of nutrients (Bender et al., 2016), or produce exopolysaccharides which offer protection against stress to all members of the consortium (Berninger et al., 2018), produce compounds which are signals that activate plant growth promotion capability of other members of the consortium, through the production of plant growth stimulating compounds, that they would otherwise not produce, for instance, in pure culture. In cases where microbes with the same mode of action are used, members may have varying tolerance to different biotic and abiotic stresses, which enhances survival of at least a member that will confer intended benefits to the plant. In the case of different modes of action, these complement each other and confer a more effective benefit to the plant. It could also be that some members of the consortium are simply helpers of the strains meant to benefit the plant. Such helper strains, for instance mycorrhiza helper bacteria, should facilitate the target strain in plant colonisation, conferring benefits to the plant. Researchers have reported inefficient strains that became efficient in a consortium. For example, Santhanam et al. (2015) observed that the inclusion of two bacterial strains with insignificant effects on mortality of sudden wilt pathogens in tobacco, in a consortium with three other bacteria improved resistance of plants to the

same pathogen, in comparison to the consortium of 3 used alone. Mycorrhizal fungi, in association with a helper bacterium, may have better established mycelia and plant root colonisation, if the bacterium produces substances that directly enhance the germination of fungal spores, or indirectly enhance the establishment of mycorrhiza through the production of antimicrobials that reduce competition from other microbes or minor pathogens (Bender et al., 2016). Because of the interaction advantage, some microbes perform better in microbial consortia than when applied individually (Khandelwal et al., 2002; Li et al., 2017). However, the reverse is true for some PGPM species, as reported by other researchers (Schuhegger et al., 2006; Santhanam et al., 2015; Li et al., 2017). Therefore, the role that single strain inoculants play cannot be written off easily, especially because microbial consortia also have their shortcomings. Coming up with effective compatible combinations in which all members actively benefit the plant can be challenging, practically given that some members of the consortium may produce compounds lethal to other members (Islam et al., 2018). Even if the produced compounds do not go to the extreme of killing other members, they may cause a shutdown of their plant growth promoting system, or interfere with their growth, as de Vrieze et al. (2017) observed in a consortium of five *Pseudomonas* strains. In such cases, it is probable that only a subset of the consortium members will actively benefit the plant, the rest being "dormant" or dead. Difficulties concerning the formulation of microbial consortia may also be associated with the variations in optimal growth conditions. For more than one species, or even genus, creating conditions that will favour all members while retaining their ability to promote plant growth may not be easy. Finally, manufacturing consortia can be challenging, as very small changes at the outset can result in very different levels of consortium members in the final product, resulting in product inconsistencies.

2.7 Microbial Compounds as "Inoculants"

The use of microbial compounds as "inoculants" is slowly gaining popularity after successful trials (Souleimanov et al., 2002; Gray et al., 2006; Prudent et al., 2015; Schwinghamer et al., 2015; Subramanian et al., 2016; Arunachalam et al., 2018; Navarro et al., 2019). To be a true inoculant, the material must contain living cells that colonize the plant. In this case, the technology may be the product of microbial growth and may be more valuable as a result of climate change were biotic and abiotic factors

may lower or completely halt the effectiveness of microbial cell-based inoculants. This practice involves the separation of cell-free supernatant from microbial cells, and the subsequent separation and purification of the compound from the cell-free supernatant, mainly through high pressure liquid chromatography (HPLC). The pure compound is then tested for its ability to promote plant growth under greenhouse and field conditions, prior to commercialisation. Before commercialisation, other tests, such as the effect of the compound on non-target organisms and humans, as well as checks regarding legal regulations, are usually carried out. The effect of the compound on non-target organisms such as plants, humans and animals ought to be substantially understood too, as with studying the residual effects of the compound (how much of it remains in the edible parts of the plant, and in the soil, following application). Therefore, before any compound can be commercialised, its ability to be purified, and produced on a large scale, should be verified (Navarro et al., 2019). The compound should be identified and characterised based on its physiological and biological properties. The efficacy and type of microbial bioactive compounds produced are influenced by microbial species and conditions to which the PGPM is exposed. Slight alterations in growth conditions may result in different compounds, produced at different levels, and with varying degrees of efficacy. For instance, varying the pH, a *Pseudomonas* sp. culture caused it to produce different phenazine compounds with varying efficacy against *Fusarium oxysporum* f. sp. radicis-lycopersici (Navarro et al., 2019). Sometimes, the PGPM must be exposed to stressful conditions before it will produce bioactive compounds, as such compounds may only be produced to enhance the survival of the microorganism under stressful conditions. Therefore, it is important to have an adequate understanding of the conditions under which a certain PGPM will produce plant growth stimulating compounds. So far, not many (compared to microbial strains) bioactive compounds have been identified for use in crop production. The Smith laboratory at McGill University has lipo-chitooligosaccharide (LCO) and thuricin 17. Thuricin 17 is a bacteriocin secreted by *Bacillus thuringiensis*, a non-symbiotic endophytic bacterium. The compound is known to have anti-microbial properties, which gives *Bacillus thuringiensis* a competitive advantage over other bacteria of the same grouping (Gray et al., 2006). After a series of experiments, thuricin 17 was discovered to have growth promoting properties for tomato, soybean, canola, arabidopsis, rapeseed and switch grass (Gray et al., 2006; Prudent et al., 2015; Schwinghamer et al., 2015; Subramanian et al., 2016a, b; Arunachalam et al., 2018;

Navarro et al., 2019). More trials are on-going, and the technology has yet to be commercialised. Lipo-chitooligosaccharide, on the other hand, is produced by rhizobia, as a signal to its host plants (Souleimanov et al., 2002). Formerly extensively studied for its role in the nodulation process, the compound is currently patented and being marketed by Novozymes as a plant growth stimulant, where its effects are greatest under abiotic stress conditions. Other compounds such as phenazine-1-carboxylic acid (PCA) have also been commercialised (Duke & Lydon, 1987; Yuan et al., 2008; Shanmugaiah et al., 2010; Chen et al., 2007; Puopolo et al., 2013; Xu et al., 2015; Zhang et al., 2015; Huang et al., 2016; Navarro et al., 2019). Table 4 shows the various compounds with potential use as agro inputs. Some of them are already commercialised.

Table 2.3: Microbial compounds of agricultural importance.

Compound	Producing Microbe	Function	Comment	Reference
LCO	*Bradyrhizobium japonicum*	Biostimulant	Stimulates plant growth under stressed and non stressed conditions.	(Yuan et al., 2008; Subramanian et al., 2016a,)
Thuricin17	*Bacillus thuringiensis*	Biostimulant	Enhances growth of different crops e.g., Soybean in stressed and non stressed conditions	(Subramanian et al., 2016a; Arunachalam et al., 2018)
Anisomycin	*Streptomyces sp.*	herbicide	Effective against *Digitaria spp*	(Duke & Lydon, 1987)
Phenazine-1-carboxyamide (PCN)	*Pseudomonas spp*	biocontrol	It is effective against; *Fusarium oxysporum* f. sp. Radicis-lycopersici, *Xanthomonas oryzae* pv. Oryzae, *Rhizoctonia solani*, *Botrytis cinerea*	(Duke & Lydon, 1987; Chen et al., 2007; Shanmugaiah et al., 2010; Navarro et al., 2019)
Phenazine-1-carboxylic acid (PCA)	*Pseudomonas spp*	biocontrol	It is effective against *Fusarium oxysporum* f. sp. *Radicis-lycopersici*,	(Duke & Lydon, 1987; Yuanet al., 2008; Puopolo et

			Colletotrichum orbiculare, *Gaeumannomyces graminis* var. tritici, *Phytophthora capsici*	al., 2013; Huang et al., 2016; Navarro et al., 2019)
Pyocyanin (PYO)	*Pseudomonas spp*	biocontrol	Effective against: *Sclerotium rolfsii*, *Macrophomina phaseolina*	(Gheorghe et al., 2017; Kare & Arora, 2011)
Pyrrolnitrin	*Burkholderia pyrrocinia 2327*	biocontrol	It has antifungal properties against; *Ralstonia solani*, *Phytophthora capsici*, and *Fusarium oxysporum*	(Jung et al., 2018; Okada et al., 2005)
Phencomycin	*Burkholderia glumae 411gr-6*	biocontrol	Effective against; *Alternaria brassicicola*, *Aspergillus oryzae*, *Cladosporium cucumerinum*, *Colletotrichum gloeosporioides*	(Han et al., 2015)
Ornibactin	*Burkholderia contaminans MS14*	biocontrol	Siderophore with biocontrol activity against *Erwinia amylovora*, *Ralstonia solanacearum*, *Pseudomonas syringae* B301, *Clavibacter michiganensis* subsp. michiganensis	(Deng et al., 2017)
Iturin A2	*Bacillus subtilis B47*	biocontrol	Effective against fungi; Bipolaris maydis	(Ye et al., 2012)
Mycosubtilin	*Bacillus subtilis*	biocontrol	Has anti fungal properties, effective against; *Bremia lactucae*	(Deravel et al., 2014)

Herboxidiene	*Streptomyces sp. A7847*	herbecide	Effective on a number of weed sp	(Isaac et al., 1992)
Phosphinothricin	*Streptomyces hygroscopicus*	herbecide		(Saxena & Pandey, 2001)
Cyanobacterin	*Scytonema hofmanni*	herbecide	Effective on cynobacteria, algae and higher plants	(Saxena & Pandey, 2001)
Avermectin	*Streptomyces avermitilis*	Insectide nematocide	Effective against Spider mites, Citrus red mite, horn worms, army worms, etc	(Tanaka & 0mura, 1993)

2.8 Microbial Cells or Microbial Compounds?

Given the current understanding, a question would be, what should a crop producer adopt, given a choice between the microbial cells and microbial compound-based products. The answer to such a question cannot be as definite as that specific factors may call for either of the two, or even the use of both simultaneously. Before one reaches the level of farmer preferences, soil, and environmental factors as well as economic implications, intended use and handling may be major considerations. For instance, in the reclamation of areas heavily affected by abiotic stress, use of microbial cells may not be a good idea, if they are not able to survive some harsh conditions. Even if they did, the efficacy of their plant growth promotion capacity may be greatly affected. Compounds, on the other hand, are less affected by such abiotic stresses, and hence have a greater chance of being successful under such conditions. The use of compounds or both compound(s) and microbial cells may be desirable, especially when an abiotic stress such as drought interrupts signaling between plant and PGPM. In such a case, external application of the signal may rectify the disruption. Prudent et al. (2015) observed a 17% increase in soybean biomass under drought conditions following co-inoculation with *Bradyrhizobium japonicum* and thuricin17, compared to inoculation with the rhizobial cells alone. The use of microbial compounds may also be a better choice in cases where the microbe is a facultative pathogen, such as *Agrobacterium spp.* (Santhanam et al., 2015). In such cases, the pathogen effect of the microbe on plants is minimised. Application of microbial compounds may also benefit a wider range of crop species compared to microbial cells, given that many microbes can be at

least somewhat species specific. A case would be that of lipo-chitooligosaccharides (LCOs), which can be utilised to enhance growth of legumes and non-leguminous crops (Chen et al., 2007), under stressed and non-stressed conditions (Wang et al., 2016; Babalola & Glick, 2017), but to a greater extent, under stress conditions. For instance, LCOs enhanced fruit and flower production in tomato (*Lycopersicon esculentum*) plants (Chen et al., 2007) and stimulated the growth of soybean and corn plants (Souleimanov et al., 2002). The compound was also reported to enhance the germination of soybean seeds subjected to high NaCl concentrations (Subramanian et al., 2016a), and canola (Schwinghamer et al., 2015). Such benefits from LCO would not be provided to these crops had *Bradyrhizobium japonicum* been applied. Compounds are also less bulky and less costly, in most cases requiring small doses to be efficient. This relieves crop producers of storage and transportation concerns. However, there are scenarios where the use of microbial cells is inevitable. For instance, the role that rhizobia play in nitrogen fixation, or mycorrhizae in P mobilisation and acquisition by plant roots could not be fulfilled by microbial compounds. Nitrogen fixing bacteria cannot be substituted by compounds in areas where N is limiting. Microbial cells have the potential to establish microbial populations in the rhizosphere, which may eliminate the need for further inoculation, a characteristic most farmers would desire, since it not only has positive financial implications, but also saves labour. Based on this, it is safe to assume that marketing companies would opt for compounds, since they guarantee continuous sales. However, the long and laborious process of isolating and purifying microbial compounds may also contribute to their scarcity and willingness of some researchers and companies to take that route.

2.9 Way Forward and Recommendations

With climate change conditions increasing, and the desperate need to come up with sustainable approaches of enhancing crop productivity to meet the food demand of the growing population microbes are a prominent source of hope. However, a great deal still needs to be done to bridge the gap between their use in developed and developing countries. More research should be done to address issues of inconsistencies observed on crop producers' fields, following the use of microbial inoculants. It is obvious that single strains and consortia, or microbial cells and microbial compounds are issues that need to be evaluated on a case-by-case basis. Therefore, a better

suggestion would be that more research be done to provide consumers with options that can address their unique needs, while being economically viable.

2.10 Conclusions

Lowering the effects of climate change on crop production, through reducing greenhouse gas emissions, is one of the major focuses of researchers in recent times. With proper manipulation, plant growth promoting microorganisms and compounds, they produce have potential to enhance growth and yield of plants exposed to biotic and biotic stress(es). This can complement other strategies, such as conservation farming and breeding for stress tolerant crop cultivars, to create an integrated approach of enhancing crop production in the face of climate change. Given that the prevalence of stress is predicted to increase with climate change, more research is needed to come up with better and more effective alternatives of utilising PGPM technology; not only to enhance plant growth, but also to reduce greenhouse gas emissions from the agricultural sector, which is a meaningful contributor.

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2.12 CONNECTING TEXT

Chapter two gave an overview on the role plant growth promoting microorganisms (PGPM) and their derivatives play in enhancing plant growth, especially in the face of climate change. Climate change has escalated the effect of biotic and abiotic stress on agricultural production, in different parts of the world. Salinity stress, in particular, is a major global constraint to crop production, which can be exacerbated by common agricultural practices like irrigation and application of fertilisers, and destructive human practices like deforestation, that are meant to increase agricultural production. PGPM and their derivatives, on the contrary, can enhance plant growth in salt affected areas, in a sustainable and environmentally friendly manner. As a result, researchers have come up with PGPM and PGPM derivatives-based inoculants technology, some of which are already on the market, to enhance crop production. The EVL Inc commercial inoculant is one such product. The product is a consortium of different microbial species, currently applied on plants, alongside NPK fertiliser, which possibly exposes the individual strains to salt stress. The performance of the strains under saline conditions has also not been studied yet. For the company to come up with new products, especially those aimed at addressing the effect of salinity stress on crop production, the tolerance of the individual strains to salt stress, and their ability to maintain crop growth enhancement traits, under saline conditions, needs to be understood. Therefore, based on this background, this project addresses three key research questions.

1. Are the members of the EVL inc commercial strains tolerant to salt stress?
2. What mechanisms, at the exoproteome level, do they employ to tolerate salt stress?
3. When exposed to salt stress, do they exude substances in their growth media that enhance plant growth?

Chapter 3 Effect of salinity stress on Growth of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H

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3.1 Abstract

Plant growth promoting microorganisms (PGPM) are affected by salinity stress, which is a major global constraint. The effect of salt on microbes varies between and within species and is dependent of the severity of the stress factor. Microbial over all growth, growth rate and generation time and loss of ability to enhance plant growth are some of the variables affected by salinity stress. This study is focused on understanding the effect of NaCl level on over all growth, growth rate and generation time of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H. The strains were cultured in M13 (*Bacillus*) and MRS (*Lactobacillus*), broth supplemented with 0 (control), 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mM (v/v) NaCl, with pH adjusted to 7.0. Microbial growth, in terms of optical density, was measured at a 2 h intervals for 48 h, using a Cytation Gen 5 imaging reader (BioTek Instruments, Inc., USA) at a wavelength of 600 nm. Results of the study showed that the two strains were tolerant to NaCl, up to 1000 mM, although their growth rate and generation time were affected, for each species, with variations observed among NaCl levels. Results of the study suggest that the two strains are tolerant to high levels of NaCl and can therefore be utilized to enhance plant growth in salt affected areas.

3.2 Introduction

Bacteria, like all other living organisms, undergo growth, which on solid media, is measured in terms of colony number and colony size, while in liquid/broth media it is based on increases in cell numbers, which is equivalent to turbidity of the growth medium being utilized. Bacteria multiply through the process of binary fission and their growth cycle involves four phases namely, the lag phase, exponential growth phase, stationary phase, and death phase (Wang et al., 2015). The duration of any of the four phases varies between and within bacterial species, depending on growth conditions at a given time. During the exponential phase, bacteria actively grow, and double their population size at specific time intervals (Wang et al., 2015). The growing population actively and competitively takes up nutrients from growth media while exuding some compounds. As the population grows, competition for resources such as substrates and space increases until growth rate is equal to death rate, at which point the stationary phase commences. Exposure to salinity stress may affect microbial growth and survival (Vriezen et al., 2007; Yan & Marschner, 2012). The effect can be lethal especially if the level of stress is beyond that which a microbe can tolerate (Somani et al., 2011). Unfortunately, PGPM are regularly exposed to salt stress, and other biotic and abiotic stresses, especially under field conditions. Therefore, screening PGPM for salt stress tolerance is essential if their potential as plant biostimulants is to be optimised for performance under saline conditions.

Bacteria may or may not require sodium chloride (NaCl), for optimum growth to be attained. Based on the NaCl requirement, microbes can be classified as halotolerant, non-halophiles or halophiles (Kushner & Kamekura, 1988; Ventosa et al; 1998; Nanjani et al., 2012). Halotolerant microbes are those that do not require NaCl to grow and are able to survive in the absence and or presence of NaCl (Ventosa et al. 1998; Reang et al., 2022). Depending on the level of NaCl they can tolerate, halotolerant microorganisms can be classified as non-tolerant, slightly tolerant, moderately tolerant, and extremely tolerant. Nontolerant halotolerant microbes can only tolerate very low concentrations of NaCl, not more than 1 % w/v, while extremely tolerant halotolerants can tolerate all levels of NaCl, up to saturation (Nanjani et al., 2012). Non-halophiles are microorganisms that cannot grow or survive in environments with high salt concentrations. They achieve optimum growth at NaCl levels of less than 1 % (Reang et al., 2022). Halophiles are microbes that require NaCl to grow, although

the level of NaCl required for optimum growth varies from one halophilic organism to another. Members of this group belong to all domains of life, namely, Archaea, Bacteria, and Eukarya (Ventosa et al., 1998; Oren, 2002b; Reang et al., 2022). Halophilic bacteria can be within gram-positive or gram-negative groups, as well as aerobic and anaerobic (Abdeljabbar et al., 2013; Mohammadipanah et al., 2015). Members of the genera *Bacillus* and *Lactobacillus* have been placed among halophilic bacteria (Chen et al., 2011; Bagheri et al.,2012; Amoozegar et al., 2013b; Karyantina et al., 2020). There are various ways of classifying halophytes although the most accepted is one which classifies them according to the amount of NaCl required to attain optimum growth (Kushner & Kamekura, 1988). Slight halophiles require a NaCl range of 1-3 %; moderate halophiles require between 3-15 % NaCl; borderline extreme halophiles require about 12 % NaCl, while extreme halophiles require 15-30 % NaCl (Mohammadipanah et al., 2015; Reang et al., 2022).

Usually, microbes that survive in salty environments possess special features and mechanisms which enable them to survive under high salt concentration (Mohammadipanah et al., 2015). For example, they can structure their cell wall in such a way that it can become more hydrophilic at high salt concentrations, which allows them access to water molecules, hence mitigating, at least to some degree, osmotic stress (Oren, 2002b). However, the major mechanisms through which microbes adapt to osmotic stress are accumulation of salt in their cytoplasm, and accumulation of compatible solutes (Oren, 2006; Oren, 2008; Kanekar et al. 2012). They expel Na^+ from their interior while accumulating K^+ and Cl^-, to maintain osmotic balance (Oren, 2002b). Maintaining a balance between NaCl levels in their growth environment and KCl levels in their cytoplasm is a challenge for the microbe since accumulating high levels of KCl requires microbial enzymes to adapt to functioning at such high salt levels (Oren, 2002b). Accumulation of compatible solutes translates to expelling as much salt as possible, from the inside of the cell, to the outside, while accumulating solutes such as proline, glycerol, glycine, betaine, and trehalose, which can be synthesised *de novo*, or be absorbed from the growth environment (Oren, 2002b; Mohammadipanah et al., 2015). Although accumulation of compatible solutes requires less adaptation on the part of microbial cells, and is less energy consuming in general, *de novo* synthesis requires a lot of energy. Exposure of microbes to salt stress can lead to changes in the expression of their gene activity, protein, and metabolite profiles, upregulating some while downregulating others (Zhang et al., 2021). This in a way may contribute to the

microbe's ability to tolerate salt stress. Sodium chloride affects microbial growth in terms of size and colony forming units (Zhang et al., 2021).

The food and agriculture organisation (FAO) estimated about 85 % of the global land area to be affected by salinity stress, out of which approximately 424 million hectares of topsoil are 85% saline, 10% sodic, and 5% saline sodic while 833 million hectares of subsoil are 62% saline, 24% sodic, and 14% saline sodic (Food Agriculture Organization of the United Nations, 2022). Halotolerant plant growth promoting microorganisms and their derivatives, such as osmoprotectants, are valuable in the agriculture sector since they can be used to enhance plant growth under saline conditions. This is especially important since the pending need to meet food requirements for all people may necessitate expanding food production to salt affected marginal land, among other interventions. The aim of this study was to elucidate the effect of varying levels of NaCl on the growth variables of two plant growth promoting microorganisms, that is *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H. The two strains are members of an EVL Inc. consortium which is made up of 5 strains in total.

3.3 Materials and Methods

3.3.1 Experimental material and growth conditions

The two strains were selected because in a preliminary study, they were able to enhance germination of corn and soybean exposed to NaCl stress. The strains and their growth media as well as growth conditions were provided by EVL Inc. (http://www.evl-global.com). Table 3.1 shows growth conditions that were used to study the two strains.

Table 3.1: Showing microbial strains for the study, growth media and incubation temperature.

Strain name	Growth medium	Temp (°C)	pH	rpm
B. amyloliquefaciens EB2003A	M-13 broth	30	7.0	120
L. helveticus EL2006H	MRS broth	37	7.0	120

3.3.2 Experimental design, set up and procedure

The methods of Singleton et al. (1982a) and Upadhyay et al. (2011) were followed, with slight modifications. Microbial broth was prepared following the

manufacturer's instructions and autoclaved at 121 °C for 20 minutes. pH was adjusted to 7.0 as advised by EVL Inc, since it's the pH at which the consortium is produced. Fifty µL of each strain were pipetted in 10 mL of broth and incubated for 48 h to obtain a working culture. For each strain, microbial medium broth supplemented with 0 (control), 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mM (v/v) NaCl was prepared and autoclaved. These were the treatments. One hundred microliters of each NaCl treatment were then pipetted in separate wells of a Cytation instrument (Cytation 5, cell imaging multi-mode reader) plate, followed by addition of 100 µL of the microbial working culture. For each strain, the corresponding medium with no microbial strains was used as the negative control for each NaCl level. The strains were then cultured in a Cytation instrument, at temperature and rpm listed in Table 3.1, for 48 h. Microbial growth, in terms of optical density (600 nm wavelength), was recorded by the Cytation instrument at 2 h intervals. Each strain was studied, and the obtained data analyzed separately. Therefore, for each strain, a completely randomized design, with four technical replicates and four biological replicates was used for the study.

Figure 3.1: The Cytation instrument connected to a computer.

3.4 Data analysis

After 48 h, data was exported to Excel, which was used to generate growth curves of OD_{600} nm (Y axis) against time, h (X-axis), combined for all 11 NaCl treatments. Microbial growth rate and generation time data were generated using R growthcurver and analysed using SAS proc GLM (SAS 9.4 software), and multiple comparisons done using Tukey's test. Statistical significance of least significant means was determined at a 5% significance level.

3.5 Results

3.5.1 *Lactobacillus helveticus* EL2006H

3.5.1.1 Growth rate

In general, growth of *L. helveticus* EL2006H was low at all NaCl levels. Growth curves for *L. helveticus* EL2006H exposed to different NaCl levels varied, as shown in Figure 3.2. There were significant variations in the effect of different NaCl levels on growth rate of *L. helveticus* EL2006H. The highest growth rate, 0.316 ± 0.084 h^{-1} was observed for *L. helveticus* EL2006H exposed to 500 mM NaCl while the lowest, 0.044 ± 0.008 h^{-1} was observed for *L. helveticus* EL2006H exposed to 1000 mM NaCl, as shown in Table 3.2. The two were significantly different from each other ($p < 0.0073$), although not significantly different from the 0 mM NaCl control.

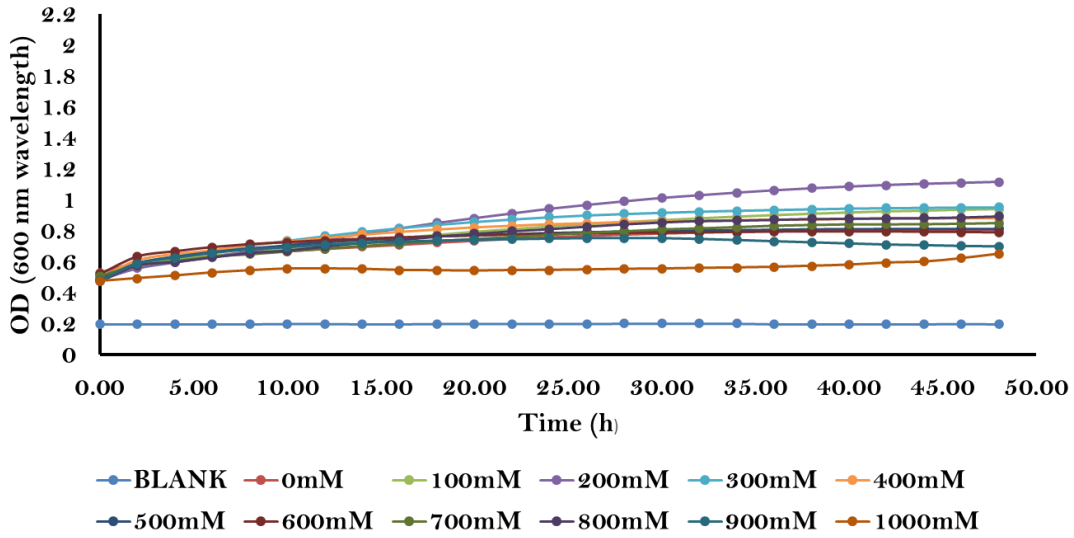

Figure 3.2: Growth curves of *L. helveticus* EL2006H treated with the varying levels of NaCl, at time intervals varying from 0 to 48 h.

Table 2.2: Effect of varying NaCl levels on growth rate of *L. helveticus* EL2006H. Data represents the mean ± SE; different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values in bold represent minimum and maximum growth rates.

Treatment	**Mean growth rate (h^{-1}) ± SEM**
0 mM NaCl	0.148 ± 0.011^{ab}
100 mM NaCl	0.128 ± 0.010^{ab}

200 mM NaCl	0.129 ± 0.006^{ab}
300 mM NaCl	0.183 ± 0.037^{ab}
400 mM NaCl	0.205 ± 0.045^{ab}
500 mM NaCl	**0.316 ± 0.084^{a}**
600 mM NaCl	0.299 ± 0.078^{a}
700 mM NaCl	0.185 ± 0.056^{ab}
800 mM NaCl	0.174 ± 0.026^{ab}
900 mM NaCl	0.289 ± 0.063^{a}
1000 mM NaCl	**0.044 ± 0.008^{b}**

3.5.1.2 Generation time

There were variations in the effect of the NaCl concentration on generation time of *L. helveticus* EL2006H, as shown in Table 3.3. The lowest, 1.095 ± 0.612 h, was observed for *L. helveticus* EL2006H treated with 600 mM NaCl while the highest, 17.320 ± 3.009 h, was observed for *L. helveticus* EL2006H treated with 1000 mM NaCl. The latter was significantly higher than the generation time (4.759 ± 0.336 h) of *L. helveticus* EL2006H treated with 0 mM NaCl.

Table 3.3: Effect of varying NaCl levels on mean generation time of *L. helveticus* EL2006H. Data represents the mean ± SE; different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values in bold represent the minimum and maximum generation times.

Treatment	**Mean generational time ± SEM**
0 mM NaCl	4.7587 ± 0.3359^{b}
100 mM NaCl	5.4932 ± 0.3932^{b}
200 mM NaCl	5.3607 ± 0.2238^{b}
300 mM NaCl	4.1896 ± 0.6886^{b}
400 mM NaCl	3.8108 ± 0.6647^{b}
500 mM NaCl	2.5879 ± 0.5136^{b}
600 mM NaCl	**1.0954 ± 0.6122^{b}**
700 mM NaCl	3.3464 ± 1.3461^{b}
800 mM NaCl	3.3434 ± 1.2268^{b}

900 mM NaCl	1.6339 ± 0.5941[b]
1000 mM NaCl	**17.3201 ± 3.0085[a]**

3.5.2 *Bacillus amyloliquefaciens* EB2003A

3.5.2.1 Growth rate

There were variations in the growth of *B. amyloliquefaciens* EB2003A. Growth curves for *B. amyloliquefaciens* EB2003A treated with different NaCl levels varied, as shown in Figure 3.3. There were significant differences in growth rate of *B. amyloliquefaciens* EB2003A, for specific NaCl levels, as shown in Table 3.4. The highest, 0.751 ± 0.089 h^{-1}, was observed in *B. amyloliquefaciens* EB2003A treated with 0 mM NaCl. This was significantly higher than the growth rate of *B. amyloliquefaciens* EB2003 A treated with 400 -1000 mM NaCl, as shown in Table 3.3. The lowest growth rate, 0.191 ± 0.033 h^{-1}, was observed for *B. amyloliquefaciens* EB200A treated with 1000 mM NaCl.

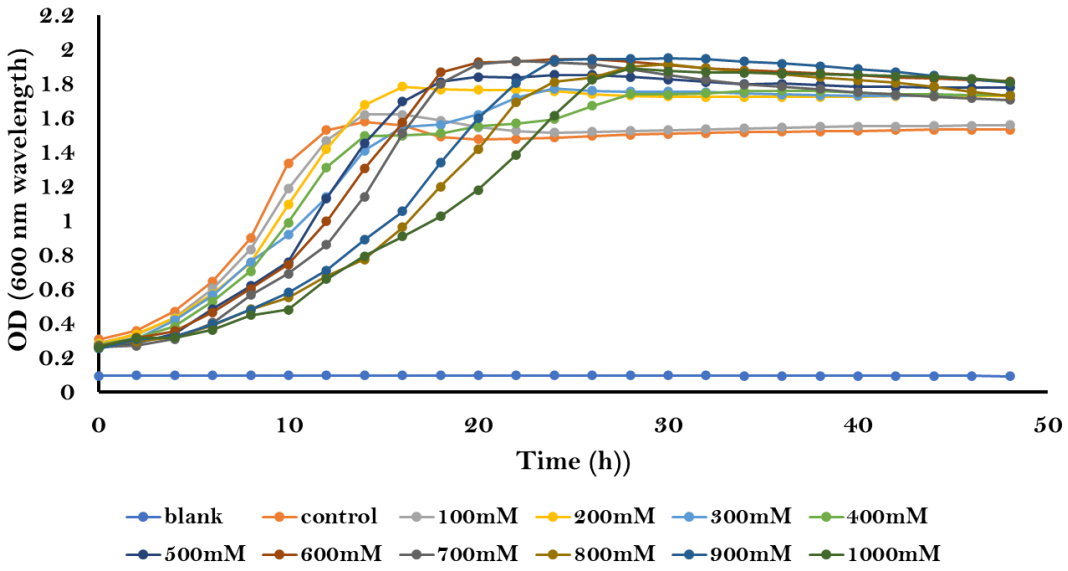

Figure 3.3: Growth curves of *B. amyloliquefaciens* EB2003A for the varying NaCl treatments, from 0 to 48 h.

Table 4.4: Effect of varying NaCl levels on growth rate of *B. amyloliquefaciens* EB2003A. Data represents the mean ± SE; different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values in bold represent minimum and maximum growth rates.

Treatment	Mean growth rate ± SEM
0 mM NaCl	**0.751 ± 0.089^{a}**
100 mM NaCl	0.704 ± 0.077^{ab}
200 mM NaCl	0.589 ± 0.094^{abc}
300 mM NaCl	0.456 ± 0.057^{abcd}
400 mM NaCl	0.485 ± 0.055^{bcd}
500 mM NaCl	0.448 ± 0.076^{bcd}
600 mM NaCl	0.333 ± 0.027^{cd}
700 mM NaCl	0.331 ± 0.058^{cd}
800 mM NaCl	0.257 ± 0.035^{d}
900 mM NaCl	0.250 ± 0.029^{d}
1000 mM NaCl	**0.191 ± 0.033^{d}**

3.5.2.2 Generation time

There were variations in the generation time of *B. amyloliquefaciens* EB2003A treated with varying NaCl levels. The highest generation time, 4.143 ± 1.023 h, was observed for *B. amyloliquefaciens* EB2003A treated with 1000 mM NaCl. It was significantly higher than the generation time of *B. amyloliquefaciens* EB2003 treated with 0 to 500 mM NaCl. The lowest was observed for *B. amyloliquefaciens* EB2003A treated with 0 mM NaCl, although it was not significantly different from the generation time of *B. amyloliquefaciens* EB2003A treated with 100 to 900 mM NaCl.

Table *3*.5: Effect of varying NaCl levels on generation time of *B. amyloliquefaciens* EB2003A. Data represents the mean ± SE; different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values in bold represent minimum and maximum generation time.

Treatment	Mean generational time ± SEM
0 mM NaCl	**0.959 ± 0.104^{b}**

100 mM NaCl	1.019 ± 0.108^{b}
200 mM NaCl	1.266 ± 0.592^{b}
300 mM NaCl	1.601 ± 0.215^{b}
400 mM NaCl	1.487 ± 0.174^{b}
500 mM NaCl	1.737 ± 0.386^{b}
600 mM NaCl	2.126 ± 0.176^{ab}
700 mM NaCl	2.334 ± 0.464^{ab}
800 mM NaCl	2.925 ± 0.554^{ab}
900 mM NaCl	2.889 ± 0.345^{ab}
1000 mM NaCl	**4.143 ± 1.023^{a}**

3.6 Discussion

Sodium chloride is one of the most dominant salts in salt affected soils, which not only affects plants but also their associated microbial counterparts' general survival and growth (Wang et al., 2016). The extent of damage on the microbe varies across microbial species and is dependent, in part, on the level of salt the microbe is exposed to, and its ability to tolerate it, among other factors (Chowdhury et al., 2011; Gandhi & Shah, 2015; Gandhi & Shah, 2016; Rath et al., 2019). In this study, treatment of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H with varying levels of NaCl affected their growth, growth rate and generation time. Salt stress affects microbial growth through direct ion toxicity, nutrient imbalance, and/or limiting water availability to the microbe due to increased extracellular osmotic potential (Egamberdieva et al., 2010; Yan and Marschner, 2013; Wang et al., 2016). Both factors affect microbial biochemical processes essential for growth and survival, such as respiration, biomass accumulation, and enzyme activity, subsequently affecting microbial diversity in salt affected soils (Marinari et al., 2012; Hou et al., 2021). Excess salt beyond what a microbe can tolerate can result in alterations and damage to microbial membranes, proteins, and nucleic acids (Gandhi & Shah, 2015).

Growth rate and generation time are important for PGPM multiplication and diversity. A higher growth rate and lower generation time are desirable for PGPM as

they allow for faster multiplication of their population. The larger the population, the higher the chances of outcompeting competitors, establishment in the soil and causing desirable effects on plants. In the current study, in general, NaCl stress lowered growth rate while increasing generation time. Findings are in part like those of other researchers. For example, Wang et al. (2016) reported an inverse relationship between increase in NaCl concentration and growth rate of *Lactobacillus plantarum*. The decrease in growth rate and increase in generational time could be attributed to salt stress effects on factors such nutrient availability, which are essential for microbial growth and multiplication (Wang et al., 2016). The effect of salt levels varied between the two species. Effect of NaCl on members of the *Bacillus* and *Lactobacillus* genera has been previously reported by other researchers. For example, Zhang et al. (2021) observed a decrease in colony length, colony forming units and changes in gene profile of the salt tolerant *Bacillus spp*. strain SX4. Wang et al, (2016) reported decreased carbohydrate metabolism and damages to the cell wall of *L. plantarum*.

For PGPM to be able to thrive to numbers sufficient to cause positive effects on plant growth, especially in NaCl affected soils, they have to be tolerant to the stress (Sharma et al., 2021). In this study, both strains exhibited a high tolerance to NaCl, up to 1000 mM NaCl. Previous reports have indicated that both strains are halotolerant and their tolerance to high levels of salt has been reported. For example, Sharma et al. (2021) reported tolerance of *B. amyloliquefaciens* strains to 10 % NaCl. The ability of a microbe to tolerate high levels of salt is dependent on several factors such as ability to exclude Na^+ from and accumulate K^+ and Cl^- in their cytoplasm, accumulation of compatible solutes such as proline, and making changes in their cell membrane, making it more hydrophilic, which allows the microbe more access to water (Oren, 2002b; Oren, 2006; Oren, 2008; Kanekar et al., 2012). Microbes can also make changes to their proteome profiles, upregulating and downregulating some proteins, depending on the role they play in the microbe's tolerance to salt stress (Wang et al., 2016; Zhang et al., 2021). Exoproteome profiles of the two strains under study, in response to NaCl, were examined in a different study, which showed changes in the exoproteome profiles of both, with some proteins upregulated and others downregulated (Naamala et al., in press). A number of proteins directly or indirectly related to salt stress tolerance in bacteria were upregulated. However, other mechanisms of tolerance have not been studied in the current or any other study, in relation to this project.

3.7 Conclusion

Treatment of *B. amyloliquefaciens* EB2003 and *L. helveticus* EL2006H with varying levels of NaCl affected their growth rate and generation time, lowering the former while increasing the later, as NaCl levels increased. However, the two strains were able to grow at all NaCl levels under study, exhibiting high tolerance to NaCl stress. Given the results, we can conclude that both strains can be studied for their ability to enhance plant growth under saline conditions.

3.8 References

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3.9 CONNECTING TEXT

In chapter three, results showed that members of the EVL Inc commercial inoculant, *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H were tolerant to high levels of NaCl, up to 1000 mM NaCl. However, the mode of action through which these strains tolerate salinity stress were not elucidated. Chapter four aims at identifying mechanisms employed by the two strains, at the proteome level, to tolerate salt stress. Given that one of the ways microbes enhance plant growth is through exuding bioactive substances in the rhizosphere or on the plant, the study focused on the exoproteome, with hope that we could as well identify exuded proteins that have been previously associated with plant growth promotion. Changes in the exoproteome profiles of both strains at 0 mM NaCl and 200 mM NaCl were elucidated. Two hundred mM NaCl was chosen because a preliminary study on the ability of the strains' CFS obtained at different NaCl concentrations showed that CFS obtained after exposing both strains to 200 mM NaCl enhanced seed germination.

Chapter 4: Effect of NaCl Stress on Exoproteome Profiles of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H

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4.1 Abstract

Salt stress can affect survival, multiplication and ability of plant growth promoting microorganisms to enhance plant growth. Changes in a microbe's proteome profile is one of the mechanisms employed by PGPM to enhance tolerance of salt stress. This study was focused on understanding changes in the exoproteome profiles of *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus* EL2006H when exposed to salt stress. The strains were cultured in 100 mL M13 (*B. amyloliquefaciens*) and 100 mL De man, Rogosa and Sharpe (MRS) (*L. helveticus*) media, supplemented with 200 and 0 mM NaCl (control), at pH 7.0. The strains were then incubated for 48 h (late exponential growth phase), at 120 rpm and 30°C (*B. amyloliquefaciens*) and 37 °C (*L. helveticus*). The microbial cultures were then centrifuged and filter sterilized, to obtain cell-free supernatants, whose proteome profiles were studied using LC-MS/MS analysis and quantified using scaffold. Results of the study revealed that treatment with 200 mM NaCl negatively affected the quantity of identified proteins in comparison to the control, for both strains. There was upregulation and downregulation of some proteins, even up to 100%, which resulted in identification of proteins significantly unique between the control or 200 mM NaCl ($p \leq 0.05$), for both microbial species. Proteins unique to 200 mM NaCl were mostly those involved in cell wall metabolism, substrate transport, oxidative stress tolerance, gene expression and DNA replication and

repair. Some of the identified unique proteins have also been reported to enhance plant growth. In conclusion, based on the results of the work described here, PGPM alter their exoproteome profile when exposed to salt stress, potentially upregulating proteins that enhance their tolerance to this stress.

4.2 Introduction

Plant growth promoting microorganisms (PGPM) and their derivatives are key technology sources for sustainable agriculture, especially with the urgent need to slow climate change and its adverse effects (Naamala and Smith, 2020). For centuries, use of PGPM based inoculants to sustainably enhance plant growth and increase yield, under stressed and ideal conditions has been practiced in different parts of the world (Babalola and Glick, 2012; Bashan et al., 2014; García-García et al., 2020; Naamala et al., 2023). The ability of PGPM and or their derivatives to enhance plant growth is associated with their ability to exude in their growth environment, proteins and metabolites with plant growth promoting characteristics (Prithiviraj et al., 2003; Gray et al., 2006b; Schwinghamer et al., 2016; Piechulla et al., 2017).

Salinity stress is a major global constraint to crop production, affecting both plant yield quality and quantity. Although PGPM can mitigate the effects of salinity stress on plants, it can also affect the ability of PGPMs to enhance plant growth and may lead to microbial death in case of exposure to levels beyond those tolerated (Zahran, 1997; Soussi et al., 2001; Nadeem et al., 2015; Naamala et al., 2022, 2023). Some microbes have developed mechanisms for surviving at high salt concentrations. The ability of microbes to tolerate saline conditions is in part dependent on their ability to regulate salt concentration in their cytoplasm, in relation to that of their growth environment. Mechanisms employed to regulate salt concentration within the microbe include accumulation of osmolytes such as glutamate and proline in their cytoplasm through de novo synthesis or uptake from their growth environment (Zahran, 1997; Soussi et al., 2001; Oren, 2008; Bojanovic et al., 2017), upregulation of iron uptake mechanisms such as production of siderophores (Bojanovic et al., 2017), alteration of their cell membrane composition (Bojanovic et al., 2017; Hachicho et al., 2017), and maintenance of a high KCl concentration in their cytoplasm to match that of their growth medium (Oren, 2002; Oren, 2008). Effecting these mechanisms may necessitate the microbe to make changes to its genome, proteome, and metabolome profiles, most

probably upregulating those components of each essential for enhancing salt tolerance mechanisms.

Protein expression occurs when genes are transcribed into messenger RNA (mRNA), which is then translated into proteins, which are major constituents of microbial cells (Karpievitch et al., 2010; Zhang et al., 2010). Protein expression and secretion are usually in response to either internal or external stimuli such as exposure to biotic and abiotic stress (Zhang et al., 2010; Armengaud et al., 2012; Schoof et al., 2022). The microbial proteome loosely translates to all proteins associated with a given microbe. The microbial exoproteome refers to proteins found in the immediate extracellular milieu of a microbe, arising from active cellular secretion, passive excretion and or cell lysis (Desvaux et al., 2010; Armengaud et al., 2012; Rubiano-Labrador et al., 2015; Schoof et al., 2022). For microbes cultured in laboratories, microbial exoproteome would refer to total proteins in spent media after removal of all microbial cells through centrifugation and filtration. Exoproteome composition reflects a microbe's physiological state at a given time and can provide insight into a microbe's interactions with its surroundings (Armengaud et al., 2012). Abiotic stresses such as salinity, acidity and alkalinity affect the quantity and quality of proteins synthesized and expressed by a microbe at a given time (Singleton et al., 1982; Soussi et al., 2001; Msimbira et al., 2022). Exploring the exoproteome of a microbe exposed to salt stress can provide insight into a set of proteins expressed in response to salt stress, which could then enhance our understanding of salt tolerance mechanisms in microbes (Rubiano-Labrador et al., 2015). In general, the 'omics' studies of biological systems have resulted in better understanding of microbes and their environment (Karpievitch et al., 2010). Advances in technology, such as invention of high through put tandem mass spectrometry and liquid chromatography have allowed for easy identification, analysis, classification, and function annotation of complex protein samples (Listgarten and Emili, 2005; Zhang et al., 2010; Armengaud, 2013; Kucharova and Wiker, 2014; Msimbira et al., 2022). It is interesting to note that while some PGPM may lose their ability to enhance plant growth following exposure to salt stress, others may gain or be more effective at enhancing plant growth, after exposure to some level of stress (Subramanian et al., 2021). Therefore, understanding how microbial exoproteome profiles change with changes in salt stress can improve utilization of CFS as plant growth biostimulants, and enhance our elucidation of mechanisms employed to enhance plant growth and or tolerate salt stress, given that some proteins such as

enzymes play a vital role in stress tolerance and plant growth stimulation (Ahmad et al., 2010).

Bacillus amyloliquefaciens are rod shaped endospore forming gram positive bacteria from the genus *Bacillus* and family *Baciliaceae* (Woldemariam et al., 2020; Ngalimat et al., 2021). *B. amyloliquefaciens* is widely used in the food, pharmaceutical and agricultural sectors (Woldemariam et al., 2020). *B. amyloliquefaciens* and its derivatives have been reported to enhance plant growth under stressed and ideal conditions (Cappellari and Banchio, 2020; Cappellari et al., 2020; Duan et al., 2021; Kazerooni et al., 2021; Naamala et al., 2022). *Lactobacillus helveticus* is a gram positive facultative anaerobic lactic acid bacterium (LAB) that is widely used in the food processing industry. However, *L. helveticus* and its derivatives have also been reported to enhance plant growth (Naamala et al., 2023). This study was focused on understanding changes in exoproteome profiles of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, at 0 mM NaCl and 200 mM NaCl. Results from previous studies have shown that CFSs of both strains, when exposed to 200 mM NaCl, enhanced germination and radicle length of soybean, and corn, as well as growth variables of potato (Naamala et al., 2022, 2023). We therefore point out some of the proteins identified in this study, that have been reported to enhance plant growth, as well as some of the mechanisms plants employ to support growth.

4.3 Materials and Methods

4.3.1 Obtaining protein samples

B. amyloliquefaciens EB2003A and *L. helveticus* EL2006H, which were generously provided by EVL Inc., were cultured in 100 mL M13 and 100 mL De man, Rogosa and Sharpe (MRS) media, respectively, supplemented with 200 and 0 mM NaCl (control), at pH 7.0. They were incubated for 48 h (late exponential growth phase), at 120 rpm and a temperature of 30 and 37°C, for *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, respectively. Four replicates per treatment per species were cultured. Microbial cultures were then centrifuged, using a Sorvall Biofuge Pico (Mandel Scientific, Guelph, ON, Canada), for 10 min, at 10,000 rpm (15,180× g; SLA-1500) and 4°C, to pellet the microbial cells and separate them from the cell-free supernatant (CFS) (Gray et al., 2006a; Subramanian et al., 2021). The CFS was further vacuum filtered using 0.22 μm nylon filters to ensure that all bacterial cells were

removed. Trichloroacetic acid (TCA; T9151, Sigma Aldrich) precipitation was used to extract total proteins from the obtained CFS replicates. The CFS was mixed with 100% (w/v) TCA, in 250 mL conical flasks to create a 25% working solution of TCA. The mixture was incubated at −20°C for 1 h, and transferred to an orbital shaker (MBI, Montreal Biotech Inc., Canada) with shaking speed of 90 rpm, in a cold room, at a temperature of - 4°C, for protein precipitation, overnight. This was followed by a 10 min centrifugation at 4°C and 10,000 rpm, to pellet the protein. The obtained protein pellet was then washed with ice-cold acetone, air-dried under a laminar flow hood, and dissolved in 2 M urea (U4883, Sigma Aldrich). The protein obtained from the four replicates of each treatment were pooled to form one sample per species. The experiment was repeated four times to get the appropriate biological replicates. Concentration of proteins obtained from the four experiments was determined, using the Lowry method (Lowry et al., 1951).

4.3.2 LC-MS/MS Protein profiling

After determining the concentration of the obtained proteins, 10 µg protein per sample was dissolved in 20 µL of 2 M urea and sent to Montreal Clinical Research Institute (IRCM), for liquid chromatography mass spectrometry (LC–MS/MS) analysis. Total proteins were digested using trypsin enzyme and injected into an LC–MS/MS equipped with Linear Trap Quadrupole Velos Orbitrap (Thermo Fisher Waltham, MA, United States). The data set obtained from the mass spectra were searched against *Bacillus* spp. and *Lactobacillus spp.* databases, using Mascot software (Matrix Science, London, United Kingdom). Scaffold Software (version 5.1.2, Proteome Software Inc., Portland OR) was used to validate the obtained MS/MS based peptides and proteins, using an equal to or greater than 95% acceptance of protein probability, with a minimum of two peptides and 95% peptide probability (Keller et al., 2002).

4.3.3 Quantitative data analysis

Proteomic data for identified proteins obtained from the LC–MS/MS analysis was quantitatively analyzed, based on spectra count values, using Scaffold 5 (Scaffold Software for MS/MS Proteomics). Spectra count values were normalized and subjected to analysis of variance, at 5% significance level, using a Benjamini-Hochberg multiple test correction, to detect significant differences between treatments. Significance was

based on both fisher's exact test ($p \leq 0.05$) and fold change of more than or equal to 1.2. FASTA files generated from Scaffold 5 were analyzed using OmicsBox for functional annotation and interpretation of the protein sequences. Volcano plots were created using OriginPro software (OriginPro learning edition, version 2023 learning edition) while Venn diagrams were generated using Scaffold software for MS/MS proteomics. Volcano plots are a form of scatter plots which show significance of proteins with high fold changes. The negative logarithm 10 of significance level (P) values are plotted on the Y axis, against the logarithm two of fold change values. Significant proteins at 0 mM NaCl and 200 mM NaCl are shown at the left and right top of the graph, respectively, while non-significant proteins, at both salt levels are towards the left and right bottom of the graph. The LC-MS/MS proteomic data are available in the **Mass** Spectrometry **I**nteractive **V**irtual **E**nvironment (*MassIVE*) at doi:10.25345/C5PG1HZ4M and PXD041778 for *B. amyloliquefaciens* EB2003A, and doi:10.25345/C54B2XF6V and PXD041177 for *L. helveticus*EL2006H.

4.4 Results

4.4.1 Exoproteome analysis for *B. amyloliquefaciens* EB2003A

Based on scaffold and OmicsBox analyses of the LC-MS data, there were variations in identified proteins for CFS of *B. amyloliquefaciens* EB2003A cultured at 0 mM NaCl and 200 mM NaCl, as shown in Table 1 and supplementary material S1. In general, NaCl lowered the quantity of identified proteins, total unique spectra, and total unique peptides, as shown in figure 1, visualised at 95 % protein threshold, 2 minimum peptides and 0.00% decoy FDR. A total of 1295 proteins, 9718 total peptides and 15283 total spectra were identified. Out of the observed proteins, 1024 were shared between both salt levels while 197 were unique to 0 mM NaCl, and 74 proteins were unique to 200 mM NaCl.

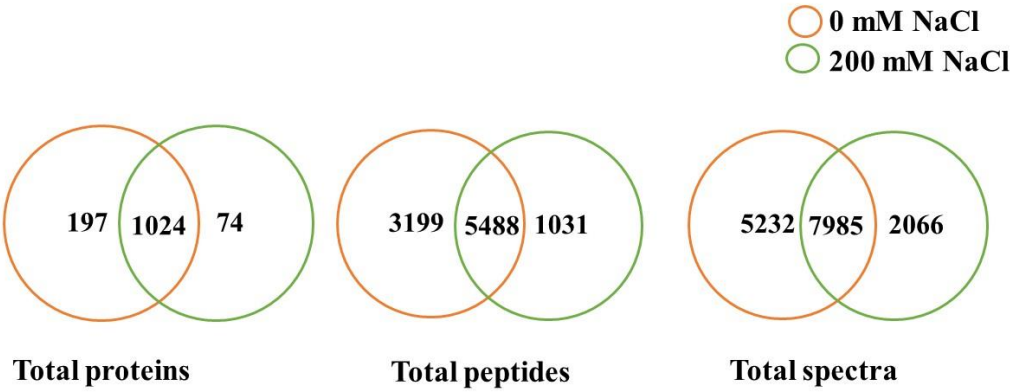

Figure 4.1: Comparison of total proteins, total peptides and total spectra identified at 0 and 200 mM NaCl, for *B. amyloliquefaciens* EB2003A ($p \leq 0.05$).

Further quantitative analysis of the LC-MS/MS data output using scaffold showed a significant decrease in the quantity of identified proteins at 200 mM NaCl in comparison to 0 mM NaCl, at $p \leq 0.05$ (Fisher's exact test). Several proteins were upregulated or down regulated at both salt levels as shown in supplementary material S1. Likewise, several proteins were unique to either 0 or 200 mM NaCl. Analysis with a ±1.2-fold change also showed significant variations in proteins identified for 0 and 200 mM NaCl, as shown in Figure 2. Supplementary S3 shows OmicsBox data for *B. amyloliquefaciens* EB2003A.

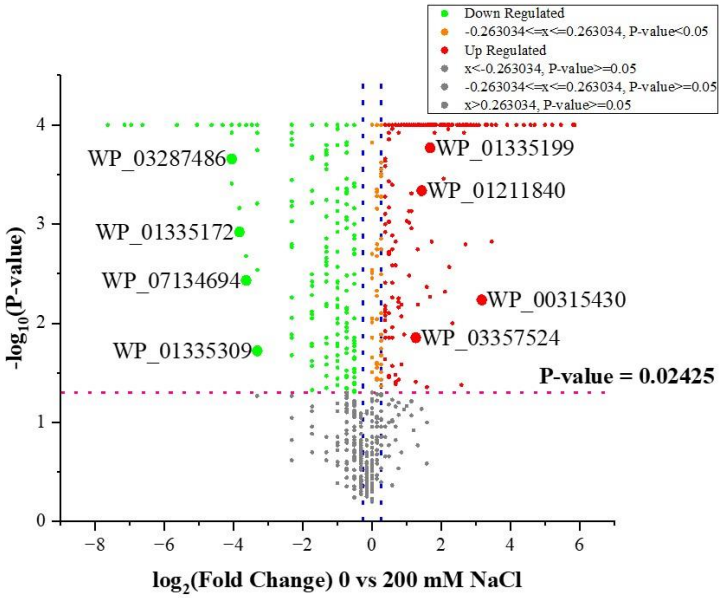

Figure 4.2: Volcano plots showing the distribution of identified proteins for *B. amyloliquefaciens* EB2003A as $-\log_{10}$ (Benjamini–Hochberg-adjusted P-values) plotted against $\log_2$ (fold change) for 0 vs 200 mM NaCl. The two blue dotted vertical lines represent a ±1.2-fold change, while the pink dotted horizontal line indicates the significance threshold (before logarithmic transformation) $p \leq 0.02425$.

4.4.2 Exoproteome analysis for *L. helveticus* EL2006H

Based on scaffold and OmicsBox analyses of the LC-MS/MS data, there were variations in identified proteins for *L. helveticus* EL2006H cultured at 0 mM NaCl and 200 mM NaCl, as shown in Table 2 and supplementary material S2. Two hundred mM NaCl greatly affected identified proteins, with the majority downregulated, even to 100 %. Figure 3 shows a comparison of the quantity of total proteins, total unique peptides, and total unique spectra for 0 and 200 mM NaCl, visualised at 95 % protein threshold, 2 minimum peptides and 0.00% decoy FDR. A total of 317 proteins, 1628 peptides and 2307 spectra were observed. Out of the observed proteins, 136 were shared between both salt levels while 178 were unique to 0 mM NaCl, and 3 were unique to 200 mM NaCl, as shown in figure 3.

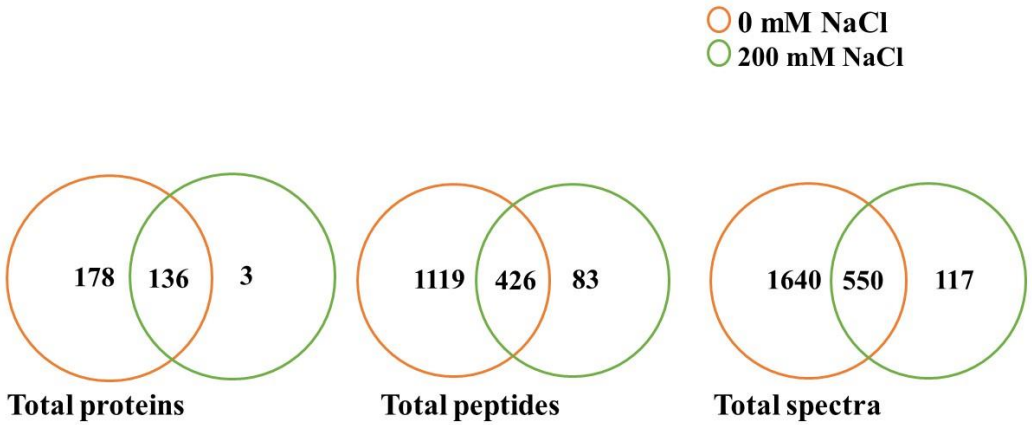

Figure 4.3: Comparison of total proteins, total peptides and total spectra identified at 0 and 200 mM NaCl for *L. helvetius* EL2006H ($p \leq 0.05$).

Further quantitative analysis of the LC-MS/MS output, using scaffold showed a significant decrease in identified proteins at 200 mM NaCl, in comparison to 0 mM NaCl, at $p \leq 0.05$ (Fisher's exact test). The majority of the proteins were significantly downregulated at 200 mM NaCl (Supplementary material S2), with only seven upregulated proteins, namely, a cluster of hypothetical proteins GFB61_00500, a

cluster of SLAP domain-containing proteins, a cluster of peptide ABC transporter substrate-binding proteins, surface proteins, fibronectin type III domain-containing proteins, a cluster of Stk1 family PASTA domain containing Ser/Thr kinases, and a cluster of metal ABC transporter substrate binding proteins. Based on the fold analysis (1.2-fold change and above), only three proteins namely, fibronectin type III domain-containing protein, cluster of hypothetical proteins and cluster of metal ABC transporter, were significantly upregulated as shown in supplementary material S2. Supplementary material S4 shows OmicsBox data for *L. helveticus* 2006H. Figure 4 is a volcano plot illustrating the distribution of identified proteins for *L. helveticus* EL2006H.

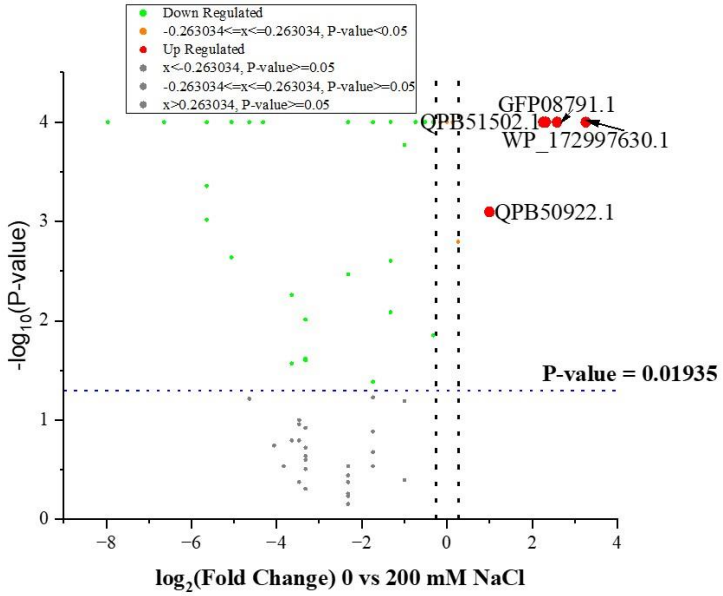

Figure 4.4: Volcano plots showing the distribution of identified proteins for *L. helveticus* EL2006H as $-\log_{10}$ (Benjamini–Hochberg-adjusted P-values) plotted against $\log_2$ (fold change) for 0 vs 200 mM NaCl. The two dotted black vertical lines represent a ±1.2-fold change, while the blue dotted horizontal line indicates the significance threshold (before logarithmic transformation) $P \leq 0.01935$.

4.4.3 Functional annotation of proteins of *B. amyloliquefaciens* CFS observed at 0 and 200 mM NaCl

Using gene ontology (GO) enrichment analysis, identified proteins were grouped into four groups, based on their functions, namely, enzyme code distribution, cellular components, biological processes, and molecular functions, as shown in Table

1. There was a variation in the effect of 200 mM NaCl on the proteins performing various functions, majority being downregulated while some were upregulated, yet others were unique to 200 mM NaCl. Worthy noting is that all proteins involved in the various listed biological processes were downregulated while proteins related to the extracellular region cellular component were unique to 200 mM NaCl. Proteins involved in catalysing proteins and nucleic acids were also upregulated by 18.8 and 17.5 %, respectively. Under the enzyme code distribution, all enzymes were downregulated except for Translocases which were upregulated by 20.6 %.

Table 4.1: Comparing the distribution of *B. amyloliquefaciens* EB2003A proteins to the different functional groups according to GO enrichment analysis, at 0 mM NaCl (control) and 200 mM NaCl (treatment).

	# Sequences	
Functional group	**0 mM NaCl**	**200 mM NaCl**
Biological process		
Organic substance metabolic process	1202	1028 (↓14.5 %)
Primary metabolic process	1061	919 (↓13.4 %)
Cellular metabolic process	1100	912 (↓17.1 %)
Nitrogen compound metabolic process	939	852 (↓9.3 %)
Biosynthetic process	637	493 (↓22.6%)
Small molecule metabolic process	631	490 (↓22.3 %)
Catabolic process	204	193 (↓5.4%)
Cellular components		
Intracellular anatomical structure	500	395 (↓21 %)
Cytoplasm	468	363 (↓22.4 %)
Membrane	162	**232 (↑30.2 %)**
Cell periphery	99	**164 (↑39.6 %)**
Intrinsic component of membrane	110	**159 (↑30.9 %)**
Extracellular region	0	**19 (↑100 %)**
Molecular function		
Ion binding	821	706 (↓14.0 %)
Organic cyclic compound binding	701	620 (↓11.6 %)
Heterocyclic compound binding	701	620 (↓(11.6 %)

Hydrolase activity	663	579 (↓12.7%)
Small molecule binding	580	463 (↓20.2 %)
Oxidoreductase activity	438	331 (↓24.4%)
Transferase activity	481	354 (↓26.4%)
Carbohydrate derivative binding	350	296 (↓15.4%)
Catalytic activity, acting on a protein	177	**218 (↑ 18.8)**
Catalytic activity, acting on a nucleic acid	160	**194 (↑17.5 %)**
Ligase activity	54	0 (↓100 %)
Enzyme code distribution		
Hydrolases	618	537 (↓13.1%)
Isomerases	114	90 (↓21.1%)
Ligases	182	148 (↓18.7%)
Lyases	134	102 (↓23.9 %)
Transferases	463	344 (↓25.7%)
Translocases	54	**68 (↑20.6 %)**
Oxidoreductases	418	310 (↓25.8%)

4.4.4 Functional annotation of proteins of *L. helveticus* EL2006H CFS observed at 0 and 200 mM NaCl

Using gene ontology (GO) enrichment analysis, identified proteins were grouped in four sets, namely, biological processes, cellular components, molecular functions, and enzyme code distribution, as shown in Table 2. There was a variation in the effect of 200 mM NaCl on the proteins performing various functions; the majority were downregulated although a few were upregulated. Proteins in some functional groups were unique to 200 mM NaCl (Table 2). For example, under biological processes, transmembrane transport and cell adhesion were unique to 200 mM NaCl while under cellular components, transporter complex and membrane protein complex were unique to 200 mM NaCl. Under molecular function, peptidoglycan muralytic activity, protein binding and structural constituents of cell walls were unique to 200 mM NaCl. Notably, all the upregulated/unique proteins perform functions related to cell wall metabolism or substrate transportation.

Table 4.2: Comparing the distribution of *L. helveticus* EL2006H CFS proteins to the different functional groups according to GO enrichment analysis, at 0 mM NaCl (control) and 200 mM NaCl (treatment).

	# Sequences	
Functional group	**0 mM NaCl**	**200 mM NaCl**
Biological process		
Organic substance metabolic process	320	50 (↓84.4 %)
Primary metabolic process	294	44 (↓85.0 %)
Nitrogen compound metabolic process	282	46 (↓83.6 %)
Cellular metabolic process	239	12 (↓94.9 %)
Biosynthetic process	149	0 (↓100 %)
Small molecule metabolic process	76	5 (↓93.4 %)
Catabolic process	60	4 (↓93.3 %)
Establishment of localization	62	51 (↓17.7 %)
ATP metabolic process	8	0 (↓100 %)
Transmembrane transport	0	**40 (↑100 %)**
Cell adhesion	0	**7 (↑100 %)**
Cellular components		
membrane	170	94 (↓44.7 %)
intracellular anatomical structure	161	0 (↓100 %)
intrinsic component of membrane	147	81 (↓44.8 %)
cell periphery	110	75 (↓31.8 %)
cytoplasm	92	0 (↓100 %)
organelle	72	0 (↓100 %)
extracellular region	17	**24 (↑29.2 %)**
external encapsulating structure	29	24 (↓17.2 %)
Transporter complex	0	**19 (↑100 %)**
Membrane protein complex	0	**19 (↑100 %)**
Molecular function		
Hydrolase activity	189	76 (↓59.7 %)
Organic cyclic compound binding	200	7 (↓96.5 %)
Heterocyclic compound binding	200	7 (↓(96.5 %)

Ion binding	138	14 (↓89.8 %)
Catalytic activity, acting on a protein	89	39 (↓56.2 %)
Small molecule binding	89	3 (↓96.6%)
Transferase activity	86	0 (↓100 %)
Structural constituent of ribosome	67	0 (↓100 %)
Carbohydrate derivative binding	53	0 (↓ 100 %)
Isomerase activity	9	0 (↓100 %)
ATP hydrolysis activity	18	0 (↓100 %)
Peptidoglycan muralytic activity	0	**9 (↑ 100 %)**
Protein binding	0	**16 (↑ 100 %)**
Structural constituent of cell wall	0	**6 (↑ 100 %)**
Enzyme code distribution		
Oxidoreductases	38	4 (↓89.5 %)
Transferases	86	4 (↓95.3 %)
Hydrolases	167	76 (↓54.5 %)
Isomerases	33	3 (↓90.1 %)
Translocases	9	6 (↓33.3 %)
Lyases	18.5	0 (↓ 100%)
Ligases	21.75	0 (↓100%)

4.5 Discussion

Uncontrollable changes in microbial environments, especially under field conditions, requires PGPM to adapt to the changes in order to survive (Gao et al., 2007; Galperin, 2010). Salinity stress is a leading global abiotic stress affecting crops and PGPM proliferation (Liu et al., 2023). When a microbe is exposed to stress, it may alter its proteome profile, upregulating proteins essential for enhancing tolerance to the stress while down regulating those that are likely not so essential (Galperin, 2010; Msimbira et al., 2022). As a result, proteome profiles of a microbe grown in different environmental conditions may vary significantly. The current study compared exoproteome profiles of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H exposed to 0 and 200 mM NaCl. Results of the study showed variations in total proteins identified for both strains at the two salt levels. Some of the identified proteins were unique to either 0 or the 200 mM NaCl. Among the proteins unique to 200 mM NaCl

were cell wall metabolic enzymes, transcription/translation regulators, potential virulence factors, phage proteins, antibiotics resistance proteins, solute transporter proteins and, of course, hypothetical proteins. These findings are to some extent like those of Pumirat et al. (2009) and Rubiano-Labrador et al. (2015) who examined the exoproteome of *Burkholderia pseudomallei* and *Tistlia consotensis* exposed to salinity stress.

The cell-wall is the outermost layer of a bacterial cell, that acts as a stress barrier and maintains cell shape (Mueller and Levin, 2020). Therefore, maintaining the integrity of the cell wall is a mechanism for stress tolerance in bacteria. This may explain why, based on GO function analysis, the different functional groups identified in the current study, protein classes performing functions related to the cell wall and the extracellular region were upregulated at 200 mM NaCl, in both *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H. Peptidoglycan is the major component of the cell wall, whose synthesis, polymerization, modification and turn over contribute to maintaining cell wall integrity (Popham and Young, 2003; Sauvage et al., 2008; Shin et al., 2020). In this study, proteins, such as LytR family transcriptional regulator, Amidases, peptidoglycan endopeptidases and penicillin binding proteins (PBPs), SLAP domain-containing protein and surface proteins were uniquely produced by either *B. amyloliquefaciens* EB2003A or *L. helveticus* EL2006H, exposed to 200 mM NaCl. They all play vital roles in peptidoglycan metabolism and maintenance of the cell wall. For example, LytR family transcriptional regulator proteins, also known as LytR-CpsA-Psr (LCP) family proteins, are in fact enzymes involved in the attachment of Glycopolymers, such as wall teichoic acids on the peptidoglycan (Kawai et al., 2011; Molloy, 2011; Gale et al., 2017; Siegel et al., 2019). Previously, this family of proteins was reported to play a transcription regulation role (Gao et al., 2007; Galperin, 2010), although in later studies, Kawai and co-authors disagreed (Kawai et al., 2011), suggesting that these regulatory roles could be bacterial genus, species, or strain specific. Bacterial amidases play a vital role in bacteria cell wall metabolism because, especially under stressful conditions, they are involved in the remodeling, turnover, recycling, and metabolism of peptidoglycan (Park, 1995; Weber et al., 2013; Senzani et al., 2017; Mueller and Levin, 2020). Endopeptidases play a major role in maintaining bacterial cell integrity and shape, through processes such as peptidoglycan turnover and modification (Shin et al., 2020). Penicillin binding protein PBP4, a peptidoglycan endopeptidase was reported to enhance tolerance of *B.*

subtilis to salt stress by modifying the peptidoglycan (Palomino et al., 2009). *B. subtilis* was reported to recycle its peptidoglycan toward the end of its exponential growth, entering stationary phase, which could enable prolonged survival of the bacteria during stationary phase (Borisova et al., 2016). Surface proteins, also known as the glycoprotein layer or S layer proteins (Engelhardt, 2007; Hynönen and Palva, 2013), and surface layer associated proteins (SLAP), in the current study, were unique to *L. helveticus* treated with 200 mM NaCl. Expression of surface proteins has been linked to the ability of some *Lactobacilli* species to tolerate changes in the human gastrointestinal tract conditions, such as bile and low pH (Sengupta et al., 2013). It should be noted that, not all prokaryotes produce surface proteins and that their role varies from one group to another, leaving no universal function of surface proteins in species that do possess them (Engelhardt, 2007). Upregulation of surface proteins was also observed in *Lactobacillus acidophilus* IBB 801 exposed to different abiotic stresses such as NaCl, bile salt and high temperature (Grosu-Tudor et al., 2016). Deletion of *IgdA* SLAP resulted in a mutant that was more sensitive to salt stress and had a visibly disrupted cell surface when compared to strains with the protein (Klotz et al., 2020). However, the mechanisms through which surface proteins and SLAP enhance tolerance to salt stress is yet to be verified. Likewise, Stk1 family PASTA domain-containing Ser/Thr kinase also upregulated in *L. helveticus* exposed to 200 mM NaCl have been reported to play a role in bacteria cell wall metabolism and cell division (Janczarek et al., 2018). Its expression was reported to enhance tolerance of *Streptococcus suis* serotype 2 to oxidative stress (Zhu et al., 2014).

Exposure to stress may be a trigger for bacteria to reprogram their gene expression, consequently resulting in new gene products that could be essential for stress tolerance. In the current study, proteins such as MarR family transcriptional regulators and rRNA pseudouridine synthase were upregulated at 200 mM NaCl. The MarR family transcriptional regulators constitute a prominent family of transcription factors involved in the reprogramming of gene expression in response to stress conditions, such as oxidative stress (Pérez-Rueda et al., 2004; Grove, 2013; Deochand and Grove, 2017). MarR are involved in metabolism and antibiotic resistance of some bacteria (Will and Fang, 2020). The enzyme rRNA pseudouridine synthase catalyzes the synthesis of RNA pseudouridine from uracil, the most common modified nucleoside in rRNA that plays a role in gene expression (Ofengand, 2002; Zhao et al., 2018). Sulfate Transporter and Anti-Sigma factor antagonist (STAS) domain-containing

proteins are produced in multiple species, including bacteria and mammals (Moy and Seshu, 2021). In bacteria, they are associated with stress tolerance among other factors, by regulating the large family of sigma factors (ρ) that bind to RNA polymerase to confer transcriptional target gene specificity (Moy and Seshu, 2021). For instance, sporulation in *B. subtilis*, which is a response to stress involves anti-anti-sigma factors, or anti-anti-ρ, which are STAS domain proteins (Sharma et al., 2011). Changes in gene products may also affect cellular biochemical composition and cellular processes, as observed in the current study.

Salt stress leads to osmotic, oxidative, and ionic stress in microbes, which, depending on their severity could cause damage to cellular components such as the cell membrane, nucleic acids, enzymes, and other proteins. A high concentration of salt ions such as Na^+ and Cl^- in the microbe's extracellular environment may cause loss of water from the microbial cell, leading to loss of cell turgor pressure as well as reduction in cellular processes such as metabolism (Krämer, 2010; Tsuzuki et al., 2011; Rubiano-Labrador et al., 2015). To counter act such effects, microbes develop mechanisms that enhance microbial tolerance to oxidative, osmotic, or ionic stress. In the current study, at 200 mM NaCl, proteins that enhance bacteria's tolerance to oxidative stress were unique to 200 mM NaCl. For example, Thioredoxins play a major role in bacterial response to oxidative stress by quenching singlet oxygen, scavenging hydroxyl radicals and donating hydrogen to peroxidases (Chae et al., 1994; Das and Das, 2000; Zeller and Klug, 2006; Lu and Holmgren, 2014; Cheng et al., 2017). Members of the xenobiotic response element (XRE) family transcriptional regulators, among other functions, have been reported to enhance oxidative stress tolerance in different bacteria species such as *Streptococcus suis* and *Corynebacterium glutamicum* (Hu et al., 2019; Si et al., 2020; Zhang et al., 2022). Flavodoxin family proteins were reported to enhance tolerance of plant growth promoting rhizobacteria, such as *Pseudomonas fluorescens* Aur6 and *Ensiifer meliloti*, to oxidative stress (Coba de la Peña et al., 2013). Heme A synthase catalyzes the synthesis of heme A from heme O (Lewin and Hederstedt, 2016). Heme A is particularly a co-factor of terminal oxidase enzymes involved in oxygen reduction during aerobic respiration (Hederstedt, 2012; Choby and Skaar, 2016). High levels of intracellular heme have been reported to activate Hap1p which subsequently induces the transcription of genes involved in oxidative stress response (Martínez et al., 2016).

Since salt stress can result in damage to essential microbial constituents such as enzymes, and nucleic acids, DNA and RNA, it is important that damaged components are repaired or replaced with new ones. Upregulation of proteins that are directly or indirectly involved in synthesis and repair of cellular proteins and nucleic acids was observed in the current study. The enzyme 2′,3′-cyclic-nucleotide 2′-phosphodiesterase plays a major role in the metabolism of purines and pyrimidines, the building blocks of DNA and RNA, and provide energy and co-factors important in cell-division (Yin et al., 2018). This is because it contains cyclic phosphodiesterase and 3′-nucleotidase activity and catalyzes the hydrolysis of 2′,3′-cyclic nucleotides to yield nucleotides and phosphate. Therefore, the enzyme plays a role in DNA and RNA synthesis and repair through provision of building blocks. The enzyme m^1A22-tRNA methyltransferase (TrmK) catalyzes N (1)-adenosine methylation to N1 of adenine 22 of bacterial tRNA (Roovers et al., 2008; Sweeney et al., 2022). Addition of a methyl group plays a role in maintaining stability of tRNA (Roovers et al., 2008). Stability of tRNA is essential in protein synthesis since they bridge the gap between mRNA and amino acids during translation. The enzyme thioredoxin plays a major role in protein repair and DNA synthesis by donating hydrogen that reduces ribonucleotide reductase and methionine sulfoxide reductase which catalyze the process (Zeller and Klug, 2006; Lu and Holmgren, 2014). The upregulation of such enzymes may also explain the high frequency of protein classes whose function annotation involves catalytic activity, acting on nucleic acid, and catalytic activity acting on proteins, that was observed in *B. amyloliquefaciens* EB2003A, at 200 mM NaCl.

When exposed to stress, its paramount that microbes maintain an even flow of substrates from their environment to the inside of the cell, and vice versa. This ensures availability of carbon sources for energy as well as metabolites required to serve purposes such as osmoregulation, enzyme co-factors, synthesis of proteins and nucleic acids. The microbe requires energy for channeling to survival mechanisms (Yan et al., 2015; Msimbira et al., 2022). Microbes respond to osmotic stress through intracellular accumulation of inorganic ions such as K^+ and organic solutes such as proline (Soussi et al., 2001; Bojanovic et al., 2017). Although some of these osmo-protectants can be synthesized *de novo*, it is less energy efficient than sourcing them from outside of the cell (Zahran, 1997; Oren, 2008; Zhang et al., 2015; Bojanovic et al., 2017; Lycklama et al., 2018). Therefore, maintaining adequate substrate transport systems is essential for microbial tolerance to stress. In this study, we observed upregulation of a number

of proteins associated with various substrate transport systems, such as the ATP binding cassette (ABC) transporter, the major facilitator superfamily (MFS) transporter and the phosphotransferase system (PTS) fructose transporter subunit IIC in *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H. The ABC transporter facilitates transportation of a wide range of substrates such as sugars, amino acids, and metals from the microbe's external environment (Du et al., 2011; Lycklama et al., 2018; Teichmann et al., 2018). Among the observed ABC transporter proteins were the amino acid ABC transporter substrate-binding protein, multispecies: ABC transporter substrate-binding protein, and multispecies: zinc ABC transporter substrate-binding protein. The MFS transporter is one of the oldest protein families, a group of secondary active transporters involved in selective transportation of substrates such as carbohydrates, amino acids, and lipids, to ions, peptides and nucleosides, across microbial membranes and plays a role in other microbial physiological processes, such as resistance to toxic compounds like antibiotics and salicylic acid, and enhanced salt tolerance (Yan, 2013; Lee et al., 2016; Pasqua et al., 2019). For instance, MFS efflux pumps VceCAB were reported to enhance the tolerance of *E. coli* to bile salts (Woolley et al., 2005). The PTS system is involved in uptake and phosphorylation of carbohydrates as well as signal transduction (Bernhard, 2012). The enzyme IIC component selectively transports sugar molecules across microbial membranes (Jahreis et al., 2008; Jason et al., 2014). This allows microbes such as bacteria to efficiently utilize carbohydrate sources of their choice, at a given time (Jahreis et al., 2008). The fructose family is a subfamily and the oldest of the glucose superfamily of PTS. The ability of an organism to utilize various carbon sources enables them to survive in varying environmental conditions. As a result, upregulation of sugar uptake systems has been linked to microbe response to stress, because microbes require nutrients and osmo-protectants for survival under stressful conditions (Pittman et al., 2014).

When exposed to stress, some members of the genus *Bacillus*, *B. amyloliquefaciens* EB2003A inclusive, form spores which are essential for survival under stressful conditions for long periods of time (Setlow, 2014; Ghosh et al., 2018). Once favorable conditions are restored, the spores germinate, giving rise to new microbial cells. The germination of spores is triggered by amino acids such as L-alanine, L- valine and L- asparagine (Setlow, 2014; Ghosh et al., 2018). In the current study, alanine containing proteins: cation symporter family protein and asparagine synthetase B were unique to the 200 mM NaCl exoproteome of *B.*

amyloliquefaciens EB2003A. The enzyme asparagine synthase catalyzes the synthesis of asparagine from aspartate and glutamine (Lomelino et al., 2017; Zhu et al., 2019). The alanine cation symporter family protein is a transporter protein that transports alanine but no other amino acids (Ma et al., 2019).

In addition to the proteins with known functions, several hypothetical proteins were also unique to 200 mM NaCl treatment. Proteins are classified as hypothetical if a corresponding mRNA sequence is available in the data base, but there is no similar protein sequence, hence, insufficient information concerning their possible functions. However, it's possible that such proteins play a role in enabling the microbe's survival in growth conditions under which they are produced.

In previous studies, CFS of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H exposed to 200 mM NaCl enhanced germination and radicle length of corn and soybean and growth variables of potato grown under NaCl stress and normal conditions (Naamala et al., 2022, 2023). The ability of a microbe to enhance plant growth is related to its ability to exude into their growth environment, bioactive substances with ability to enhance plant growth. Among the proteins upregulated at 200 mM NaCl, in the current study, are those that have been reported to enhance plant growth under stressed and ideal conditions. Whether these proteins were in part responsible for the bioactivity observed in our previous study needs to be investigated further. However, application of exogenous heme has been reported to enhance plant tolerance to stress such salt stress (Woodson et al., 2011; Zhang et al., 2013; Wu et al., 2022). The heme precursor 5-aminolevulinic acid (ALA) was reported to enhance growth of plants exposed to salt stress (Hui et al., 2006; Daneshmand and Oloumi, 2015; Genişel and Erdal, 2016; Wu et al., 2022). Exogenous application of ALA, resulted in an increase in heme content, an indication that heme is involved in the role of ALA in alleviating salinity stress (Wu et al., 2022). Heme is involved in the transformation of superoxide anions in the antioxidant system, hence, potentially playing a pivotal role in mitigating the effects of oxidative stress on plants (Wu et al., 2022). Esterases have been reported to play a role in plant growth and development, involved in such crucial stages as seed germination, pollen development, lateral root, and overall root development (Takahashi et al., 2010; Clauss et al., 2011; Dolui and Vijayaraj, 2020; Zhang et al., 2020; Ursache et al., 2021; Shen et al., 2022). In fact, esterases are believed to have played a role in the evolutionary colonization of land by plants, through the conservation of water in a desiccating environment (Niklas et al.,

2017; Philippe et al., 2020). MarR homologs were involved in symbiotic plant microbe interactions. For example, the MarR homolog ExpG *Sinorhizobium meliloti* activates transcription of three exp. operons that are involved in the production of galactoglucan, which it needs for plant root nodulation (Becker et al., 1997; Bartels et al., 2003). Proteins such as the MFS efflux pumps were reported to be involved in the interaction of plants and symbiotic microbes, such as rhizobia through enhancing nodulation and enhancing tolerance to flavonoids (Pasqua et al., 2019). Thioredoxin, another protein (enzyme) that was unique to 200 mM NaCl exoproteome of *B. amyloliquefaciens* EB2003A is also found in higher plants where it is classified as a disulfide regulatory protein, belonging to a complex of regulatory proteins consisting of types *f, m, x, y, h,* and *o* (Meng et al., 2010). Thioredoxin proteins play major roles in the regulation of carbon metabolism, embryogenesis, chloroplast development and mobilization of seed reserves, in plants (Jedelská et al., 2020). They also play a role in plant responses to biotic and abiotic stresses through protection from reactive oxygen species (Dos Santos and Rey, 2006; Meyer et al., 2012; Geigenberger et al., 2017). Thioredoxin *h* ortholog Trx *h*9, was reported play a role in the germination of wheat (Li et al., 2009). It is believed that Trx h regulates seed germination by reducing the disulfide proteins stored in the dry seed to the sulfhydryl state, following the addition of water (Maeda et al., 2003, 2005; Rhazi et al., 2003; Zahid et al., 2008). Meng et al. (2010) observed chlorotic leaves, short and smaller roots in *Arabidopsis thaliana Trx h*9 mutants. Furthermore, mutant plants were dwarf, with small and irregular mesophyll cells, as well as lower chloroplast numbers and less chlorophyll, in comparison to the wild type control, suggesting that Trx *h* plays a role in plant growth (Meng et al., 2010). High expression of thioredoxin h8 was observed in tobacco plants whose growth was enhanced when treated with *Bacillus aryabhattai* (Xu et al., 2022). Amidases are involved in the biosynthesis of indole acetic acid, a phytohormone that plays major roles in plant growth and development (Spaepen et al., 2007). Other PGPM such as *Pseudomonas putida* have also been reported to produce amidase (Chacko et al., 2009). Because they are involved in nitrogen metabolism, amidases increase nitrogen use efficiency in plants, which subsequently enhances plant growth under both stressed and non-stressed conditions (Unkefer et al., 2023).

There are several mechanisms through which the identified proteins can enhance plant growth. These include regulation of the anti-oxidant system, regulation of the photosynthetic system, ion balance, hydrolysis of compounds that affect plant

quality, mobilization of nutrients during seed germination, biosynthesis of phytohormones involved in metabolic pathways such as nitrogen metabolism and maintenance of plant fertility (Spaepen et al., 2007; Takahashi et al., 2010; Clauss et al., 2011; Dolui and Vijayaraj, 2020; Zhang et al., 2020; Unkefer et al., 2023).

Based on these studies, it's possible that some of the proteins upregulated at 200 mM NaCl stress were responsible for enhancing radicle length, germination of corn and soybean, and growth variables of potato, as observed in our previous studies.

4.6 Conclusions

Salinity stress affects survival, growth, and ability of PGPM to enhance plant growth. However, some PGPM have developed mechanisms of tolerating high levels of salt, altering their exoproteome profile being one. In the current study, *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, exhibited unique proteins when exposed to 200 mM NaCl, some of which have also been reported to enhance plant growth. Results of the study are in line with previous reports that when exposed to stress, microbes alter their exoproteome profile. To the best of our knowledge, this is the first study to report on the effect of NaCl on *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H exoproteome profiles. Findings of this study will expand knowledge regarding mechanisms through which *Bacillus* spp. and *Lactobacillus spp.* tolerate salt stress at the protein level.

4.7 References

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4.8 CONNECTING TEXT

Chapter four showed that there were changes in the exoproteome profile of *Bacillus amyloliquefaciens* EB2003A, when exposed to 200 mM NaCl, in comparison to the 0 mM NaCl control. Among the expressed proteins, at 200 mM NaCl, were those that have been reported by other researchers, to enhance plant growth. Chapter five therefore aimed at understanding whether the CFS of *B. amyloliquefaciens* EB2003A, exposed to 200 mM NaCl could enhance plant growth. In case of positive results, perhaps that could in part be related to the identified proteins, and also prove that indeed, *B. amyloliquefaciens* EB2003A, when exposed to 200 mM NaCl, exudes plant growth promoting substances in its growth environment.

Chapter 5 Cell-Free Supernatant Obtained from A Salt Tolerant *Bacillus Amyloliquefaciens* Strain Enhances Germination and Radicle Length Under NaCl Stressed and Optimal Conditions

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5.1 Abstract

Seed germination and early plant growth are key stages in plant development, that are susceptible to salinity stress. Plant growth promoting microorganisms (PGPMs) produce substances, in their growth media, that could enhance plant growth under more optimal conditions, and or mitigate abiotic stresses, such as salinity. This study was carried out to elucidate the ability of a NaCl tolerant *Bacillus amyloliquefaciens* strain's cell-free supernatant to enhance germination and radicle length of corn and soybean, under optimal and NaCl stressed growth conditions. Three NaCl levels (0, 50, and 75 mM) and four cell-free supernatant concentrations (1.0, 0.2, 0.13, and 0.1% [v/v]) were used to formulate treatments that were used in the study. There were observed variations in the effect of treatments on mean radicle length and mean percentage germination of corn and soybean. Overall, the study showed that *Bacillus amyloliquefaciens* (BA) EB2003's cell-free supernatant could enhance mean percentage germination and or mean radicle length of corn and soybean. At optimal conditions (0 mM NaCl), 0.2% BA, 0.13% BA, and 0.1% BA concentrations resulted in 36.4, 39.70, and 39.91%, increase in mean radicle length of soybean, respectively. No significant observations were observed in mean radicle length of corn, and mean

percentage germination of both corn and soybean. At 50 mM NaCl, 1.0% BA resulted in 48.65% increase in mean percentage germination of soybean, at 24 h. There was no observed effect of the cell-free supernatant on mean radicle length and mean percentage germination, at 72 and 48 h, in soybean. In corn however, at 50 mM NaCl, treatment with 0.2% BA and 0.13% BA enhanced mean radicle length by 23.73 and 37.5%, respectively. The resulting radicle lengths (43.675 and 49.7125 cm) were not significantly different from that of the 0 mM control. There was no observed significant effect of the cell-free supernatant on mean germination percentage of corn, at 50 mM NaCl. At 75 mM NaCl, none of the treatments enhanced mean radicle length or mean percentage germination to levels significantly higher than the 75 mM NaCl control. Treatment with 1.0% BA, however, enhanced mean percentage germination to a level not significantly different from that of the 0 mM control, at 72 h. Likewise, in corn, none of the treatments enhanced radicle length to lengths significantly higher than the 75 mM control, although treatment with 1.0% BA, 0.13% BA, and 0.1% BA elongated radicles to lengths not significantly different from the 0 mM NaCl control. Treatment with 0.2% BA, 0.13% BA, and 0.1% BA resulted in mean percentage germination significantly higher than the 75 mM NaCl by 25.3% (in all 3), not significantly different from that of the 0 mM NaCl. In conclusion, concentration of cell-free supernatant and NaCl level influence the effect of *Bacillus amyloliquefaciens* strain EB2003A cell-free supernatant on mean percentage germination and mean radicle length of corn and soybean.

5.2 Introduction

Germination and seedling establishment are significant stages of plant growth and development, which if compromised, can have significant effects on overall crop growth, including, quality and quantity of yield. Fast and uniform germination is a desirable characteristic of seed, for commercial purposes. Salinity stress can cause physiological and biochemical changes in germinating seeds, such as inhibition of water uptake during imbibition, which may subsequently affect the germination process, establishment of seedlings and general plant growth (Ibrahim, 2016; El Moukhtari et al., 2020). Na^+, Cl^-, Mg^{2+}, and $SO_2{}^{-4}$ ions are the most dominant in saline soils, due their high solubility, and hence, ease of deposition by water, of minerals such as NaCl, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl, Na_2CO_3, Na_2SO_4 and $[Na_2Mg(SO_4)_2]$ (Tanji,

2002), NaCl, the dominant in most saline environments (Forni et al., 2017). Salinity may cause osmotic, ionic, and oxidative stresses in the seed, which may delay or cause complete failure of a seed to germinate (Rouhi et al., 2011; Ansari and Sharif Zadeh, 2012; Ilangumaran et al., 2021). Osmotic stress may result in reduced activity or denaturation of plant cytosolic and organelle proteins (Forni et al., 2017), decrease of cytosolic and vacuolar volumes which may negatively impact plant growth, due to reduced photosynthesis and increased production of reactive oxygen species (ROS), which may be detrimental to plant cell components and physiology (Forni et al., 2017). Ionic stress, due to accumulation of ions such as Na^+, interferes with plant nutrient availability, which may also affect plant growth and yield (Forni et al., 2017). Oxidative stress occurs when there is increase in the production of ROS such as, singlet oxygen, superoxide ions and peroxides, above what is scavenged by plant cells, causing toxicity and subsequent damage to vital plant cells and their components such as proteins, membrane lipids and nucleic acids (Ahmad et al., 2010, 2019; Kohli et al., 2019). Corn and soybean are important crops worldwide and are both considered moderately sensitive to salinity (Mozafariyan et al., 2016; Bai et al., 2018; Diouf et al., 2018), although variation occurs inter and intra-species.

Irrigation and use of chemicals has led to increased secondary salinity, which is detrimental to seed germination and subsequent plant growth (Li et al., 2015; Mozafariyan et al., 2016; Bai et al., 2018). A soil is classified as saline when the electrical conductivity of a saturated soil paste extract (ECe) is $\geq$4 dS m^{-1}, which is equivalent to 40 mM NaCl (US Salinity Laboratory Staff, 1954; Shrivastava and Kumar, 2015; Forni et al., 2017). Unfortunately, globally, due to agricultural practices and climate change, which has resulted in changing rainfall patterns, evapotranspiration, and landscape hydrology (Bui, 2013), soil salinization is expected to expand at a rate of 10% annually (Shrivastava and Kumar, 2015), hence an estimated 50% of arable land is projected to be salinity affected by 2050.

Use of PGPM technology, is a potentially sustainable and ecofriendly mechanism of mitigating salinity stress effects on crops (Ilangumaran et al., 2021; Naamala and Smith, 2021a,b). Use of pure microbial derived active compounds is especially a section of PGPM technology, that has currently gained popularity due to ease of patent and certification process compared to live cells (García-García et al., 2020; Naamala and Smith, 2020). Microorganisms produce metabolites and proteins in their growth environment (exometabolome and exoproteome), for various reasons, such

as adaptation mechanism to stressful conditions, signaling purposes, etc. (Zhao et al., 2020; Kazerooni et al., 2021; Subramanian et al., 2021). Improvement in technology has allowed for isolation and independent use of pure active compounds, as plant growth stimulants (García García et al., 2020; Naamala and Smith, 2021b; Subramanian et al., 2021). A series of steps are usually followed before a compound is discovered. The first step is to test microbial-cell free supernatant for bioactivity, on a crop species of choice. If the cell-free supernatant exhibits bioactivity, the biologically active compound(s) can then be isolated using techniques such as high-pressure liquid chromatography (HPLC) and identified using techniques such as mass spectrometry (Gray, 2005; Subramanian et al., 2021). Through such steps, the microbe-to-plant signal compounds thuricn17 and lipo-chitooligosaccharide (LCO) were isolated in the Smith laboratory and have been reported to successfully enhance plant growth, under stressed and optimal conditions (Souleimanov et al., 2002; Schwinghamer et al., 2015; Subramanian et al., 2016a, b). Research on microbe-derived compounds has gained popularity among researchers, with hopes that compounds could complement, supplement or at least, address some of the inconsistencies associated with the use of PGPM cells, especially under field conditions (Naamala and Smith, 2021a; Subramanian et al., 2021).

This study, therefore, focused on the bioactivity on corn and soybean, of a cell-free supernatant obtained from the salt tolerant *Bacillus amyloliquefaciens* EB2003A strain, under NaCl stressed and optimal conditions. This strain, a property of EVL Inc., was isolated in their laboratories and is a component of the EVL Enhancer solution, a unique plant growth biostimulant developed by the same company (Macouzet, 2016). The aim of the study was to understand whether, when exposed to salinity stress, *Bacillus amyloliquefaciens* EB2003A produces plant growth promoting substances and excretes these into its growth media.

5.3 Materials and methods

5.3.1 Obtaining microbial cell-free supernatant

Bacillus amyloliquefaciens strain EB2003A and its growth media (M13) were provided by EVL Inc. (http://www.evl-global.com). To obtain the cell-free supernatant, the *Bacillus amyloliquefaciens* EB2003A cells were cultured in sterilized M13 medium, supplemented with 200 mM NaCl, and incubated for 48 h, at 30 ºC and 120 rpm. At 48

h, the bacteria culture was centrifuged for 10 min, at 10,000 rpm and 4 ºC, to pellet the bacterial cells and separate them from the cell-free supernatant (Gray, 2005; Gray et al., 2006; Subramanian et al., 2021). The cell-free supernatant was further filtered using 0.22µm nylon filters to remove any microbial cells remaining after centrifugation. Three NaCl (0, 50 and 75 mM NaCl) and four cell-free supernatant concentrations [1.00, 0.20, 0.13, and 0.10% (v/v)] were dissolved in distilled water to formulate treatments (30 mL each) that were used in the study. Table 5.1 shows the quantity of water, microbial cell-free supernatant and NaCl, mixed to formulate 30 mL of each of the different treatments used in the study. For each microbial cell-free supernatant concentration, a similar concentration of M13 bacterial growth medium (not inoculated with bacteria), was used as a positive control. The different treatments and controls per experiment are presented in Table 5.2.

Table 5.1: Quantity of water, microbial cell-free supernatant and NaCl, mixed together to obtain 30 mL of each treatment.

NaCl level	Treatment #	Treatment name	Water (ml)	Supernatant (ml)	NaCl (g)
0 mM NaCl	1	0 mM NaCl	30	0	0
	2	1.0% BA	29.7	0.3	0
	3	0.2 % BA	29.94	0.06	0
	4	0.13 % BA	29.96	0.04	0
	5	0.1 % BA	29.97	0.03	0
50 mM NaCl	1	0 mM NaCl	30	0	0
	2	50 mM NaCl	30	0	0.08766
	3	1.0 % BA	29.7	0.3	0.08766
	4	0.2 % BA	29.94	0.06	0.08766
	5	0.13 % BA	29.96	0.04	0.08766
	6	0.1 % BA	29.97	0.03	0.08766
75 mM NaCl	1	0 mM NaCl	30	0	0
	2	75 mM NaCl	30	0	0.13149
	3	1.0% BA	29.7	0.3	0.13149
	4	0.2 % BA	29.94	0.06	0.13149
	5	0.13 % BA	29.96	0.04	0.13149
	6	0.1 % BA	29.97	0.03	0.13149

Table 5.2: The treatments and controls used in the study. BA refers to *Bacillus amyloliquefaciens* cell-free supernatant, M13 refers to M13 bacterial growth medium.

Treatments	Control
Normal conditions	
0 mM NaCl (control)	
0 mM NaCl + 1.0% BA	0 mM NaCl + 1.0 % M13
0 mM NaCl + 0.2 % BA	0 mM NaCl + 0.2 % M13

0 mM NaCl + 0.13 % BA	0 mM NaCl + 0.13 % M13
0 mM NaCl + 0.1 % BA	0 mM NaCl + 0.1 % M13

NaCl stressed conditions	
0 mM NaCl	
50 mM NaCl	
50 mM NaCl + 1.0% BA	50 mM NaCl + 1.0 % M13
50 mM NaCl + 0.2 % BA	50 mM NaCl + 0.2 % M13
50 mM NaCl + 0.13 % BA	50 mM NaCl + 0.13 % M13
50 mM NaCl + 0.1 % BA	50 mM NaCl + 0.1 % M13

0 mM NaCl (control)	
75 mM NaCl (control)	
75 mM NaCl + 1.0% BA	75 mM NaCl + 1.0 % M13
75 mM NaCl + 0.2 % BA	75 mM NaCl + 0.2 % M13
75 mM NaCl + 0.13 % BA	75 mM NaCl + 0.13 % M13
75 mM NaCl + 0.1 % BA	75 mM NaCl + 0.1 % M13

5.3.2 Percentage germination and total radicle length

Soybean (cultivar P0962X) and corn (Hybrid 25M75) were used to study the effect of the cell-free supernatant on germination and radicle length, under optimal (0 mM NaCl) and NaCl stressed conditions (50 and 75 mM NaCl). The ability of microbial derived compounds to enhance plant growth varies between and within crop species, hence, more than one crop was studied, to increase the possibility of observing bioactivity. A different experiment was carried out for each crop species and even then, every NaCl level was studied separately. In summary, 3 separate experiments, corresponding to each NaCl level, were conducted per plant species and data for each experiment were analyzed separately. Ten seeds per species were surface sterilized using 2% sodium hypochlorite, for 2 min, rinsed with 5 changes of sterilized distilled water and placed on petri-plates (Cat.no. 431760, sterile 100 × 15 mm polystyrene Petri dish, Fisher Scientific Co., Whitby, ON, Canada), lined with filter article (09-795D, QualitativeP8, porosity coarse, Fisher Scientific Co., Pittsburg, PA, USA). Petri plates with seeds were then randomly applied to the treatments as shown in Table 2, with four replicates per treatment, in a completely randomized design (CRD). The Petri plates were then sealed with parafilm and incubated for 7 days in the dark, at a temperature of 25 ºC. Total number of germinated seeds per plate was recorded at 24 h intervals, for 72 h, as a percentage of the total number of seeds in the plate. i.e., $(\times/10) * 100$. After 7 days, radicle length was measured, in centimeters (cm). For each replicate, radicle

length for all the germinated seeds, was summed, to obtain total length of germinated seeds per plate. Each experiment was repeated twice. Percentage germination data for each time interval (24, 48, and 72 h) were analyzed separately.

5.4 Data analysis

Data from the two sets of experiments were pooled and analyzed using PROC GLM (SAS 9.4). Type III tests were used to determine effects of treatments on seed germination and radicle length while differences between the treatments were assessed using a student t-test with the least square means (LSMEANS) statement, with Tukey's adjustment for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

5.5 Results

5.5.1 0 mM NaCl (Optimal Conditions)

5.5.1.1 Soybean

There were observed variations in the effect of treatments on mean percentage germination and mean radicle length of soybean. Treatment with different concentrations of microbial cell-free supernatant resulted in increase in observed mean radicle length of soybean seedlings. Concentrations 0.2% BA, 0.13% BA, and 0.1% BA resulted in 36.4, 39.7, and 39.91% increases in mean radicle length, respectively, significantly higher than the negative control. Although not significantly different, the concentration 1.0% BA, resulted in mean radicle length higher than that of the negative control by 24.74%. The highest mean radicle length (109.08 cm) was observed in seedlings treated with 0.1% BA, while the lowest (67.35 cm) was observed in seedlings treated with 1.0% M13. Positive controls 0.13 and 0.1% M13 also resulted in radicle lengths significantly longer than the negative control. Treatment 0.2% BA (103.04 cm) was significantly higher than the negative control and its corresponding positive control, 0.2% M13 as shown in Figure 5.1. Table 5.3 shows the effect of treatments on mean radicle length and mean percentage germination of soybean exposed to 0, 50 and 75 mM NaCl, at 72 h.

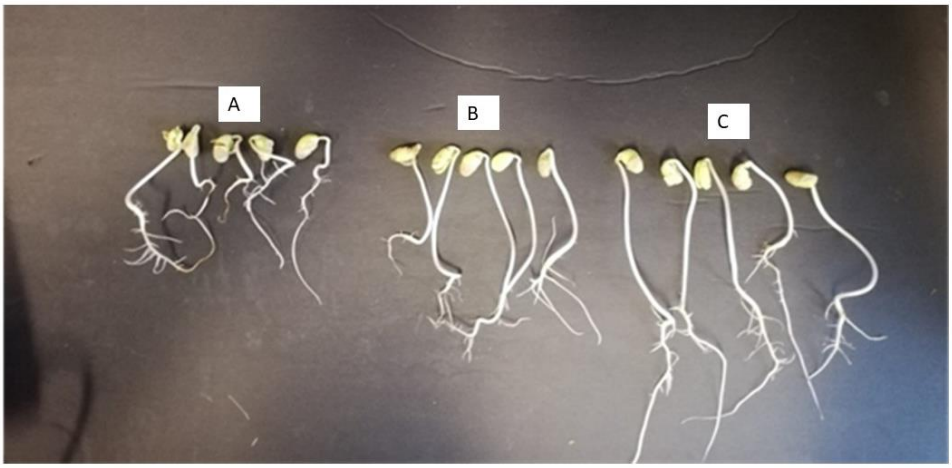

Figure 5.1: Radicle length of soybean seedlings treated with 0 mM NaCl (A), 0 mM NaCl + 0.20% M13 (B), and 0 mM NaCl + 0.2% BA (C).

Treatments had no significant effect on mean percentage germination, at 24, 48, and 72 h. The mean percentage germination of the positive control was highest but not significantly different from that of the other treatments. However, it is worth noting that, at 72 h, the mean germination percentage of soybean treated with BA cell free supernatant was more uniform (91.25, 91.25, 90, and 93.75%,) compared to the positive controls (76.25, 87.5, 93.75, and 78.75%,). A similar observation was also made at both 48 and 24 h. Germination percentage of soybean treated with BA cell free supernatant was higher and more uniform (ranging between 85 and 92.5%), compared to that of soybean treated with M13 media, which ranged between 73.75 and 87.5%, at 48 h. The highest percentage was observed in soybean treated with 0.1% BA while the lowest was observed in soybean treated with 1.0 M13. At 24 h, the highest mean percentage germination (58.75%) was observed in soybean treated with 1.0% BA while the lowest (37.5%) was observed in soybean treated with 0.1% M13. Overall, result suggest that, at 0 mM NaCl, *Bacillus amyloliquefaciens* cell-free supernatant significantly enhances mean radicle length and may enhance uniformity, and higher mean percentage germination, in soybean. Tables 5.4 and 5.5 show the effect of treatments on mean percentage germination of soybean exposed to 0, 50 and 75 mM NaCl, for 48 and 24 h, respectively.

Table 5.3: Effect of treatment on mean radicle length and mean percentage germination of soybean exposed to 0, 50 and 75 mM NaCl, for 72 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	Mean Radicle length (cm)	SEM	Mean % germination	SEM
0 mM NaCl	65.55^{a}	3.658	95^{a}	1.889
0 mM NaCl + 1.0 % BA	87.1^{abc}	2.256	91.25^{a}	7.778
0 mM NaCl + 0.2 % BA	103.04^{bc}	3.314	91.25^{a}	2.500
0 mM NaCl + 0.13 % BA	108.71^{c}	2.866	90^{a}	4.119
0 mM NaCl + 0.1 % BA	109.08^{c}	3.886	93.75^{a}	1.637
0 mM NaCl + 1.0 % M13	67.35^{a}	3.483	76.25^{a}	5.154
0 mM NaCl + 0.2 % M13	69.2375^{a}	4.797	87.5^{a}	4.795
0 mM NaCl + 0.13 % M13	82.525^{bc}	2.679	93.75	2.631
0 mM NaCl + 0.1 % M13	73.625^{bc}	4.834	78.75^{a}	3.508
0 mM NaCl	63.4^{b}	3.128	96.25^{a}	1.829
50 mM NaCl	43.6125^{a}	2.259	88.75^{a}	3.981
50 mM NaCl + 1.0 % BA	52.6625^{ab}	2.747	95^{a}	2.673
50 mM NaCl + 0.2 % BA	57.6^{ab}	3.396	96.25^{a}	1.829
50 mM NaCl + 0.13 % BA	46.1875^{a}	3.269	87.5^{a}	3.659
50 mM NaCl + 0.1 % BA	46.3^{a}	3.286	88.75^{a}	3.504
50 mM NaCl + 1.0 % M13	44.55^{a}	3.448	88.75^{a}	2.950
50 mM NaCl + 0.2 % M13	42.5875^{a}	1.513	90^{a}	3.274
50 mM NaCl + 0.13 % M13	45.325^{ab}	3.876	90^{a}	3.779
50 mM NaCl + 0.1 % M13	42.325^{a}	2.984	91.25^{a}	2.673
0 mM NaCl	57.65^{b}	2.513	96.25^{b}	3.658
75 mM NaCl	43.4125^{ab}	2.358	83.75^{ab}	7.483
75 mM NaCl + 1.0 % BA	47.7875^{ab}	2.759	91.25^{b}	4.797
75 mM NaCl + 0.2 % BA	51.3375^{ab}	2.298	92.5^{b}	2.679
75 mM NaCl + 0.13 % BA	45.9625^{ab}	1.296	91.25^{b}	2.867
75 mM NaCl + 0.1 % BA	42.625^{ab}	1.703	86.25^{ab}	3.659
75 mM NaCl + 1.0 % M13	42.8625^{ab}	0.723	87.5^{ab}	4.256
75 mM NaCl + 0.2 % M13	40.95^{ab}	1.324	66.25^{a}	6.314
75 mM NaCl + 0.13 % M13	39.325^{a}	0.831	86.25^{ab}	2.867
75 mM NaCl + 0.1 % M13	41.8375^{ab}	2.345	88.75^{ab}	6.455

Table 5.4: Effect of treatment on mean percentage germination of soybean exposed to 0, 50 and 75 mM NaCl, for 48 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean % germination**	**± SEM**
0 mM NaCl	86.25[a]	7.055
0 mM NaCl + 1.0 %BA	88.75[a]	5.806
0 mM NaCl + 0.2 % BA	85[a]	5.669
0 mM NaCl + 0.13 % BA	86.25[a]	4.199
0 mM NaCl + 0.1 % BA	92.5[a]	3.659
0 mM NaCl + 1.0 % M13	73.75[a]	8.004
0 mM NaCl + 0.2 % M13	87.5[a]	3.750
0 mM NaCl + 0.13 % M13	87.5[a]	2.266
0 mM NaCl + 0.1 % M13	76.25[a]	5.324
0 mM NaCl	95[a]	1.889
50 mM NaCl	82.5[a]	3.659
50 mM NaCl + 1.0% BA	92.5[a]	3.134
50 mM NaCl + 0.2 % BA	95[a]	2.673
50 mM NaCl + 0.13 % BA	83.75[a]	4.978
50mM NaCl + 0.1 % BA	85[a]	3.273
50 mM NaCl + 1. 0 % M13	82.5[a]	5.590
50 mM NaCl + 0.2 % M13	87.5[a]	3.659
50 mM NaCl + 0.13 % M13	85[a]	3.273
50 mM NaCl + 0.1 % M13	83.75[a]	5.324
0 mM NaCl	93.75[a]	2.500
75 mM NaCl	80[a]	5.957
75 mM NaCl + 1.0 % BA	83.75[a]	3.779
75 mM NaCl + 0.2 % BA	87.5[a]	2.631
75 mM NaCl + 0.13 % BA	83.75[a]	3.504
75 mM NaCl + 0.1 % BA	78.75[a]	3.981
75 mM NaCl + 1.0 % M13	81.25[a]	6.105
75 mM NaCl + 0.2 % M13	83.75[a]	5.261
75 mM NaCl + 0.13 % M13	82.5[a]	2.673
75 mM NaCl + 0.1 % M13	77.5[a]	2.500

Table 5.5: Effect of treatment on mean percentage germination of soybean exposed to 0, 50 and 75 mM NaCl, for 24 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean % germination**	**± SEM**
0 mM NaCl	55[a]	8.018
0 mM NaCl + 1.0 % BA	58.75[a]	10.928
0 mM NaCl + 0.2 % BA	56.25[a]	9.246
0 mM NaCl + 0.13 % BA	50[a]	9.636
0 mM NaCl + 0.1 % BA	57.5[a]	5.590
0 mM NaCl + 1.0 % M13	46.25[a]	8.647
0 mM NaCl + 0.2 % M13	45[a]	7.791
0 mM NaCl + 0.13 % M13	52.5[a]	6.748
0 mM NaCl + 0.1 % M13	37.5[a]	8.606
0 mM NaCl	48.75[c]	3.504
50 mM NaCl	23.75[ab]	2.631
50 mM NaCl + 1.0 % BA	46.25[c]	6.250
50 mM NaCl + 0.2 % BA	17.5[a]	2.500
50 mM NaCl + 0.13 % BA	17.5[a]	3.134
50 mM NaCl + 0.1 % BA	37.5[bc]	5.261
50 mM NaCl + 1.0 % M13	28.75[ab]	2.266
50 mM NaCl + 0.13 % M13	18.75[a]	2.266
50 mM NaCl + 0.13 % M13	18.75[a]	5.154
50 mM NaCl + 0.1 % M13	16.25[a]	1.829
0 mM NaCl	56.25[c]	3.239
75 mM NaCl	26.25[ab]	4.978
75 mM NaCl + 1.0 % BA	32.5[bc]	7.258
75 mM NaCl + 0.2 % BA	21.25[ab]	2.266
75 mM NaCl + 0.13 % BA	20[ab]	2.673
75 mM NaCl + 0.1 % BA	28.75[ab]	4.795
75 mM NaCl + 1.0 % M13	21.25[ab]	3.981
75 mM NaCl + 0.2 % M13	15[a]	1.889
75 mM NaCl + 0.13 % M13	11.25[a]	2.266
75 mM NaCl + 0.1 % M13	17.5[a]	2.500

5.5.1.2 Corn

There was an observed significant difference in the effect of the treatments on mean radicle length of corn. None of the treatments raised mean radicle length to levels significantly higher than the negative control. However, treatment with 0.1% BA resulted in radicles longer than the control by 5.36%. Interestingly, treatment with 1.0% M13 lowered mean radicle length by 15.4875%, significantly lower than the negative control. Radicle length of corn treated with 1.0% BA was significantly lower than that of corn treated with 0.1% BA by 25.95%, suggesting that the lower the concentration of the cell free supernatant, the more effective it is at enhancing radicle length in corn. Note that the radicle length of corn treated with 0.13% BA (58.2875 cm) was higher than that of corn treated with 1.0% BA and lower than that of corn treated with 0.1% BA. Overall, there was no significant effect of microbial cell-free supernatant on mean radicle length of corn. Table 5.6 shows the effect of treatment on mean radicle length and mean percentage germination of corn exposed to 0, 50 and 75 mM NaCl, for 72 h.

Table 5.6: Effect of treatment on mean radicle length and mean percentage germination of corn exposed to 0, 50 and 75 mM NaCl, for 72 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean Radicle length (cm)**	**± SEM**	**Mean % germination**	**± SEM**
0 mM NaCl	56.6375^{bcd}	2.365	97.5^{ab}	1.637
0 mM NaCl + 1.0 % BA	45.9125^{abc}	1.185	96.25^{ab}	1.829
0 mM NaCl + 0.2 % BA	$52.6a^{bcd}$	4.204	97.5^{ab}	1.637
0 mM NaCl + 0.13 % BA	58.2875^{cd}	3.083	100^{b}	0.000
0 mM NaCl + 0.1 % BA	62^{d}	2.053	95^{ab}	1.889
0 mM NaCl + 1.0 % M13	41.15^{a}	2.903	98.75^{ab}	1.251
0 mM NaCl + 0.2 % M13	43.8625^{ab}	1.78	96.25^{ab}	1.829
0 mM NaCl + 0.13 % M13	$53.425a^{bcd}$	3.276	100^{b}	0.000
0 mM NaCl + 0.1 % M13	46.4375^{abc}	2.958	91.25^{a}	2.950
0 Mm NaCl	48.725^{d}	2.994	97.5^{a}	1.637
50 mM NaCl	33.3125^{ab}	1.704	93.75^{a}	3.239
50 mM NaCl + 1.0 % BA	38.6625^{abc}	1.913	98.75^{a}	1.25

50 mM NaCl + 0.2 % BA	43.675^{cd}	2.049	96.25^{a}	1.829
50 mM NaCl + 0.13 % BA	49.7125^{d}	1.019	96.25^{a}	1.829
50 mM NaCl + 0.1 % BA	37.25^{abc}	2.247	100^{a}	0.00
50 mM NaCl + 1.0 % M13	30.7125^{ab}	2.911	96.25^{a}	1.829
50 mM NaCl + 0.2 % M13	37.025^{abc}	1.819	100^{a}	0.00
50 mM NaCl + 0.13 % M13	40.5625^{bcd}	1.321	93.75^{a}	1.829
50 mM NaCl + 0.1 % M13	33.55^{ab}	1.236	95^{a}	2.673
0 Mm NaCl	58.5375^{b}	3.585	93.75^{ab}	1.829
75 mM NaCl	37.75^{a}	4.378	92.5^{ab}	2.500
75 mM NaCl + 1.0 % BA	40.4625^{ab}	4.769	100^{b}	0.000
75 mM NaCl + 0.2 % BA	30.2875^{a}	5.500	98.75^{b}	1.250
75 mM NaCl + 0.13 % BA	39.8875^{ab}	5.019	100^{b}	0.000
75 mM NaCl + 0.1 % BA	47.825^{ab}	3.122	98.75^{b}	1.250
75 mM NaCl + 0.13 % M13	39.075^{a}	4.869	85^{a}	5.976
75 mM NaCl + 1.0 % M13	37.5125^{a}	3.609	86.25^{a}	1.829
75 mM NaCl + 0.1 % M13	34.1625^{a}	5.229	92.5^{ab}	3.134
75 mM NaCl + 0.2 % M13	30.4625^{a}	3.159	98.75^{b}	1.250

The effect of treatments on mean percentage germination varied with time. At 72 h, there was no observed significant difference in the effect of treatments on mean percentage germination of corn. The highest mean percentage germination (100%) was observed in corn treated with 0.13% BA and 0.13% M13, while the lowest was observed in corn treated with 0.1% M13. At 48 h, the effect of treatments on mean percentage germination was not significantly different. The highest mean percentage germination was 96.25%, observed in corn treated with 0.13% M13 while the lowest was 86.25%, observed in corn treated with 0.2% M13. Table 5.7 shows the effect of treatment on mean percentage germination of corn exposed to 0, 50 and 75 mM NaCl, for 48 h.

Table 5.7: Effect of treatment on mean percentage germination of corn exposed to 0, 50 and 75 mM NaCl, for 48 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean % germination**	**± SEM**
0 mM NaCl	93.75^{ab}	2.631
0 mM NaCl + 1.0 % BA	91.25^{ab}	1.25
0 mM NaCl + 0.2 % BA	95^{ab}	1.889
0 mM NaCl + 0.13 % BA	95^{ab}	1.889
0 mM NaCl + 0.1 % BA	93.75^{ab}	2.631
0 mM NaCl + 1.0 % M13	93.75^{ab}	2.631
0 mM NaCl + 0.2 % M13	86.25^{a}	2.631
0 mM NaCl + 0.13 % M13	96.25^{b}	1.829
0 mM NaCl + 0.1 % M13	90^{ab}	2.673
0 mM NaCl	92.5^{a}	2.500
50 mM NaCl	86.25^{a}	4.605
50 mM NaCl + 1.0 % BA	95^{a}	2.673
50 mM NaCl + 0.2 % BA	93.75^{a}	1.829
50 mM NaCl + 0.13 % BA	92.5^{a}	2.500
50 mM NaCl + 0.1 % BA	98.75^{a}	1.250
50 mM NaCl + 1.0 % M13	91.25^{a}	2.950
50 mM NaCl + 0.2 % M13	96.25^{a}	2.631
50 mM NaCl + 0.13 % M13	88.75^{a}	3.504
50 mM NaCl + 0.1 % M13	90^{a}	3.779
0 mM NaCl	81.25^{ab}	2.266
75 mM NaCl	70^{a}	3.273
75 mM NaCl + 1.0 % BA	85^{ab}	3.273
75 mM NaCl + 0.2 % BA	93.75^{b}	2.631
75 mM NaCl + 0.13 % BA	93.75^{b}	3.239
75 mM NaCl + 0.1 % BA	93.75^{b}	1.829
75 mM NaCl + 1.0 % M13	77.5^{ab}	5.59
75 mM NaCl + 0.2 % M13	76.25^{a}	3.75
75 mM NaCl + 0.13 % M13	81.25^{ab}	4.407
75 mM NaCl + 0.1 % M13	82.5^{ab}	4.532

At 24 h, significant differences were observed in the effect of treatments on mean percentage germination. Corn treated with 1.0% BA, 0.2% BA, and 0.13% BA enhanced mean percentage germination by 36.842, 46.66, and 48.94%, respectively. The resulting mean percentage germinations were significantly higher than the control. Likewise, corn treated with 0.2% M3, 0.13% M13, and 0.1% M13 exhibited mean percentage germination significantly higher than the control by 47.83, 47.83, and 36.842%, respectively. The highest mean percentage germination was 58.75%, observed in corn treated with 0.13% BA while the lowest was 33.75%, observed in corn treated with 1.0% M13. Table 5.8 shows effect of treatments on mean percentage germination of corn exposed to 0 and 50 mM NaCl, for 24 h. Figure 5.2 below shows the effect BA cell-free supernatant on mean percentage germination of corn and soybean, at 24 h while Figure 5.3 shows the effect of BA cell-free supernatant on the mean radicle length (cm) of corn and soybean under optimal conditions.

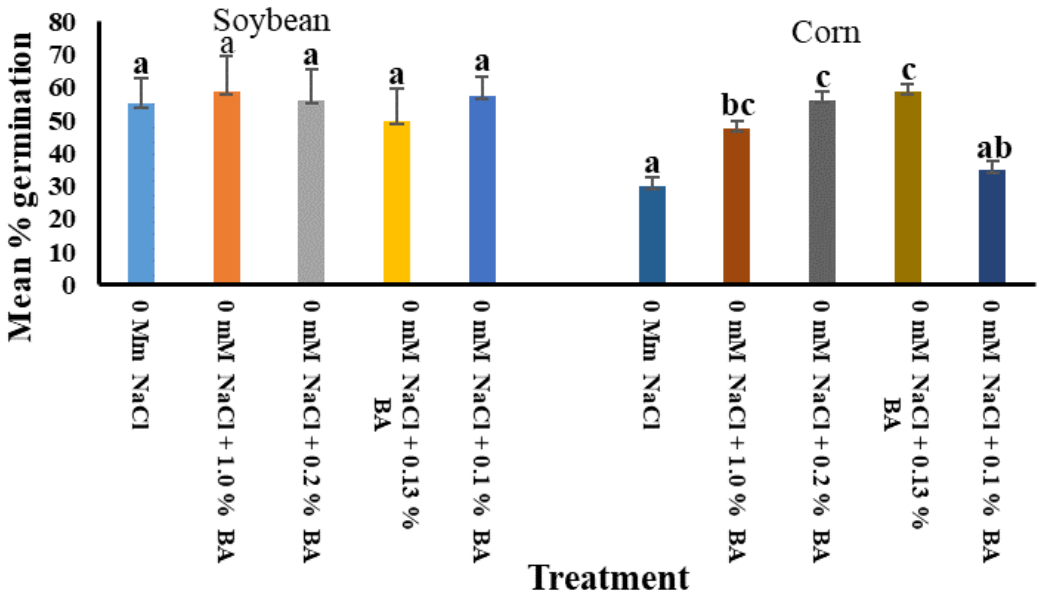

Figure 2.2: Effect of treatment on the mean percentage germination of corn and soybean, at 24 h, under optimal conditions. Different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

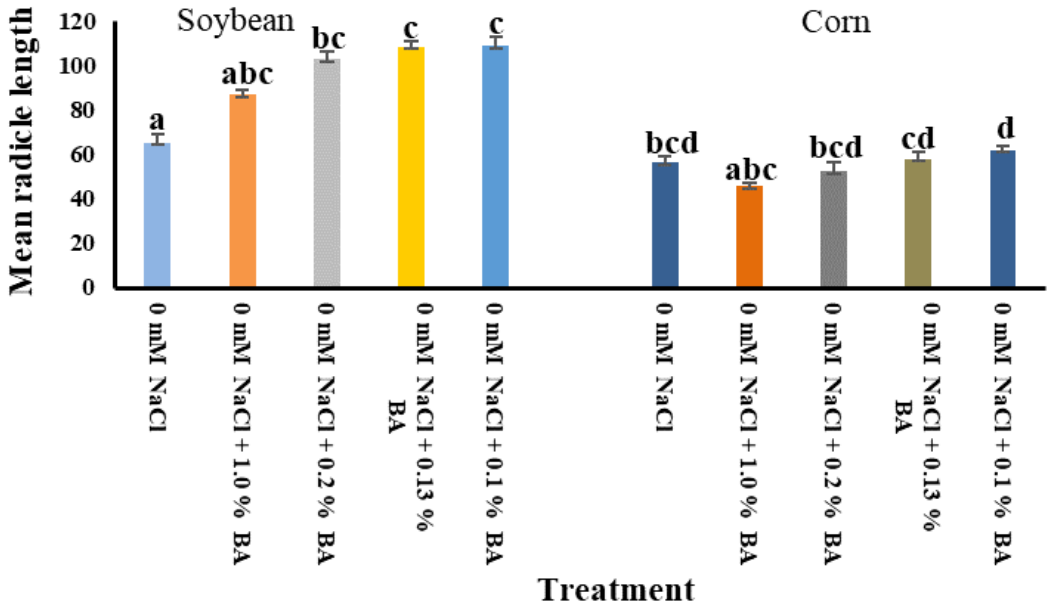

Figure 2.3: Effect of treatment on the mean radicle length (cm) of soybean and corn under optimal conditions. different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

5.5.2 50 mM NaCl

5.5.2.1 Corn

There were observed significant differences in the effect of treatments on mean radicle length of corn. Treatment with 50 mM NaCl significantly lowered mean radicle length by 31.63%, compared to mean radicle length of corn at 0 mM NaCl. However, treatment with concentrations of 0.13% BA and 0.2% BA significantly enhanced mean radicle lengths by 32.99 and 23.73%, to lengths not significantly different from that of the 0 mM control. This implies that, the two BA concentrations may have mitigated the limitations of 50 mM NaCl on corn radicle growth, hence, enhancing radicle length growth. Other treatments also enhanced radicle length but, to lengths not significantly different from that on the 50 mM control, although, the effect of 0.13% M13 was also not significantly different from that of the 0 mM control (Figure 5.8).

The effect of treatments on mean percentage germination varied across time intervals. At 72 h, there was no observed significant difference in the effect of treatments on mean percentage germination. In fact, the effect of all treatments on mean percentage germination was not significantly different from that of the 0 mM NaCl control. The highest mean percentage germination (100%) was observed in corn treated with 0.1% BA and 0.2% M13, while the lowest was observed in corn treated with 0.13%

M13 and 50 mM NaCl. Results suggest that germination of corn was tolerant to 50 mM NaCl. At 48 h, still no significant difference was observed in the effect of treatments on mean percentage germination, although treatment with 0.1% BA resulted in a rise in mean percentage germination, from 86.56% observed at 50 mM NaCl, to 98.75%, the highest observed at 48 h. In fact, the mean percentage germination of all treatments was higher than that of the 50 mM NaCl control. This suggests a possibility that the treatments may have had an effect, but it was not significant because of the already high percentages of the control. At 24 h, there were observed significant differences in the effect of treatments on mean percentage germination. Like at 72 and 48 h, there was no significant difference in the mean percentage germination at 0 mM NaCl and 50 mM NaCl, although the latter resulted in a 23.81% decrease in mean percentage germination. Treatment with 0.13% M13, 0.1% M13, and 1.0% BA resulted in 18.75, 21.2, and 21.25% mean percentage germination, respectively, significantly lower than that of the 50 mM control.

Table 5.8: Effect of treatment on mean percentage germination of corn exposed to 0 and 50 mM NaCl, for 24 h. Data represents the mean ± SE; in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean % germination**	**± SEM**
0 mM NaCl	30^{a}	2.673
0 mM NaCl + 1.0 % BA	47.5^{bc}	2.500
0 mM NaCl + 0.2 % BA	56.25^{c}	2.631
0 mM NaCl + 0.13 % BA	58.75^{c}	2.266
0 mM NaCl + 0.1 % BA	35^{ab}	2.673
0 mM NaCl + 1.0 % M13	33.75^{ab}	2.631
0 mM NaCl + 0.2 % M13	57.5^{c}	4.532
0 mM NaCl + 0.13 % M13	57.5^{c}	2.500
0 mM NaCl + 0.1 % M13	47.5^{bc}	4.196
0 mM NaCl	52.5^{e}	2.500
50 mM NaCl	40^{cde}	1.889
50 mM NaCl + 1.0 % BA	21.25^{ab}	2.795
50 mM NaCl + 0.2 % BA	45^{de}	2.629

50 mM NaCl + 0.13 % BA	26.25[abc]	3.239
50 mM NaCl + 0.1 % BA	52.5[e]	3.134
50 mM NaCl + 1.0 % M13	13.75[a]	3.239
50 mM NaCl + 0.2 % M13	30[bcd]	2.673
50 mM NaCl + 0.13 % M13	18.75[ab]	3.504
50 mM NaCl + 0.1 % M13	21.2[ab]	3.504

5.5.2.2 Soybean

50 mM NaCl lowered mean radicle length of soybean by 19.79%, in comparison to mean radicle length of soybean grown under optimal conditions. At 50 mM NaCl, there was no observed significant difference in the effect of treatments on the mean radicle length of soybean. The highest mean radicle length, 57.67 cm, was observed in soybean treated with 0.2% BA, while the least, 42.35 cm, was observed in soybean treated with 0.1% M13. Although the effect of all treatments was not significantly different from the negative control (50 mM NaCl), microbial cell-free supernatant concentrations of 1.0% BA (52.67 cm) and 0.2% BA, resulted in mean radicle length that was not significantly different from that of soybean grown under optimal conditions (63.4 cm). The effect of treatments on mean percentage germination varied across time. At 72 h, there was no observed significant difference in the effect of treatments on mean percentage germination. There was an observed 7.5% decline in the mean percentage germination at 50 mM NaCl, compared to 0 mM NaCl. The mean percentage germination of all treatments was not significantly different from that of soybean grown under normal conditions. The highest mean percentage germination (96.25%) was observed in soybean treated with 0 mM NaCl and 0.2% BA while the lowest (87.5%) was observed in soybean treated with 0.13% M13. At 48 h, there was no significant difference in the effect of treatments on mean percentage germination. At 50 mM NaCl, the observed mean germination percentage was 12.5% lower than the mean percentage germination at 0 mM NaCl. The observed mean percentage germination of soybean treated by all the treatments was not significantly different from that of the 0 mM control. The highest mean percentage germination was observed in soybean treated with 0.2% BA (95%) while the lowest was observed in soybean treated with 1.0% M13 (82.5%). At 24 h, there was an observed difference in the effect of treatments on mean percentage germination. 50 mM NaCl decreased mean percentage germination of soybean by 51.28%, compared to mean germination percentage at 0 mM

NaCl. Treatment with 1.0% BA increased mean percentage germination by 48.64%, significantly higher than that of 50 mM NaCl and not significantly different from that of 0 mM NaCl. Observed mean percentage germination of soybean treated with 0.1% BA, was also noticeably high (37.5%), not significantly different from that observed at 0 mM NaCl. The least mean germination percentage was observed in soybean treated with 0.1% M13. Figure 5.4 below shows the effect of BA cell-free supernatant on mean percentage germination of corn and soybean exposed to 50 mM NaCl for 24 h. Figure 5.5 shows the effect BA cell-free supernatant on mean radicle length of corn and soybean exposed to 50 mM NaCl.

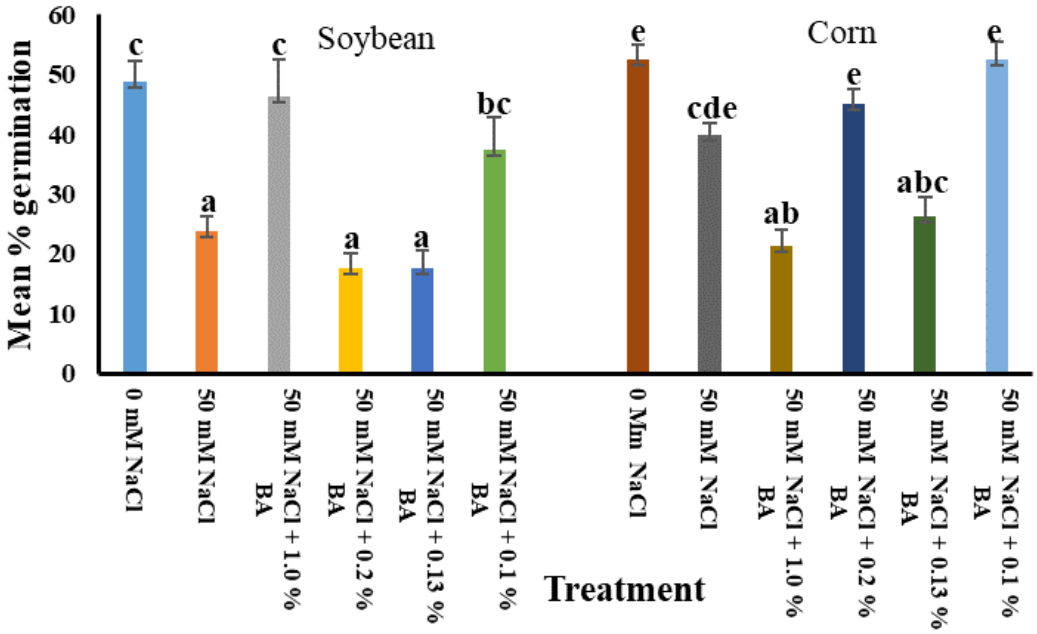

Figure 5.4: Effect of BA cell-free supernatant on mean percentage germination of corn and soybean exposed to 50 mM NaCl for 24 h. Different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

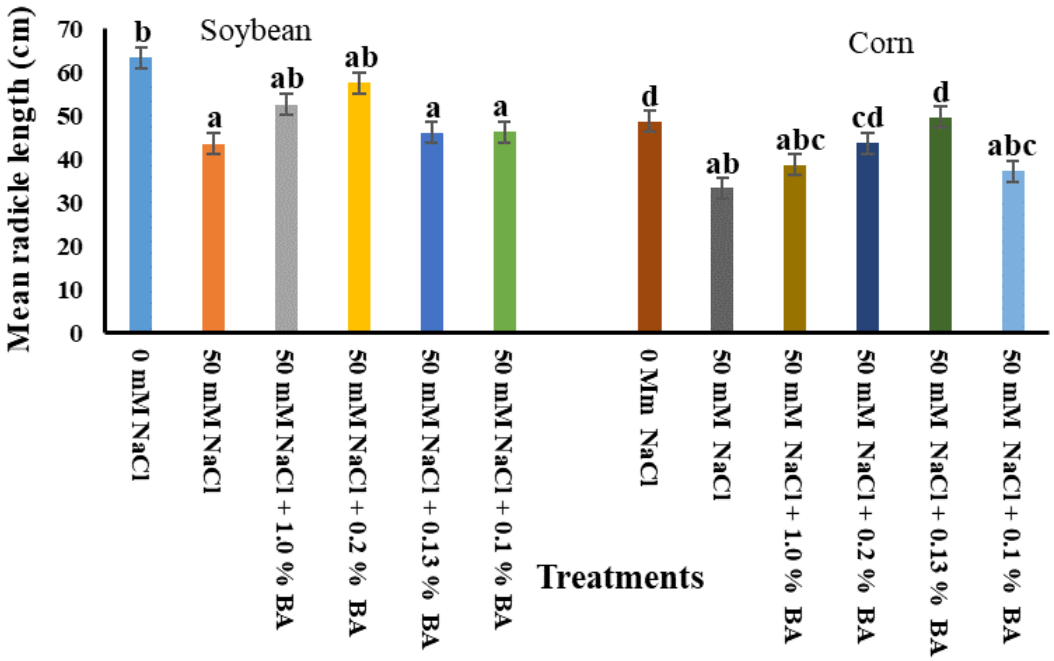

Figure 5.5: Effect of BA cell-free supernatant on mean radicle length of corn and soybean exposed to 50 mM NaCl. Different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

5.5.3 75 mM NaCl

5.5.3.1 Corn

There was an observed significant difference in the effect of treatments on mean radicle length of corn. 75 mM NaCl significantly lowered mean radicle length by 35.51%, compared to the control. Radicle length of corn treated with both BA and M13 cell-free supernatant was not significantly different from that of the 75 mM NaCl. However, 1.0% BA, 0.13% BA, and 0.1% BA increased mean radicle length to levels not significantly different from the 0 mM NaCl control. The highest mean radicle length was observed in the 0 mM control while the lowest was observed in corn treated with 0.2% BA (30.29 cm).

There was a significant difference in the effect of treatments on mean percentage germination. At 72 h, there was no significant effect of 75 mM NaCl on mean percentage germination of corn. Mean percentage germination of corn treated with 1.0% BA (100%), 0.2% BA (98.75%), 0.13% BA (100%), and 0.1% BA (98.75%) were significantly higher than that of corn treated with 1.0% M13 (85%) and 0.2% M13 (86.25%). At 48 h, 75 mM NaCl lowered mean percentage germination by 13.46%.

Overall, BA cell-free supernatant enhanced mean percentage germination. Mean percentage germination of corn treated with 0.2% BA, 0.13% BA, and 0.1% BA was significantly higher than that of 75 mM NaCl by 25.33%. The three also exhibited the highest observed mean percentage germination. The lowest was observed in corn treated with 0.13% M13 (81.25%). At 24 h, there was no observed germination at 75 mM NaCl, even to corn treated with BA and M13 cell-free supernatant.

5.5.3.2 Soybean

There was an observed difference in the effect of treatments on mean radicle length of soybean. 75 mM NaCl lowered mean radicle length by 24.7% although the difference was not significant. Mean radicle length of soybean treated with 0.13% M13 was significantly lower than the 0 mM NaCl control. Other treatments were not significantly different from 0 mM and 75 Mm NaCl.

The effect on treatments on mean percentage germination varied across time. At 72 h, there was an observed significant difference in the effect of the treatments on mean percentage germination. It was lowered by 12.98 % at 75 mM NaCl, compared to mean percentage germination under optimal conditions, although the difference was not significant. Mean percentage germination of soybean treated with 0.2% M13 was significantly lower than that of the 0 mM control. The two exhibited the lowest and highest mean percentage germination, respectively. At 48 h, mean percentage germination was reduced by 14.67% at 75 mM NaCl, compared to the mean percentage germination of soybean grown under optimal conditions (93.75%), which was also the highest. There was no significant difference in the effect of the treatments on mean percentage germination. The lowest mean percentage germination (95.75%) was observed in soybean treated with 0.1% M13. At 24 h, mean percentage germination was significantly reduced at 75 mM NaCl, by 53.3% compared to the mean percentage germination of soybean under optimal conditions. There was no significant difference between mean percentage germination of soybean treated with microbial derived compounds and the 75 mM NaCl control. However, the mean percentage germination of soybean treated with 1.0% BA was also not significantly different from that observed under optimal conditions. Mean percentage germination of soybean treated with M13 except 1.0% M13, was significantly lower than the 75 mM control. The lowest mean percentage germination was observed in soybean treated with 0.13% M13. Figure 5.6

below shows the effect of BA cell-free supernatant on corn and soybean exposed to 75 mM NaCl for 72 h. Figure 5.7 shows the effect of BA cell-free supernatant on mean radicle length of corn and soybean exposed to 75 mM NaCl.

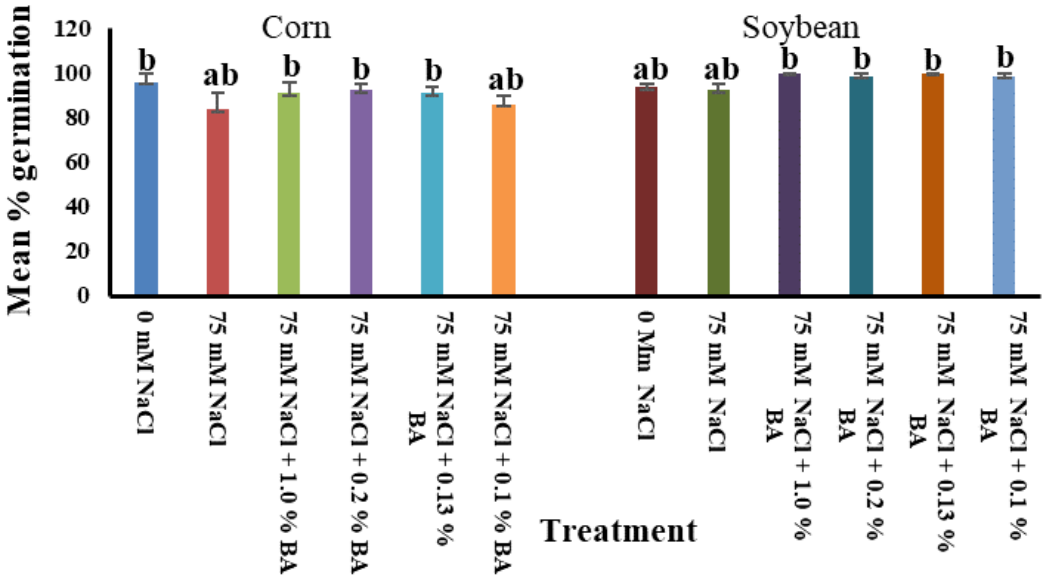

Figure 5.6: Effect of BA cell-free supernatant on mean percentage germination of corn and soybean exposed to 75 mM NaCl for 72 h. different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

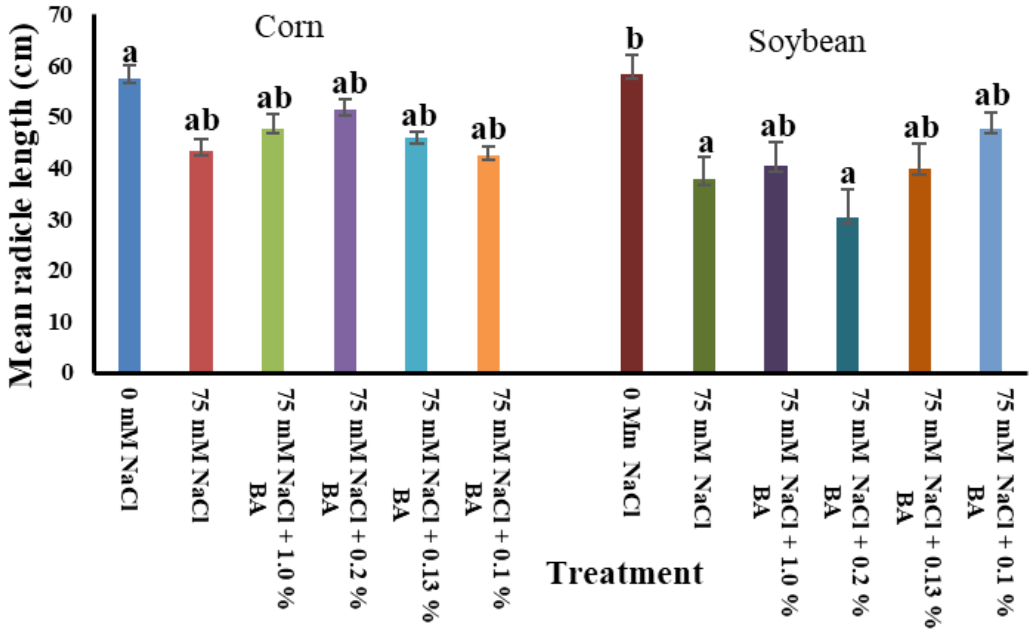

Figure 5.7: Effect of BA cell-free supernatant on mean radicle length of corn and soybean exposed to 75 mM NaCl. Different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

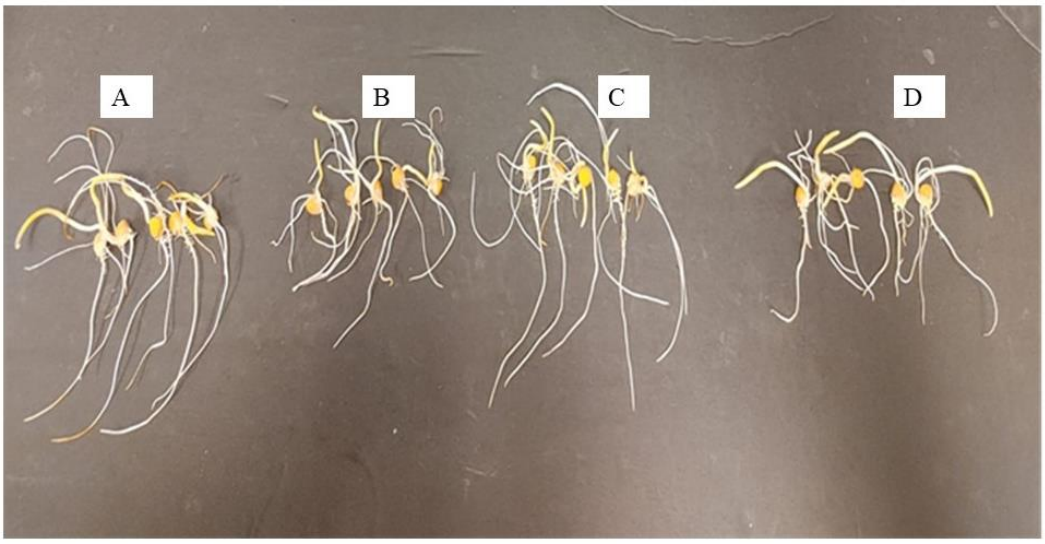

Figure 5.8: Radicle length of corn with 0 mM NaCl (A), 50 mM NaCl (B), 50 mM NaCl + 0.13% BA (C), and 50 mM NaCl + 0.13% M13 (D).

5.6 Discussion

Seed germination and root establishment are essential stages of plant growth and development, which when compromised, may have a significant effect on overall quantity and quality of crop yield (Rifna et al., 2019). A high and uniform germination percentage is a sought-after trait by seed companies and farmers. There are several seed and soil related factors that could affect seed germination and root establishment, even under supposedly ideal conditions (Rifna et al., 2019). Delay in seed germination may increase susceptibility of seedlings to soil borne pathogens, decrease plant vigor, delay plant maturity and lower crop yield (Schwinghamer et al., 2015). The radicle grows into the primary root/tap which anchors the seedling in the soil. A poorly developed radicle may negatively influence plant establishment and water uptake. Therefore, a technology to enhance seed germination and seedling establishment is essential. Use of PGPM and their derived compounds is an environmentally sustainable approach that enhances seed germination and plant growth. In this study, *Bacillus amyloliquefaciens* EB2003A's cell free supernatant enhanced mean percentage germination and mean radicle length of corn (*Zea mays*) and soybean (*Glycine max* L. merill), under optimal

and NaCl stressed conditions. There are no previous studies on the effect of BA cell-free supernatant on plant growth. However, previous studies have shown that compounds derived from cell-free supernatant of other PGPM enhance seed germination and plant growth. Subramanian et al. (2016) observed an increase in germination of soybean treated with thuricin 17 and lipo-chitooligosaccharide (LCO). The two compounds were isolated from cell-free supernatant of *Bacillus thuringiensis* NEB17 and *Bradyrhizobium japonicum* 532C, respectively. Souleimanov et al. (2002) also observed an increase in root length of soybean seedlings treated with LCO. *Bacillus amyloliquefaciens* strains have been reported to mitigate salt effects in different plants through production of volatile organic compounds (Chen et al., 2016; Cappellari and Banchio, 2020; Liu et al., 2020). For instance, Liu et al. (2020) observed a significant increase in the biomass and maintenance of chlorophyll content of *Arabidopsis thaliana* plants treated with *Bacillus amyloliquefaciens* FZB42 VOCs, compared to the control. Chen et al. (2016) observed increases in chlorophyll content, production of antioxidant enzymes and K^+/Na^+ ratio, in 100 mM NaCl stressed corn plants exposed to *Bacillus amyloliquefaciens* SQR9 VOCs. The same strain also enhanced salt tolerance through production of spermidine (Chen et al., 2017). In rice, tested *Bacillus amyloliquefaciens* strains enhanced salt tolerance through production of phytohormones such as auxins, abscisic acid, and gibberellic acid (Shahzad et al., 2016, 2017). All these findings are evidence that, indeed some *Bacillus amyloliquefaciens* strains can produce substances which mitigate salt stress in a range of crop species. In this study, there were observed variations in the effect of the cell-free supernatant on mean percentage germination and mean radicle length, at different NaCl levels, cell free supernatant concentration and plant species. Previous studies have reported that indeed the effectiveness of a given microbial derived compound may vary across plant species, concentration of compound, soil conditions and different biotic and abiotic stresses (Naamala and Smith, 2021a).

5.6.1 Effect of Cell-Free Supernatant Concentration Level

In this study, BA cell-free supernatant enhanced mean radicle length and mean percentage germination, under optimal and NaCl stressed conditions. There were variations observed in the effect of the different concentrations on both mean percentage germination and mean radicle length of corn and soybean. It is not surprising

that such observations were made, given the fact that the bioactivity of the cell-free supernatant is presumed to be due to compounds exuded by *Bacillus amyloliquefaciens*, in the growth media. Majority of such microbial derived compounds are signaling molecules such as phytohormones, whose concentration has been for long known to either enhance or inhibit plant growth (Lyu et al., 2020; Antar et al., 2021; Naamala and Smith, 2021a). Some compounds will enhance plant growth at very low concentrations while others will be required in relatively high concentrations for positive significant effects to be observed. The same observation was made in this study, where at a given concentration, mean radicle length and or mean percentage germination were significantly enhanced, significantly lowered or no effect observed. For instance, in soybean, results of mean radicle length at optimal conditions showed an increase in mean radicle length of soybean as concentration of the cell-free supernatant reduced from 1.0% BA to 0.1% BA. Results on mean radicle length in corn, under optimal conditions seem to suggest the same as concentration 1.0% BA exhibited a significantly lower radicle length than that of 0.1% BA. In a different scenario, at 75 mM NaCl, concentration 1.0% BA significantly enhanced mean percentage germination while 0.2% BA, 0.13%, and 0.1% BA did not, at 24 h. However, the effect of 0.1% BA was not significantly different from that of 1.0% BA. Whether it is the same potential compound inducing these significant effects is a question that cannot be answered at this stage of the study. Overall, our results suggest that concentration plays a significant role in the effectiveness of the cell-free supernatant. The findings are in line with previous studies which also showed that effectiveness of some microbe-derived compounds can be dependent on concentration of the compound (Schwinghamer et al., 2015; Gautam et al., 2016). In their study on canola, Schwinghamer et al., observed a variation in the effect of LCO concentration on germination. A concentration of 10^{-6} enhanced early canola germination while a concentration of 10^{-9} delayed germination but enhanced uniformity in germination (Schwinghamer et al., 2015). Souleimanov et al. (2002) studied the effect of different concentrations of LCO on soybean and observed differences in the effect of different concentrations on soybean growth. Concentration of 10^{-7} to 10^{-9} enhanced soybean growth while concentrations of 10^{-11} did not (Souleimanov et al., 2002). A thuricin 17 concentration of 10^{-9} enhanced soybean germination at 100 mM NaCl, while a concentration of 10^{-11} did not (Subramanian et al., 2016).

5.6.2 Effect of NaCl Level

In this study, the role NaCl level played on seed germination and radicle development cannot be ignored. NaCl (75 mM NaCl and 50 mM NaCl) lowered mean percentage and mean radicle length of both soybean and corn, in some cases, significantly, in others not. Salinity stress is a major global abiotic stress that affects crops, at all stages of development, including seed germination, radicle length and general plant growth (Subramanian et al., 2016; Ilangumaran et al., 2021). As little as 0.1 M NaCl caused visible reductions in plant height in some corn cultivars, compared to corn grown under optimal conditions (Farooq et al., 2015). For soybean, high salt concentrations affect germination (Kondetti et al., 2012), early growth and the nitrogen fixation process (Zahran, 1997; Zaharan, 1999; Egamberdieva and Lugtenberg, 2014). Microbial derived compounds have been reported to enhance plant growth under saline conditions (Naamala and Smith, 2021b). In this study, the effect of BA cell-free supernatant on mean radicle length and mean percentage germination varied at different NaCl levels, in corn and soybean. In soybean, BA cell-free supernatant was more effective at 0 mM NaCl, and not 50 and 75 mM NaCl. In corn however, significant results were observed at 50 and 75 mM NaCl, but not 0 mM NaCl. It should also be noted that effectiveness was higher at 50 mM NaCl than at 75 mM NaCl. For instance, in corn, concentrations 0.2% BA and 0.13% resulted in a significantly higher mean radicle length at 50 mM NaCl while at 75 mM NaCl the effect of the two concentrations was not significantly different from that of the 75 mM control. In fact, at 75 mM NaCl, mean radicle length reduced with the decrease in BA cell free supernatant. Previous studies have reported that level of stress plays a significant role in the effect of microbial derived compounds on growth of different plants species, in some being effective under stressed conditions, while in others under optimal conditions, yet, in others at both. Subramanian and co observed that thuricin 17 and LCO enhanced germination in soybean under NaCl stressed and optimal conditions (Subramanian et al., 2016a). Canola subjected to low temperature stress responded to treatment with LCO while that grown under optimal temperature conditions did not (Schwinghamer et al., 2015). Results also seem to suggest that higher NaCl levels require higher cell-free supernatant concentrations to have a chance at effectiveness. For instance, at 0 mM NaCl, in soybean and corn, lower concentrations seemed to be more effective at enhancing radicle length than, higher concentrations. However, the reverse is true at both 50 and 75 mM NaCl. For instance, at 50 mM NaCl, radicle length of soybean treated with the

four BA concentrations was not significantly different from that of 50 mM NaCl, but radicle length of 1.0% BA and 0.2% BA was also not significantly different from that of the 0 mM NaCl control. The mean percentage germination was also higher in soybean treated with the two concentrations. In corn, at 75 mM NaCl, mean radicle length reduced with the decrease in BA cell free supernatant. However, at 50 mM NaCl, 0.2% BA and 0.13% induced higher than the control. Plants have a stress defend system, that is activated when a plant is exposed to stress. Perhaps, at lower concentrations, the plant stress defensive system can mitigate a great deal of NaCl stress, requiring only a little boost from the microbial derived cell-free supernatant, to exhibit significant effects on plant growth. However, it should be noted that as stress builds up in and around the plant, some signals involved in the plant defensive system, such as ethylene can also accumulate to levels toxic to the plant. In addition to that, high stress levels can damage the plant proteins and nucleic acids, perhaps, to levels that even microbial derived compounds cannot rehabilitate, hence, the effect on plant growth processes such as germination and radicle length. Subramanian and co observed that a concentration of 10^{-7} LCO enhanced soybean germination at 100 mM and not at 150, 175, and 200 mM NaCl (Subramanian et al., 2016a). Previous reports show that PGPM and PGPM derived compounds enhance plant growth under saline conditions by mitigating the effect of salt on the plant, through employing one of the following mechanisms: upregulation of the plant's defense system through induced systemic resistance or production of antioxidants that degrade ROS (Amna et al., 2019). Production of exopolysaccharides which can act to improve water holding capacity and bind Na^+ hence, lowering osmotic and ionic stress in plants (Tewari and Arora, 2014; Shrivastava and Kumar, 2015; Amna et al., 2019). Production of ACC deaminase which breaks down ethylene, there by lowering its concentration in plant tissue to levels less toxic (Hayat et al., 2010; Nautiyal et al., 2013; Amna et al., 2019).

5.6.3 Effect of Plant Species

In this study, there were variations in the effect of treatments on soybean and corn. For instance, at optimal conditions, soybean radicle length was more responsive to treatments while at 50 mM NaCl, corn radicle length was more responsive. The effect of microbial derived compounds on plants growth has been reported to vary between and within plant species. For example, LCO was reported to exhibit varying effects on canola varieties, enhancing growth in cultivar polo and not the other cultivars under

study (Schwinghamer et al., 2015). Souleimanov et al. (2002) observed varying effect of LCO on corn and soybean. In soybean, significant effects on root length and root dry weight were observed while in corn, significant increases in shoot dry weight were observed. This study supports findings of these two studies. Corn and soybean have different structural and genetic components which may explain the differences in their response to treatments. Also, the two crops may respond to different microbial signals differently, some being compatible and others not. It is possible that a single potential bioactive compound causes both effects in corn and soybean. It is also possible that the bioactive compound which caused the effect in soybean is different from what caused effects in corn.

5.7 Conclusion

The cell-free supernatant obtained from salt tolerant *Bacillus amyloliquefaciens* EB2003A strain exposed to 200 mM NaCl, enhanced germination and radicle length of corn, under NaCl stressed and optimal conditions. The effect of treatments varied across plant species, concentration of BA cell free-supernatant and NaCl level. Results of the study suggest that that *Bacillus amyloliquefaciens* EB2003A produces in its growth media, bioactive compound(s), with ability to enhance mean radicle length and mean percentage germination in corn and soybean. The identity and quantity of the potential compound are yet to be known. Findings of this study can be used as a baseline to further study the cell-free supernatant for isolation and identification of potential compounds with ability to enhance plant growth, independently.

5.8 References

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5.9 CONNECTING TEXT

In chapter four, *Lactobacillus helveticus* EL2006H, following exposure to 200 mM NaCl expressed proteins that are yet to be associated with enhancement of plant growth. However, other than proteins, microbes exude other substances such as metabolites, which can enhance plant growth under stressed and non-stressed conditions. Therefore, it is possible that the cell-free supernatant of *L. helveticus* EL2006H contains such metabolites, that could influence plant growth. The next chapter, six, aimed at understanding whether the strain's CFS, after exposure to 200 mM NaCl could enhance growth of plants exposed to NaCl stress.

Chapter 6 *Lactobacillus helveticus* EL2006H Cell-Free Supernatant Enhances Growth Variables in *Zea mays* (maize), *Glycine max* L. Merill (soybean) and *Solanum tuberosum* (potato) exposed to NaCl stress.

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6.1 Abstract

Plant growth promoting microorganisms and their derived compounds, such as cell-free supernatant (CFS), enhance plant growth under stressed and non stressed conditions. Such technology is sustainable and environmentally friendly, which is desirable amidst the climate change threat. The current study evaluated the effect of CFS obtained from *Lactobacillus helveticus* EL2006H on its ability to enhance mean percentage germination and mean radicle length of corn and soybean, as well as growth variables of potato, using treatment formulations that consisted of 0.2 and 1.0% [v/v] *L. helveticus* EL2006H CFS concentrations and 100 mM NaCl and 150 mM NaCl levels. Results show that treatment with 100 mM NaCl lowered percentage germination of corn by 52.63%, at 72 h, and soybean by 50%, at 48 h. Treatment with 100 NaCl + 0.2% EL2006H enhanced percentage germination of soybean by 44.37%, at 48 h, in comparison to that of the 100 mM NaCl control. One hundred mM NaCl lowered radicle length of corn and soybean by 38.58 and 36.43%, respectively. Treatment with 100 Mm NaCl + 1.0% EL2006H significantly increased radicle length of corn by 23.04%. Treatment with 100 mM NaCl + 0.2% EL2006H significantly increased photosynthetic rate, leaf greenness and fresh weight of potato. Increasing NaCl

concentration to 150 NaCl lowered the effectiveness of the 0.2% EL2006H CFS on the same growth variables of potato. In general, the lower CFS concentration of 0.2% was more efficient at enhancing germination in soybean while the higher concentration of 1.0% was more efficient at enhancing radicle length of corn. There was an observed variation in the effectiveness of *L. helveticus* EL2006H CFS across the different CFS concentrations, NaCl levels and crop species studied. In conclusion, based on findings of this study, CFS obtained from *L. helveticus* can be used as a bio stimulant to enhance growth of corn, soybean, and potato. However, further studies need to be conducted, for validation, especially under field conditions, for commercial application.

6.2 Introduction

Plant growth promoting microorganisms (PGPM) live in close association with their host plants, forming a holobiont (Hartmann et al., 2014; Lyu et al., 2021); these relationships have existed for at least half a billion years (Knack et al., 2015). A plant's exudates into its surroundings are a major determinant of the phytomicrobiome composition in its rhizosphere (Zhang et al., 2017). The association between PGPM and their host plants can enhance the latter's growth and development, through mechanisms such as biostimulation, mitigation of abiotic stress effects, bioremediation, and biocontrol (Glick, 2012; Ahemad and Kibret, 2014; Backer et al., 2018; Naamala and Smith, 2020), in a sustainable and environmentally friendly manner (Naamala and Smith, 2021a,b). PGPM and their derived compounds can be utilised in singular or consortium forms, results varying in such a way that some strains may be more effective when applied as single cells while others, in a consortium (Giassi et al., 2016; Subramanian et al., 2016a,b; Shah et al., 2022). *Lactobacillus helveticus* is a gram positive facultative anaerobic lactic acid bacterium (LAB) that is mostly known for its role in the food processing industry. Use of *L. helveticus* in plant agriculture, especially as biostimulants is not widely documented although the use of members of the genera *Lactobacillus* in crop production, as biostimulants and biocontrol agents, among other uses, has been practiced (Hamed et al., 2011). Members of the genus *Lactobacillus* are endophytic to a variety of plants species (Baffoni et al., 2015; Minervini et al., 2015; Lamont et al., 2017) while others have been isolated from the rhizosphere of plants. Examples of LAB species that have been used in plant agriculture include *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *L. helveticus*

(Hamed et al., 2011; Caballero et al., 2020; Msimbira et al., 2022). LAB play an important role in fermentation of organic matter to form organic fertilisers used in crop production (Lamont et al., 2017; Caballero et al., 2020). Lactic acid, a by-product of LAB has been reported to enhance plant growth (Rodríguez-Morgado et al., 2017). LAB strains have been reported to solubilise phosphate, produce siderophores (Shrestha et al., 2014; Giassi et al., 2016), produce antimicrobial compounds (Stoyanova et al., 2012), produce phytohormones such as indole −3- acetic acid [IAA] (Shrestha et al., 2014; Giassi et al., 2016) and enhance systemic acquired resistance (Hamed et al., 2011), all of which are desirable characteristics of PGPM.

Microbes, such as bacteria and fungi, exude into their growth environment, secondary metabolites such as hormones, enzymes, organic acids, bacteriocins, oligopolysaccharides and siderophores, among others (Piechulla et al., 2017; Schulz-Bohm et al., 2017; Lemfack et al., 2018). In laboratory-based experiments, such metabolites are exuded into the microbe's growth medium. Recent studies have focused on the possibility that such metabolite rich media, also known as cell-free supernatant (CFS) can enhance plant growth without the presence of microbial cells. Experimentation with CFSs has previously yielded interesting results, showing that such CFSs can enhance the growth of different crop species, such as corn, soybean, canola, and tomato, at germination and seedling stages (Msimbira et al., 2022; Naamala et al., 2022, Shah et al., 2022). Earlier studies went further and extracted bioactive compounds from the CFS, resulting in the discovery of compounds such as lipo-chitooligosaccharide (LCO) from *Bradyrhizobium japonicum* CFS and thuricin17 from *Bacillus thuringiensis* NEB17 CFS (Prithiviraj et al., 2003; Gray et al., 2006a,b; Lee et al., 2009), which are already on the market as plant growth promoting biostimulants. Both compounds have shown efficacy in enhancing plant growth under growth chamber and field conditions (Subramanian et al., 2016a,b; Arunachalam et al., 2018). They were reported to enhance plant growth under normal and salt-stress conditions (Subramanian et al., 2016a,b). It has been reported that the nature of metabolites exuded vary with varying conditions of growth media in which the microbe is growing (Subramanian et al., 2021). For instance, the metabolic profile of CFS of a microbe grown under ideal conditions may significantly vary from that of the same microbe exposed to some level of stress, such as low pH or high salinity levels. Not all metabolites exuded in growth media enhance plant growth, although some are the bioactive ingredients that do so. The effectiveness of a bioactive metabolite/CFS also

varies across plant species, concentration of the compound, level of stress to which a plant is exposed and growth stage of the plant (Msimbira et al., 2022; Naamala et al., 2022; Shah et al., 2022). This, in a sense, complicates the process of discovering novel microbe derived plant growth promoting compounds as it may require trials on several crop species under different growth conditions, at different concentrations, before bioactivity may be detected. The seemingly long and possibly complicated process is however potentially worth the effort since microbial derived compounds can overcome some issues associated with using microbial cells as inoculants if they are produced in fairly large quantities and are economical to isolate for application. For instance, compounds are less prone to diminished effects under harsh field conditions, are generally required in low concentrations and are easier to store compared to live microbial cells (Naamala and Smith, 2021a).

Salinity stress is a major abiotic stress of agricultural crops, resulting in decreased yield quantity and quality which subsequently causes economic losses estimated at US$ 12 billion. per year (FAO, 2020). It affects leaf area, chlorophyll content, plant vigour, plant height, root length, plant dry matter, nutrient, metabolite, and protein contents, can delay plant development and at severe stress levels may lead to plant death (Bistgani et al., 2019; Garcia et al., 2019). Unfortunately, with current climate change projections, reduced rainfall, excess and improper application of inorganic fertilisers and other chemicals, arable land affected by salinity stress is projected to increase by 50% by 2050 (Jamil et al., 2011). PGPM and their derived compounds can mitigate the effect of salt stress on plants, hence, allowing better growth, yield quality and yield quantity, in salt affected fields (Naamala and Smith, 2021b). The aim of this study therefore was to elucidate the ability of *L. helveticus* EL2006H CFS to enhance germination and radicle length of corn and soybean, and growth parameters of potato, under saline conditions. Results of the study will be a baseline for further studies, with a possibility of isolating and identifying bioactive compounds. This study is part of a broader study that is studying CFSs of the EVL Inc., consortium strains, which comprises *L. helveticus* EL2006H and four other microbial species, to improve the product and or come up with new product combinations.

6.3 Materials and Methods

6.3.1 Obtaining microbial CFS

L. helveticus EL2006H was cultured in De man, Rogosa and Sharpe (MRS) medium at pH 7.0, and incubated for 48 h, at 120 rpm and 37°C. At 48 h, the microbial culture was centrifuged for 10 min, at 10,000 rpm and 4°C, to pellet the microbial cells and separate them from the CFS (Gray et al., 2006a,b; Subramanian et al., 2021). The CFS was further filtered using 0.22 µm nylon filters to remove any microbial cells that could have remained after centrifugation. The obtained CFS was then used in the formulation of treatments used in the study.

6.3.2 Formulation of treatments

Treatments were formulated by mixing known quantities of distilled water, NaCl and CFS. Two NaCl levels, (100 mM NaCl and 150 mM NaCl) and two CFS levels (1.0 and 0.20% [v/v]) were used in the mixtures to formulate treatments. The two CFS concentrations were chosen because they exhibited positive results with *Bacillus amyloliquefaciens* EB2003 CFS, in our previous study (Naamala et al., 2022). 0, 100 and 150 mM NaCl, with no addition of CFS were used as negative controls. In addition, for each microbial CFS concentration, a similar concentration of microbial growth medium (not inoculated with microbe), was used to formulate positive controls. A treatment name ending in MRS or EL2006H implies that MRS medium and *L. helveticus* EL2006H CFSs were used, respectively.

6.3.3 Set up of germination and radicle length experiments

The germination experiments were carried out in a phytorium located at the Macdonald Campus of McGill University, Sainte Anne de Bellevue, Quebec, Canada. Soybean (cultivar P0962X) and corn (Hybrid 25 M75) were used for the study. The two crop species were chosen because they are widely consumed in Canada and the world over. In our previous study, the two species' germination and radicle lengths were stimulated by *B. amyloliquefaciens* CFS. The following treatments were used for the study: 0 mM NaCl (control), 100 mM NaCl (control), 100 mM NaCl +0.2% MRS (control), 100 mM NaCl +0.2% EL2006H, 100 mM NaCl +1.0% MRS (control) and 100 mM NaCl +1.0% EL2006H. Treatments with 0.2% EL2006H and 1.0% EL2006H, with their corresponding controls, were studied separately. It should also be noted that

each crop was studied separately. Therefore, because each treatment, within each crop species, was studied in a separate experiment and the data obtained analysed separately, a completely randomised design (CRD) was used for each experiment, to randomly apply experimental units to treatments. For each experiment, ten seeds of the crop species under study were surface sterilized using 2% sodium hypochlorite, for 2 min, rinsed with 5 changes of sterilized distilled water and placed on petri-plates (Cat. no. 431760, sterile 100 × 15 mm polystyrene Petri dish, Fisher Scientific Co., Whitby, ON, Canada), lined with filter paper (09-795D, QualitativeP8, porosity coarse, Fisher Scientific Co., Pittsburg, PA, United States). Petri plates with seeds then randomly received the treatments with ten replicates per treatment, hence, 40 samples per experiment. The Petri plates were then sealed with parafilm and incubated for 7 days in the dark, at 25°C. Total number of germinated seeds per plate was recorded at 24 h intervals, for 72 h, as a percentage of the total number of seeds in the plate. i.e., $(x/10)*100$, where X is the total number of germinated seeds per petri plate. After 7 days, radicle length was measured, in centimeters (cm). For each replicate, radicle length for all the germinated seeds, was summed, to obtain total radicle length of germinated seeds per plate. Each experiment was repeated twice. Percentage germination data for each time interval (24, 48, and 72 h) were analyzed separately.

6.3.4 Set up of greenhouse experiment

Potato cultivar goldrush was used for the study. Potato is grown and widely consumed in Canada, with a sizable fresh market area of production in Quebec. EVL Inc., the source of the bacterial strains in collaboration with SynAgri, focus on cultivation of potato cultivars. Hence this part of the study was focused on potato's response to treatment with the CFS, under greenhouse conditions. Treatments used for this experiment were: 0 mM NaCl (control), 100 mM NaCl (control), 100 mM NaCl + 0.2% MRS (control), 100 mM NaCl + 0.2% EL2006H, 150 mM NaCl (control), 150 mM NaCl + 0.2% MRS (control) and 150 mM NaCl + 0.2% EL2006H. Twelve L pots were filled with G7 growth medium were used for plant growth. The rooting medium in each pot was fully saturated with water before sowing one potato seed per pot. At emergence, pots were allocated to treatments following a CRD, with four replicates per treatment, hence, a total of 28 samples. The experiment was repeated twice. A number of excess pots were sown with seed so that on the day of treatment application, only

pots with seeds that emerged on the same day were applied to treatments, to minimize initial variation. Two L of treatment were applied twice a week, per pot, for 4 weeks after emergence, at which time harvesting was conducted. Data on variables: greenness, photosynthetic rate, leaf area, plant height and plant fresh weight were taken. Leaf greenness was measured in SPAD units, using a SPAD-502 chlorophyll meter at 3 weeks after treatment application. Greenness of ten leaves was randomly measured and average greenness recorded. Photosynthetic rate was measured in μmol CO_2 m^{-2} S^{-1} using a LI-COR 6400 portable photosynthesis meter (Lincoln, NE, United States of America), and recorded, 3 weeks after treatment application. Plant height was measured using a meter ruler, first, at emergence, just before the first application of treatments and 3 weeks after emergence. The difference in height was then recorded. Fresh weight was measured in grams, using a weighing scale balance (ME4001E, CH), 4 weeks after emergence. Leaf area was measured in cm^2, using a leaf area meter (LI-3100 C, Lincoln, NE, United States of America), 4 weeks after emergence.

6.4 Data analysis

Data obtained from all samples were analyzed using PROC GLM (SAS 9.4 software). Type III tests were used to determine effects of treatments on seed germination and radicle length while differences between the treatments were assessed using a student t-test with the least square means (LSMEANS) statement, with Tukey's adjustment for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

6.5 Results

6.5.1 Mean radicle length

6.5.1.1 Corn

6.5.1.1.1 100 mM NaCl + 1.0% EL2006H

There was a significant effect of *L. helveticus* CFS on radicle length of corn, as shown in Figure 6.1. One hundred mM NaCl significantly lowered radicle length of corn by 38.58% ($p < 0.0001$) in comparison to the 0 mM NaCl control. Treatment with 100 mM NaCl +1.0% EL2006H significantly increased mean radicle length of corn by 23.04% ($p < 0.0001$). The greatest radicle length was for the 0 mM control (55.38 cm) while the smallest was for the 100 mM NaCl control (35.205 cm), which was also not

significantly different from the 100 mM NaCl +1.0% MRS control (36.735 cm). There was no significant effect of the 0.2% *L. helveticus* CFS on radicle length of corn.

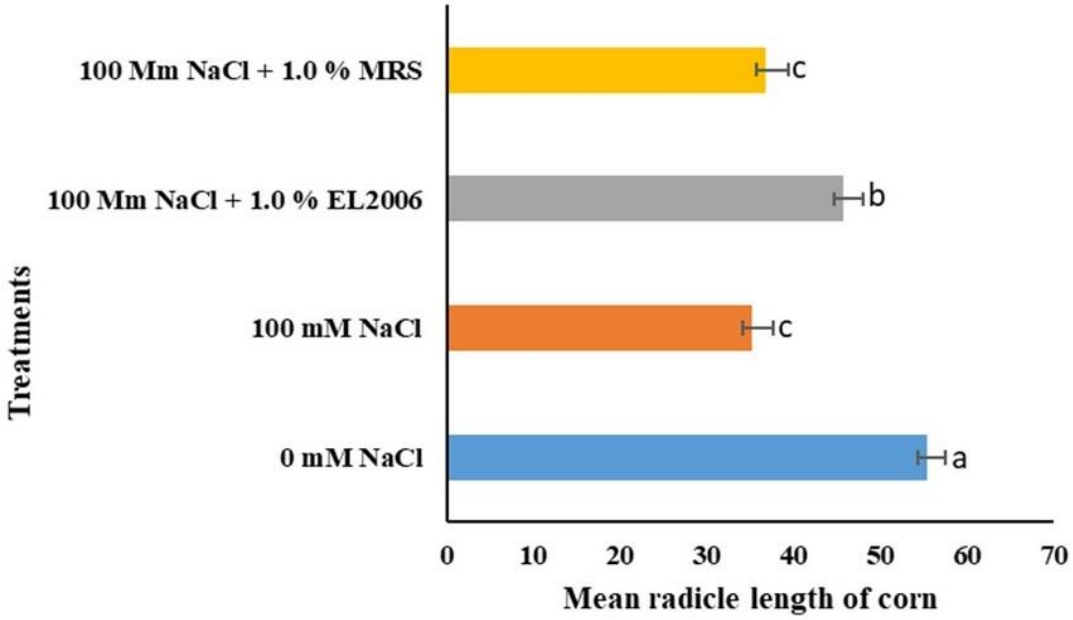

Figure 6.1: Effect of treatments on radicle length of corn at 1.0% [v/v]. Data represents the mean ± SE (n = 80); different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

6.5.1.2 Soybean

There was no significant effect of *L. helveticus* CFS on radicle length of soybean at both 0.2 and 1.0% concentrations, as shown in Table 6.1. Figure 6.2 shows the effect of treatments on radicle length of corn (1) and soybean (2).

Table 6.1: Effect of treatments on mean radicle length of soybean and corn. Data represents the mean ± SE (n=80); in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	Mean radicle length of soybean (cm)	Mean radicle length of corn (cm)
0 mM NaCl	57.98 ± 2.636[a]	49.81 ± 1.879[a]

100 mM NaCl	37.43 ±1.766[b]	35.15 ±1.232[b]
100 Mm NaCl + 0.2% EL2006	38.05 ±1.773[b]	38.76 ±1.193[b]
100 Mm NaCl + 0.2% MRS	37.12 ±2.088[b]	40.26 ±1.705[b]

0 mM NaCl	63.355 ± 4. 230[a]	55.38 ± 2.084[a]
100 mM NaCl	38.910 ± 2.213[b]	35.205 ± 2.491[c]
100 Mm NaCl + 1.0 % EL2006	41.195 ± 2.393[b]	45.745 ± 2.233[b]
100 Mm NaCl + 1.0 % MRS	44.445 ± 3.600[b]	36.735 ± 2.66[c]

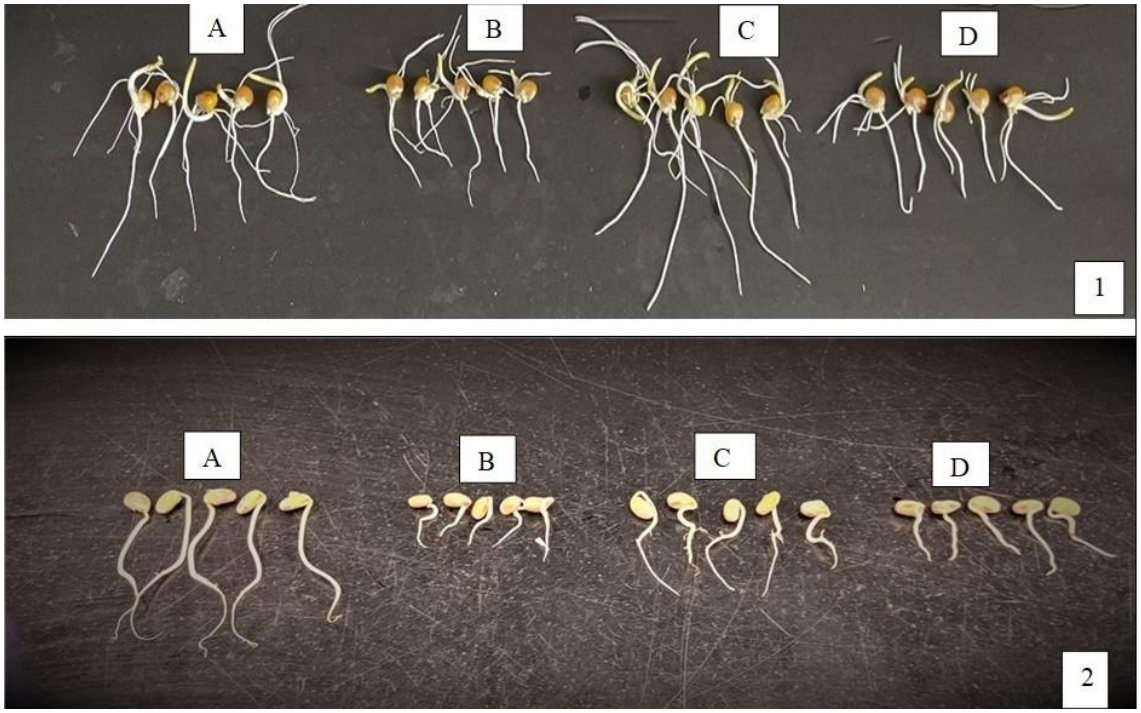

Figure 6.2: Effect of treatments on germination and radicle length of corn (1) and soybean (2). From left to right: 0 mM NaCl (A), 100 mM NaCl (B), 100 mM NaCl +0.2% EL2006H (C) and 100 mM NaCl +0.2% MRS (D).

6.5.2 Mean percentage germination

6.5.2.1 Soybean

6.5.2.1.1 100 mM NaCl +0.2% EL2006

At 24 h, there was a significant effect of *L. helveticus* EL2006H CFS on soybean germination. At 48 h, CFS significantly enhanced percentage germination of soybean. Treatment with 100 mM NaCl lowered percentage germination by 50% in comparison

to the 0 mM NaCl control. The highest percentage germination was observed in the 0 mM NaCl control (84%) while the lowest was observed in soybean treated with 100 mM NaCl (42%). Percentage germination of soybean treated with 100 mM NaCl +0.2% EL2006H and 100 mM NaCl +0.2% MRS were 75.5 and 67.5%, respectively. The two were significantly higher than that observed for the 100 Mm NaCl control ($p < 0.0001$). In fact, the percentage germination of soybean treated with 100 mM NaCl +0.2% EL2006H CFS was significantly higher than that of the 100 mM NaCl control, by 44.37% ($p < 0.0001$) when treated with CFS and not different from that of the 0 mM NaCl control.

At 72 h, treatment with microbial CFS did not result in significant differences in the germination of soybean, when compared to the 100 mM NaCl control. However, treatments 100 mM NaCl +0.2% EL2006H increased percentage germination by 16.1, to a percentage not significantly different from the 0 mM NaCl control. Table 6.2 show the effect of treatments on percentage germination of soybean (Figure 6.3).

Table 6.2: Effect of treatments on mean percentage germination of soybean, at 72, 48 and 24 h, respectively. Data represents the mean ± SE (n=80); in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean % germination at 72 h ± SEM**	**Mean % germination at 48 h ± SEM**	**Mean % germination at 24 h ± SEM**
0 mM NaCl	89.5 ± 1.697[a]	86.0 ± 1.835[a]	34.5 ± 2.563[**b**]
100 mM NaCl	82.5 ± 3.898[**a**]	66.0 ± 4.834[b]	1.00 ± 0.688[**a**]
100 mM NaCl + 1.0 % EL2006	88.5 ± 3.647[a]	70.5 ± 5.452[ab]	0.50 ± 0.500[**a**]
100 mM NaCl + 1.0% MRS	85.0 ± 3.591[**a**]	74 ± 4.995[**ab**]	1.00 ± 0.688[**a**]

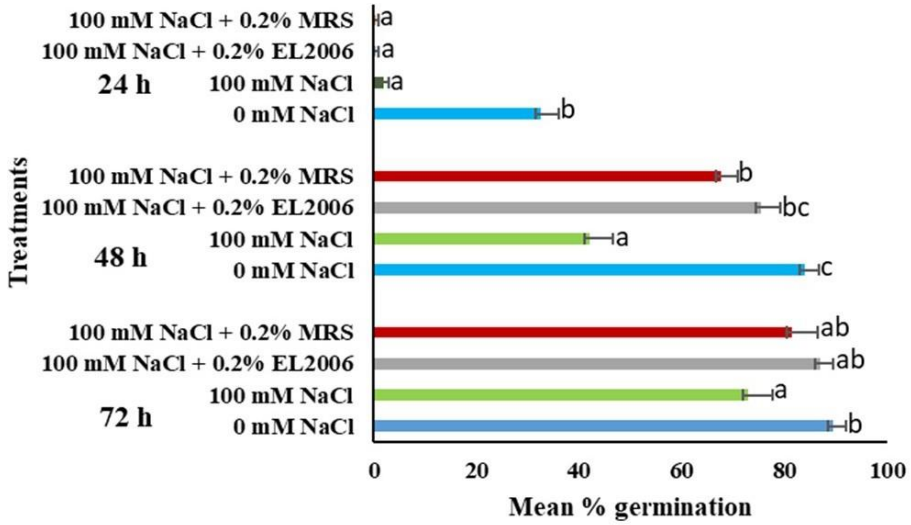

Figure 6.3: Effect of treatments on mean percentage germination of soybean. Data represents mean ± SE (n = 80); different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

6.5.2.1.2 100 mM NaCl +1.0% EL2006

At 24 h, microbial CFS had not significant effect on germination of soybean. At 48 h, soybean treated with 100 mM NaCl +1.0% EL2006H and 100 mM NaCl +1.0% MRS exhibited percentages of 70.5 and 74%, respectively; both values were not significantly different from that of the 0 mM control, and higher, though not significantly different from the percentage germination observed for the 100 mM NaCl control. At 72 h there was no observed significant difference among the percentage germination of the different treatments.

6.5.2.2 Corn

There was no significant effect of *L. helveticus* CFS on germination percentage of corn at both 0.2 and 1.0% concentrations, as shown in Tables 6.3, 6.4.

Table 6.3: Effect of treatments on mean percentage germination of corn at 72, 48 and 24 h, respectively. Data represents the mean ± SE (n=80); in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean percentage germination at 72 h ± SEM**	**Mean percentage germination at 48 h ± SEM**	**Mean percentage germination at 24 h ± SEM**
0 mM NaCl	57.0 ± 3.332[a]	16.0 ± 2.938[a]	0.00 ± 0.000[a]
100 mM NaCl	27.0 ± 4.110[**b**]	3.5 ± 1.817[b]	0.00 ± 0.000[**a**]
100 mM NaCl + 0.2 % EL2006	28.5 ± 3.789[**b**]	4.0 ± 1.338[b]	0.00 ± 0.000[**a**]
100 mM NaCl + 0.2 % MRS	32.0 ± 3.742[**b**]	0.00 ± 0[**b**]	0.00 ± 0.000[**a**]

Table 6.4: Effect of treatments on mean percentage germination of corn at 72, 48 and 24 h, respectively. Data represents the mean ± SE (n=80); in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Mean percentage germination at 72 h ± SEM**	**Mean percentage germination at 48 h ± SEM**	**Mean percentage germination at 24 h ± SEM**
0 mM NaCl	71.5 ± 5.144[a]	15.5 ± 1.846[a]	0.00 ± 0.000[a]
100 mM NaCl	30.0 ± 3.770[b]	1.0 ± 0.688[b]	0.00 ± 0.000[**a**]
100 mM NaCl + 1.0 % EL2006	34.5 ± 3.515[b]	1.5 ± 0.819[b]	0.00 ± 0.000[**a**]
100 mM NaCl + 1.0% MRS	26.5 ± 3.185[b]	2.0 ± 0.918[**b**]	0.00 ± 0.000[**a**]

6.5.3 Greenhouse experiment

While the above germination experiments were to establish the effects of *L. helveticus* CFS on seed/tuber germination as a possible positive plant growth promoter, the greenhouse experiment with potato was to add more value to the commercial application of the CFS, which is one of the goals of SynAgri/EVL's mandate and the crop of importance. Hence, experiments were carried out using potato variety goldrush

(as per company's recommendation), to elucidate the effect of *L. helveticus* EL2006H CFS on the growth variables of potato. Results regarding variables varied between treatments.

6.5.3.1 Mean leaf greenness

The effect of treatments on mean leaf greenness was significantly different among treatments. Treatment with 100 mM and 150 mM NaCl resulted in 27.014 and 19.88% decreases in mean leaf greenness, respectively, in comparison to the 0 mM NaCl control, the two becoming significantly lower than the later ($p < 0.0001$). Treatment with 100 mM NaCl +0.2% EL2006 resulted in leaf greenness significantly higher than the 100 mM NaCl control by 13.56% ($p < 0.0001$) and not significantly different from the 0 mM NaCl control, as shown in Figure 6.4. Although not significantly different, it was also higher than the 100 mM NaCl +0.2% MRS control. There was no significant difference between treatment 150 mM NaCl +0.2% EL2006 and its corresponding controls 150 mM NaCl and 150 mM NaCl +0.2% MRS. *L. helveticus* EL2006H CFS enhanced leaf greenness in potato treated with 100 mM NaCl but not 150 mM NaCl.

6.5.3.2 Mean photosynthetic rate

There was a significant effect of *L. helveticus* CFS on mean photosynthetic rate. as shown in Figure 6.4. Treatment with 100 and 150 mM NaCl resulted in 13.76 and 26.6% decreases in photosynthetic rate, respectively, in comparison to the 0 mM NaCl control. Treatment with 100 mM NaCl +0.2% EL2006 resulted in the highest photosynthetic rate (21.00 μmol CO_2 m^{-2} S^{-1}), significantly higher than that of the 100 mM NaCl control, by 17.97% ($p < 0.0001$), and higher but not significantly different from the 0 mM NaCl control, as shown in Figure 6.4. The lowest photosynthetic rate was observed in potato treated with 150 mM NaCl (14.863 μmol CO_2 m^{-2} S^{-1}).

6.5.3.3 Mean fresh weight

There was a significant effect of *L. helveticus* CFS on fresh weight of potato. One hundred mM NaCl and 150 mM NaCl lowered fresh weight of potato by 19.62 and 23.61%, respectively, in comparison to the fresh weight observed for the 0 mM NaCl control. Treatment with 100 mM NaCl +0.2% EL2006 resulted in potato with fresh

weight higher but not significantly different from the 0 mM control. It was also significantly higher than the fresh weight of potato treated with 100 mM NaCl +0.2% MRS control (Figure 6.5). There was no significant effect of *L. helveticus* CFS on leaf area and plant height of potato, as shown in Table 6.5.

Table 6.5: Effect of treatments on selected growth variables of potato. Data represents the mean ± SE (n=48); in a column, different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$. Values with the same letters are not significantly different at $p < 0.05$.

Treatment	**Height gain (cm)**	**Leaf area (cm^2)**
0 mM NaCl	37.275±7.531[a]	2851.261±158.736[a]
100 mM NaCl	32.263±5.739[a]	2951.652±180.931[a]
100 mM NaCl + 0.2% EL2006	34.838±6.937[a]	3358.082±156.557[a]
100 mM NaCl + 0.2% MRS	33.113±6.129[a]	2943.797±234.763[a]
150 mM NaCl	38.9±2.282[a]	3045.75±157.209[a]
150 mM NaCl + 0.2% EL2006	40.825±1.723[a]	2960.841±87.894[a]
150 mM NaCl + 0.2% MRS	40.063±2.233[a]	3076.615± 211.951[a]

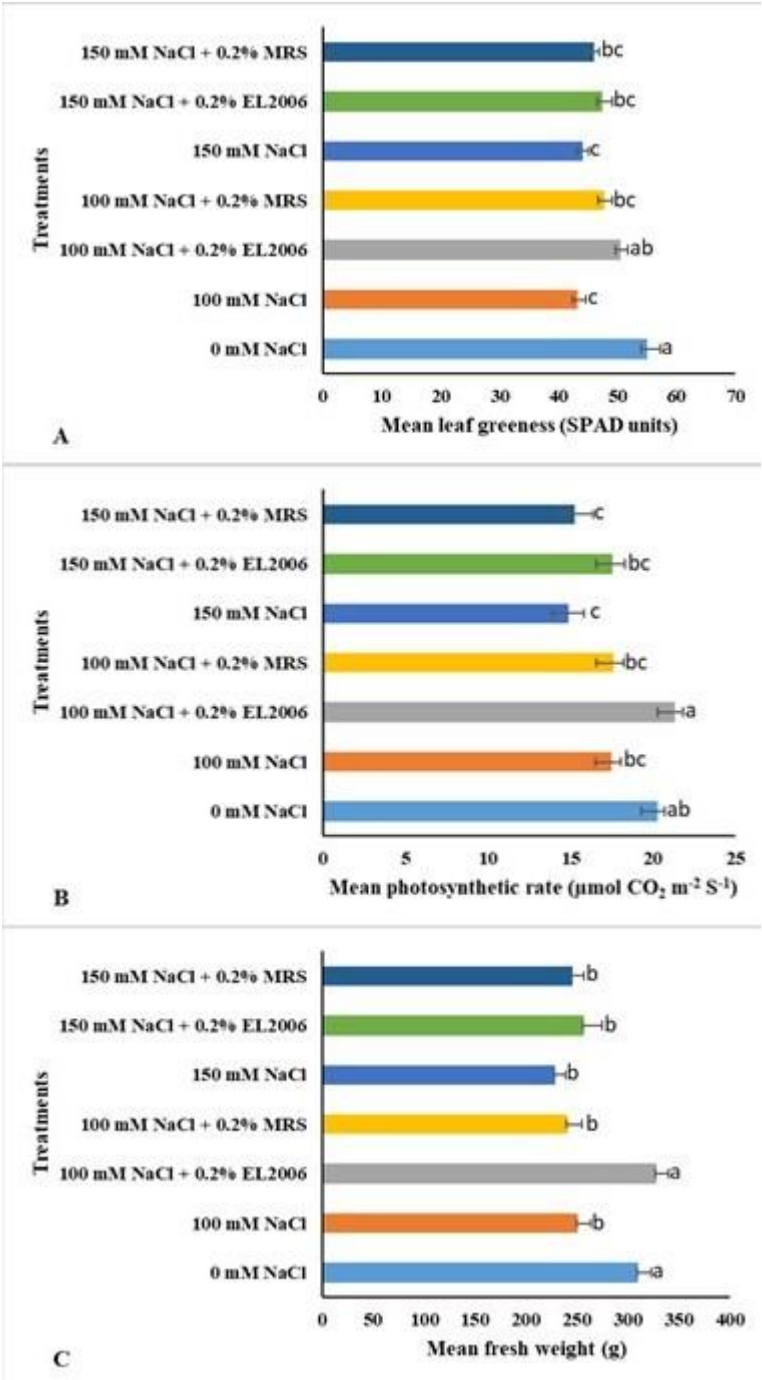

Figure 6.4: Effect of treatments on leaf greenness (A), photosynthetic rate (B) and fresh weight (C) of potato. Data represents the mean ± SE (n = 48); different letters indicate values determined by Tukey's multiple mean comparison significantly different at $p < 0.05$.

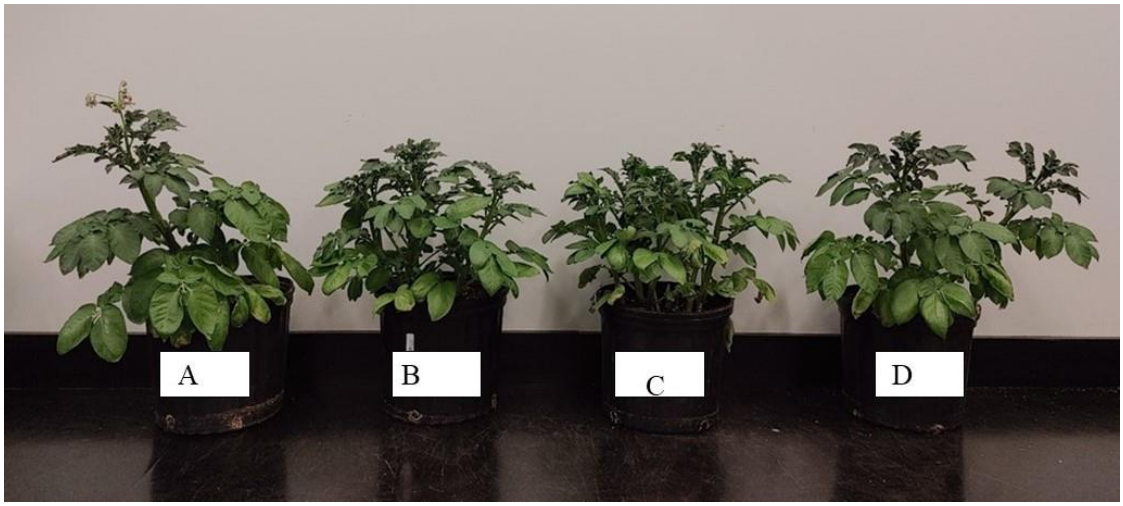

Figure 6.5: Potato treated with 0 mM NaCl (A), 100 mM NaCl (B), 100 mM NaCl +0.2% MRS (C) and 100 mM NaCl +0.2% EL2006H (D).

6.6 Discussion

There is a continuous need to increase food production to quantities sufficient to feed the growing human population, without compromising quality, using sustainable and environmentally friendly approaches such as plant growth promoting microorganisms (PGPM) and their derived compounds (Lyu et al., 2020; Shah et al., 2021). NaCl stress is a major global constraint to food production (Naamala and Smith, 2021b). Plant growth promoting microorganisms (PGPM), or their derived compounds have been reported to enhance plant growth under saline conditions (Schwinghamer et al., 2016; Subramanian et al., 2016a,b; Ilangumaran et al., 2021; Naamala et al., 2022). However, use of microbial CFS as plant growth biostimulants is less explored, with just a handful of publications available (Tewari et al., 2020; Naamala et al., 2022; Shah et al., 2022). It is possible that CFS could enhance plant growth because microbes exude metabolites into their growth media in response to various signals, such as those related to biotic or abiotic stress (Subramanian et al., 2021). Among the exuded metabolites are some that possess phyto-stimulation properties, such as IAA, LCO and thuricin17 (Prithiviraj et al., 2003; Mohite, 2013; Subramanian et al., 2016a,b; Antar et al., 2021). In the laboratory setting, such signals/metabolites are exuded into the microbe's growth medium, which when filtered of microbial cells, will still contain the metabolites that can then enhance plant growth (Gray et al., 2006a). Consequently, the possible modes of action through which CFS could enhance plant growth include presence of phytohormones such as jasmonic acid; presence of enzymes such as ACC deaminase; presence of osmoprotectants such as proline and presence of volatile

organic compounds and exopolysaccharides, all of which may function to mitigate osmotic, oxidative and ionic stress associated with salinity (Forni et al., 2017; Khan et al., 2019; Cappellari and Banchio, 2020; Kumar et al., 2020; Fincheira et al., 2021; Lopes et al., 2021). There is limited publication on the role of members of the genus *Lactobacillus* and/or their CFS as plant growth biostimulants (Hamed et al., 2011; Lamont et al., 2017; Msimbira et al., 2022). The modes of action through which members of the genus *Lactobacillus* and their CFSs enhance plant growth are not fully understood (Lamont et al., 2017). However, plant growth promotion by LAB species has been attributed to production of metabolites such as IAA and siderophores (Omer et al., 2010; Mohite, 2013; Shrestha et al., 2014; Limanska N.V. et al., 2015; Limanska N. et al., 2015), and solubilisation of phosphorus (Shrestha et al., 2014), among other mechanisms. In general, PGPM and or their derived compounds can mitigate salinity stress by employing one or more of the following mechanisms: Production of antioxidants, production of enzymes such as ACC deaminase, production of exopolysaccharides, inducing systemic resistance in plants (Shrivastava and Kumar, 2015; Amna et al., 2019) and production of microbe-to-plant signal compounds (Backer et al., 2018).

The current study focused on the ability of CFS obtained from *L. helveticus* EL2006H to enhance growth of three crop species: corn, soybean, and potato, exposed to NaCl stress, under controlled conditions. Results of the study highlight the role CFS concentration, NaCl level, crop species and growth level play in the effectiveness and efficacy of CFS as plant growth biostimulants. The effect of CFS on mean radicle length varied among crop species, concentration of the CFS, and level of NaCl in the treatment. For instance, treatment with 100 mM NaCl +1.0% EL2006H resulted in a significant increase in corn radicle length but not soybean, suggesting crop specific responses. Although some PGPM, such as some *Rhizobium* species, can be promiscuous, enhancing growth in a wide range of crop species, others are host specific, enhancing growth of just one or two crop species (Lyu et al., 2020). PGPM and their host plants communicate through signals, which vary depending on the host plant needs (Antar et al., 2021). Sometimes, such signals will limit the host range of a particular PGPM. However, such PGPM can produce metabolites that enhance growth in a wide range of crops. For instance, LCO, can enhance growth of non-legumes although it is produced by *B. japonicum*, and plays a major role in nodulation of soybean. It is such advantages that make CFS, and microbial derived compounds relevant in PGPM

technology (Naamala and Smith, 2020). Even then, there is not yet a single microbial derived compound or PGPM that enhances growth of all crop species. It is possible that the varied responses to CFS observed in corn, soybean and potato are in part due to variation in ways through which the three crops perceive and respond to the bioactive signals in the CFS. In another study, we also observed variation in soybean and corn responses to *B. amyloliquefaciens* CFS (Naamala et al., 2022). The *B. japonicum* derived LCO also exhibited variation in its effect on corn and soybean (Souleimanov et al., 2002). At a lower concentration, variations were observed in the response of canola varieties treated with LCO (Schwinghamer et al., 2015).

Lowering CFS concentration from 1.0% (v/v) to 0.2% EL2006H CFS resulted in no significant effect on both corn and soybean mean radicle length. This seems to suggest that the concentration and quantity of CFS applied to a plant is vital in determining efficacy and effectiveness of the applied CFS in enhancing plant growth. In this case, especially for corn, higher concentrations resulting in more effective results than lesser concentrations. The same cannot be said about soybean. Its possible that in soybean, perhaps minute quantities of the supernatant were enough to enhance growth. For example, Msimbira et al. (2022) observed variation in germination of corn, in response to *Bacillus subtilis* CFS, where a concentration of 0.1% (v/v) yielded better results than higher concentrations of 0.2, 0.4 and 1.0%. It should also be noted that high concentrations may sometimes inhibit growth of the crop in question (Naamala et al., 2021b), although there is no universal description of how much is sufficient and this could vary among crop species. Published studies on *L. helveticus* CFS as a plant biostimulant are currently limited to a study by Msimbira et al. (2022). In our previous study on *B. amyloliquefaciens* EB2003 CFS, we observed the effect of CFS concentration on its effectiveness in enhancing germination and radicle length of corn and soybean (Naamala et al., 2022). Studies on other members of the genus *Lactobacillus* have also reported concentration as a major determinant of effectiveness and efficacy in plant growth promotion. For instance, radish plants responded differently to varying concentrations of *L. plantarum* (Higa and Kinjo, 1991).

Results on mean percentage germination varied between corn and soybean at the two CFS concentrations and three different time frames studied. For instance, in soybean, following treatment with 100 mM NaCl +0.2% EL2006H, greatest significance was observed at 48 h, where percentage germination of soybean was not only significantly higher than that of the 100 mM NaCl control, but was also not

significantly different from that of the unstressed 0 mM NaCl control. At 24 h, there was no observed difference among the effects of treatments for percentage germination. It is however possible that the CFS was already working on the physio-chemical properties of the seed to mitigate the effect of NaCl on the plant, hence the higher percentage germination observed at 48 h. The plant also naturally attempts to put in place a defence against stress, but can be slower, which could explain why at 72 h, there was no significant difference in the percentage germination of all the treatments on soybean. Therefore, it seems likely that at 48 h, CFS mitigated delays in germination due to 100 mM stress, resulting in percentage germination levels higher than the stressed control and not significantly different from the unstressed control, which is desirable because slow germination exposes seed to attack by pathogens in the soil, among other disadvantages. Similar results were obtained by Msimbira et al. (2022) who observed variation in the effect of EL2006H CFS on germination of corn and tomato across time. Naamala et al. (2022) also observed variation in effect of *B. amyloliquefaciens* CFS among 24, 48 and 72 h sampling times. In corn however, there was no significant effect of the EL2006H CFS on mean percentage germination at all time intervals. The mean percentage germination of corn was significantly lower than the unstressed control at both 48 and 72 h. This again takes us back to the effect of crop species on effectiveness of the CFS. The effect of PGPM CFS and other PGPM derived compounds on seed germination has been reported by other researchers (Tallapragada et al., 2015; Subramanian et al., 2016a,b; Shah et al., 2022). *Devosia sp*. CFS enhanced germination of canola and soybean under NaCl stressed and optimal conditions (Shah et al., 2022).

Increasing concentration from 0.2 to 1.0% of CFS yielded less desirable results in soybean as there was no significant difference between mean percentage germination of soybean treated with 100 mM NaCl + 0.2% EL2006H and 100 mM NaCl, at all measurement times, although the percentage germination of the latter was not significantly different from that observed in the 0 mM control at 48 h. Results seem to suggest that when it comes to mean percentage germination, lesser quantities of the CFS are required to mitigate the effect of NaCl stress on germination of soybean. In corn, CFS had no significant effect on mean percentage germination, with the unstressed control still significantly higher at both 48 and 72 h. Shah et al. also observed variations in the germination of canola and soybean treated with a range of concentrations of *Devosia sp*. CFS (Shah et al., 2022).

In the potato experiment, results exhibited effects of stress level on effectiveness of EL2006H CFS. The supernatant enhanced some growth variables but not all. Specifically, CFS enhanced fresh weight, photosynthetic rate and leaf greenness but not leaf area and plant height. Among the variables enhanced, levels varied across NaCl levels. Better results of the CFS were observed in potato treated with 100 mM NaCl than 150 mM NaCl. This implies that increasing NaCl level by 50 mM reduced effectiveness of the CFS. This is not surprising as its possible that the potential bioactive substance was less effective at 150 mM NaCl so that it/ they could not function as efficiently as they would at lower concentrations, or, at 150 mM NaCl, potato plant cell components could have been damaged (Ilangumaran et al., 2021) to a point where even the CFS could not fully mitigate these effects on growth. The effect of NaCl level on the effectiveness of CFS was also observed by Naamala et al., (2022) on percentage germination and radicle length of soybean and corn treated with different concentrations of NaCl. The CFS enhanced radicle length in soybean not stressed with NaCl but not in soybean exposed to NaCl while the reverse was true for corn (Naamala et al., 2022). Subramanian et al. (2016a) also observed that LCO enhanced germination of soybean exposed to 100 mM NaCl but not higher NaCl concentrations of 150 mM NaCl and 175 mM NaCl. Shah et al. observed significant increases in germination of canola and soybean treated with *Devosia sp*. CFS (Shah et al., 2022).

The ability of PGPM, LAB included, to enhance plant growth can be affected by plant growth stage and growth variables, in which case a microbial strain or its CFS can enhance plant growth at a certain plant growth stage and not at another or enhance growth of one variable but not the other. For instance, under field conditions, Shrestha et al. (2014) observed an increase in growth of pepper plants treated with each of the three LAB they were studying, 1 week after transplanting. However, after that stage, only one strain of the three was able to enhance plant height. This, in a way, points out the complexity of depending on the plant-microbe interactions for plant growth stimulation.

Published research on *L. helveticus* as a plant growth biostimulant remains minimal. However, other members of the genus have been reported to enhance plant growth. For example, *L. plantarum* exhibited antimicrobial activity against *Fusarium spp*. in agar plate assays and a consortium consisting of the same and *B. amyloliquefaciens* reduced severity of *Fusarium spp*. in wheat (Baffoni et al., 2015). *L. plantarum* ONU 12 expressed antimicrobial properties in carrot, kalanchoe and grapes

exposed to *Agrobacterium tumefaciens*, protection ranging from 72.7 to 100% of wounded kalanchoe tissues, depending on mode of application (Limanska et al., 2015). LAB species KLF01, KLC02 and KPD03 had antagonistic effects against *Xanthomonas campestris pv. vesicatoria* (Shrestha et al., 2014) while strains LB-1, LB-2 and LB-3 increased total fresh weight of tomato plants by 348, 260, and 390%, respectively (Hamed et al., 2011). LAB species identified as KLF01, KLC02 and KPD03 were reported to enhance chlorophyll content in pepper (Shrestha et al., 2014). The same strains, except KLC02, were reported to enhance shoot length in pepper (Shrestha et al., 2014). Recently, research on PGPM has extended to their CFSs, attempting to elucidate whether it can enhance plant growth in the absence of the microbial cell. The ability of *L. helveticus* CFS at varying pH levels to enhance plant growth was recently reported (Msimbira et al., 2022). The effectiveness and efficacy of PGPM or their CFS, including LAB is dependent on several soil, plant and microbe factors, such soil conditions and plant species. A PGPM can enhance growth of one crop species but not another or enhance growth under certain soil conditions, such as under stressed conditions but not under optimal conditions.

At this stage we do not know whether the effect of CFS on the different variables is from a single bioactive compound or more than one; both scenarios are possible. As mentioned earlier, knowledge on the mechanisms employed by members of the genus *Lactobacillus* and or their CFSs to enhance plant growth remain poorly understood.

6.7 Conclusion

Based on the results of this study, it seems likely that *L. helveticus* EL2006H exudes into its growth media substances which enhance radicle length in corn, mean percentage germination in soybean and photosynthetic rate, greenness and mean fresh weight in potato. However, the effect varies depending on crop species, concentration of CFS and level of NaCl stress. *L. helveticus* CFS concentration of 0.2 and 1.0% were more effective at enhancing radicle length in corn and percentage germination in soybean, respectively. One hundred and fifty mM NaCl lowered the effectiveness of the 0.2% concentration in enhancing leaf greenness, mean photosynthetic rate and mean fresh weight of potato. Findings of this study can be used as a basis to further study of *Lactobacillus* CFSs and possibly identify the bioactive substances therein. Findings of this study are promising in the field of microbial inoculants where new ways of

improving efficacy and effectiveness of the technology, especially under field conditions, are constantly sought. However, further studies need to be done, especially under field conditions, with trials on different crop species and varying soil conditions.

6.8 References

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Chapter 7: General Discussion

As world population continues to grow, there is a need to increase food production in order to meet the food requirements of the resulting larger number of people (Barea, 2015). Unfortunately, the green revolution approaches to increasing yield and crop productivity, such as the use of inorganic fertilisers and pesticides, although effective must be minimised in the longer term to keep greenhouse gas accumulation and its subsequent effects on climate to the bare minimum (Barea, 2015; Gupta et al., 2015; Bender et al., 2016). Further, some of these approaches, together with other natural and artificial factors have led to increases in arable land affected by abiotic stress, notably, salinity stress, which is a major global constraint to crop production (Metternicht and Zinck, 2003; Yensen, 2008; Bui, 2013). There are other sustainable and environmentally suitable approaches that are currently being used to enhance growth of crops in both more optimal and salt affected areas, such as breeding for salt tolerant crop cultivars, genetic engineering and use of plant growth promoting microorganisms (Cheng et al., 2012; Smith et al., 2015a; Zhou et al., 2016). The current study focused on the later, with major emphasis on two strains, *Bacillus amyloliquefaciens* EB2003A and *Lactobacillus helveticus EL2006H.*

In chapter three, the effect of sodium chloride (NaCl) on survival and growth of the two strains was elucidated. Results showed that both strains were tolerant to levels of NaCl, up to 1000 mM, although some concentrations significantly lowered growth rate, and increased generation time, in relation to the 0 mM NaCl control. Na^+ and Cl^- are among the most dominant ions in saline soils, due their high-water solubility, and hence, ease of deposition by water, of NaCl (Tanji, 2002). Once a microbe is exposed to high concentrations of such ions, osmotic, ionic, and or oxidative stress are likely to affect the microbe. Oxidative stress results from excessive accumulation of reactive oxygen species (ROS), such as peroxides, beyond the microbe's anti oxidant defensive system (Seixas et al., 2022). This then causes damage to the microbial cell membranes and other cell components such as nucleic acids and proteins (Farr & Kogoma, 1991; Fasnacht & Polacek, 2021). This then affects microbial processes such as respiration and cell division, which are essential for microbial growth (Ferjani et al., 2003; Yan & Marschner, 2012; Yan & Marschner, 2013; Seixas et al., 2022). However, some microbes are able to counteract the effect of oxidative stress through upregulation of specific genes, proteins and metabolites that enhance their anti oxidant defensive

systems. Osmotic stress on the other hand results from a disruption in the osmotic pressure of the bacterium's surrounding environment, where a decrease causes excessive uptake of water by the microbe which may result in swelling and possible lysis of the microbial cell, while an increase results in loss of water from the microbial cell, resulting in possible dehydration and death (Wood, 2015). Disruption of the osmotic pressure may then affect cell shape, loss of turgor pressure, cause cytoplasmic tension and affect cellular processes such as metabolism (Rubiano-Labrador et al., 2015; Wood, 2015). Microbes counteract osmotic stress by maintaining their cell wall integrity to control what comes in and out of the cell, accumulation of compatible solutes, as well as other mechanisms essential for osmoadaptation (Oren 2008; Wood, 2015). Accumulation of compatible solutes is the most common mechanism employed by gram positive bacteria, such as *B. amyloliquefaciens* and *L. helveticus*, for osmoadaptation (Sleator & Hill, 2002). Ionic stress, on the other hand, results from the accumulation of excess ions in the microbial cell, resulting in ion toxicity which then affects normal functioning of the cell. Ions such as Na^{+}, K^{+}, Ca^{+2}, Mg^{+2}, are essential for normal microbial growth and functioning, except when they are in excess, that they affect processes such as substrate transport and enzyme activity (Lin et al., 2021). Microbes have developed mechanisms for regulating ion accumulation in their cytoplasm, which involve restructuring their cell membrane, as well as pumping them out using efflux pumps (Shabala et al., 2009). Exposure to NaCl requires bacteria to activate tolerance mechanisms that enables them to tolerate osmotic, ionic, and oxidative stresses (Csonka, 1989; Ivey et al., 1993; Ferjani et al., 2003; Oren 2008). Some bacteria, such as *B. amyloliquefaciens*, form spores, which enable them to be in a dormant state and survive stressful conditions for long periods of time (Ghosh et al., 2018). Effects of osmotic, ionic, and oxidative stresses could in part explain the decrease in growth rate and increase in generation time of both *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H. However, establishment of tolerance mechanisms could in part be responsible for the ability of the two strains to survive high concentrations of NaCl. Tolerance mechanisms enable some microbes to not only adjust to high concentrations of NaCl but even thrive in environments with high salt concentrations (Mohammadipanah et al., 2015). Such mechanisms work together to counter act the effects of osmotic stress, ionic stress, and oxidative stress on microbial growth (Oren, 2002b). This may also explain why there was no observed significant difference in the growth rate and generation time of both *B. amyloliquefaciens*

EB2003A and *L. helveticus* EL2006H, at some NaCl levels, in comparison to the control. Other bacteria have a NaCl requirement, hence, they exhibit better growth when exposed to some level of NaCl, in comparison to none, while halotolerant bacteria can survive in the presence or absence of NaCl (Oren, 2002b; Reang et al., 2022). In this study, such mechanisms were not studied. However, changes in the exoproteome profiles of both *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H were elucidated.

Several proteins such as enzymes and proteins involved in transport systems play major roles in the microbe's physiochemical processes, as do those involved in respiration, DNA synthesis and cell division, which are essential for microbial survival and growth (Ferjani et al., 2003). Therefore, examining changes in the exoproteome profiles of *L. helveticus* El2006H and *B. amyloliquefaciens* EB2003A could provide insight into possible ways the two strains tolerate high concentrations of NaCl, at protein level. In chapter 4, examining the exoproteome profiles of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, showed differences in protein profiles of the NaCl stressed treatment units and the control. Several proteins were downregulated while others were upregulated. The majority of proteins that were upregulated play essential roles in mitigating effects related to salinity stress, such as mitigation of oxidative stress, DNA and RNA synthesis, cell wall maintenance and repair, substrate transportation and spore formation. Proteins such as thioredoxins, XRE family transcriptional regulators, flavodoxin family proteins, and heme A synthase, which are involved in enhancing microbial defense against oxidative stress (Coba de la Peña et al., 2013; Lewin & Hederstedt, 2016; Cheng et al., 2017; Zhang et al., 2022), were upregulated at 200 mM NaCl (Supplementary material S1). Alanine containing proteins: cation symporter family protein and asparagine synthetase B, that play roles in the formation of spores (Ghosh et al., 2018; Zhu et al., 2019), were also upregulated at 200 mM NaCl. The upregulation of such proteins could potentially be in part responsible for the tolerance of *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, to high NaCl levels. Taken together, downregulation and upregulation of proteins work together to aid microbial tolerance to salt stress.

Several upregulated proteins, especially for *B. amyloliquefaciens* EB2003 were also reported to play a role in plant growth promotion, which prompted the assumption that perhaps one or more of these proteins were responsible for the enhancement in plant growth that was observed following treatment of potato, corn, and soybean with

B. amyloliquefaciens EB2003 CFS. Proteins such as esterases, heme, MarR homologs, MFS efflux pumps, thioredoxin and amidases have been reported to enhance plant growth, both under stressed and ideal conditions (Bartels et al., 2003; Shen et al., 2022; Wu et al., 2022; Xu et al., 2022; Unkefer et al., 2023). For example, esterases are enzymes involved in the hydrolysis of short chained esters, and hence play a vital role in many biological processes in plants (Pereira et al., 2017). They are known to be stress tolerant which makes them suitable candidates for enhancement of plant growth in a wide range of environmental conditions (Wang et al., 2020). Being diverse, esterases play vital work to enhance plant growth and plant quality. For example, sinapine esterase (BnSCE3) enhanced the nutritive quality of oil seed rape by breaking down sinapine, a phenolic compound with antinutritive activity that makes oil seed rape protein unsuitable for consumption (Clauss et al., 2011). The gene *CDEF1*, which encodes an esterase cutinase was involved in the degradation of the cuticle to enhance lateral root development in *Arabidopsis thaliana* (Takahashi et al., 2010). Some esterases play a role in maintaining plant fertility by playing a role in pollen development (Zhang et al., 2020). The ability of several PGPM derivatives, such as phytohormones, microbial volatile organic compounds (mVOCs), cell free supernatants (CFS), bacteriocins and lipo-chitooligosaccharides to enhance plant growth has been reported by several researchers (Schwinghamer et al., 2016; Subramanian et al., 2016a, b). To alleviate salt stress in plants, microbial derivatives have been reported to employ mechanisms such as osmoregulation, ROS scavenging, upregulation of plant antioxidant system, upregulation of salt responsive genes, enhance nutrient mobilisation, increase chlorophyll content and photosynthetic rate, improve ion homeostasis and enhance plant root systems (Timmusk et al., 2014; Vaishnav et al., 2015; Subramanian et al., 2016a; Forni et al., 2017; Cappellari et al., 2020; Liu et al., 2020; Zhao et al., 2020). For example, application of mVOCs in pepper mint increased concentration of 2,2-diphenyl−1-picrylhydrazyl, with radical scavenging activity (Cappellari et al., 2020). Treatment of salt stressed soybean with thuricin17 enhanced mobilization of carbon and nitrogen (Subramanian et al., 2016a). Upregulation of PEP carboxylase, rubisco-oxygenase, pyruvate kinase, and proteins of the light harvesting complex, energy and antioxidant pathways were observed in *A. thaliana* treated with thuricin17 and resulted in alteration of carbon and energy metabolism pathways (Subramanian et al., 2016b). The upregulated proteins in the current study employ similar mechanisms, which in turn enhance plant tolerance to salinity stress. It should

also be noted that the role of PGPM derivatives in enhancing plant growth extend beyond mitigation of effects of salinity on plants, to mitigation of other abiotic and biotic stresses (Schwinghamer et al., 2015; Schwinghamer et al., 2016; Schulz-Bohm et al., 2017; Thakur et al., 2019).

The success of PGPM in a new environment is dependent on their ability to survive in the rhizosphere while maintaining their ability to produce plant growth promoting substances (Martínez-Viveros et al., 2010). Salt tolerant PGPM strains can be pivotal in enhancing plant growth under salt stressed and non-salt stressed environments. Where strain performance is limited by exposure to salt, PGPM derivatives could be used to supplement and or complement PGPM strains in salt affected areas. This is because salt stress may either lower a PGPM efficacy or cause complete loss of ability to promote plant growth (Zahran, 1997; Soussi et al., 2001; Nadeem et al., 2015). For example, salt stress affects the entire nitrogen fixation process in rhizobia (Singleton et al., 1982a; Zaharan, 1999). Therefore, identification of proteins in microbial CFS, that can enhance plant growth makes it possible for CFSs to be used to enhance plant growth. Besides proteins, several metabolites and genes can also be found in CFS. Chapters five and six elucidated the ability of CFSs of *L. helveticus* EL2006H and *B. amyloliquefaciens* EB2003A to enhance germination and rootlength of corn and soybean. Chapter six further elucidated the effect of *L. helveticus* CFS on growth variables of potato exposed to NaCl stress, under greenhouse conditions. When treated with NaCl, seed germination and rootlength were significantly lowered, especially in corn. Water, temperature, oxygen, and availability of nutrients are essential factors for proper seed germination. The germination process involves imbibition of water by the seed and eventual protrusion of the radicle to the outside of the seed, were it growths into the primary root. Salinity stress creates a low water potential around the seed which may affect the imbibition process (Mwando et al., 2020). Moreover, Na^+ and Cl^- ions taken up by the seed could cause ion toxicity which may affect seed metabolism and energy production, leading to nutrient and phytohormone imbalances, and subsequent delays or hinderance of seed germination (Uçarlı, 2021). NaCl could result in accumulation of ROS, which in turn may influence seed nucleic acids, proteins, and carbohydrate contents, hence negatively affecting seed germination (Ibrahim, 2016). The two strains' CFSs were able to mitigate the effect of NaCl stress on seed germination and root length, possibly by balancing hormones, aiding nucleic acids and DNA repair, enhancing water uptake and elimination of

oxidative stress, among others. This is because the study of the exoproteome composition of the CFSs used in the study showed presence of antioxidant proteins, Nucleic acids and protein repairing proteins and substrate transport proteins, among others, which have been reported to enhance seed germination and plant growth. Boosting water uptake, nutrient metabolism, and enzyme activity from bioactive constituents of the CFS could explain enhancement of seed germination and root length in non-stressed corn and soybean seeds. Treatment with *B. amyloliquefaciens* EB2003A CFS resulted in a significantly higher percentage germination of corn and soybean, at 24 h. Early germination is a desirable characteristic for seed since it lowers risks of seed being attacked by soil born pathogens. Overall, it also resulted in a more uniform germination, which is also desirable especially for large scale production where mechanisation is common, as it eases agronomic practices like weeding and harvesting.

Salt stress also affected growth variables of potato, in comparison to plants treated with 0 mM NaCl. It should be noted that salinity stress lowers stomatal conductance, water, and carbon dioxide uptake, affects the activity and expression of enzymes essential for chlorophyll biosynthesis, and diminishes nutrient availability in plants, all of which work together to affect leaf greenness, photosynthesis, and overall plant growth (Agastian et al., 2000). Treatment of NaCl stressed potato with *L. helveticus* CFS significantly enhanced leaf greenness, photosynthetic rate, and plant fresh weight, in comparison to the salt stressed controls. Proteins expressed by *L. helveticus*, in the current study, are yet to be associated with plant growth promotion. It's possible that this stimulation resulted from other bioactive metabolites, that were exuded by the strain in its CFS. Several researchers have reported that microbes exude, in their growth environment, metabolites, such as phytohormones, VOCs, enzymes, exopolysaccharides, bacteriocins and osmoprotectants, and even microbe-to-plant signals, some of which have been reported to enhance plant growth (Souleimanov et al., 2002; Brígido et al., 2013; Egamberdieva et al., 2013; Gautam et al., 2016; Schwinghamer et al., 2016; Cappellari et al., 2020). Such compounds have also been associated with the ability of several PGPM's CFSs to enhance plant growth. For example, CFS of *Pseudomonas putida, Bacillus licheniformis, Pseudomonas fluorescens, Serratia marcescens* and *B. amyloliquefaciens* lowered the accumulation of Pepper mild mottle virus in chill pepper (Gangireddygari et al., 2022). Analysis of the CFSs using gas chromatography linked to mass spectrometry showed that they contained alkanes, ketones, alcohols, and aromatic ring containing compounds, varying

from one microbial CFS to another. In another study, CFSs of different phytopathogens such as *Rhizoctonia solani* and *Phytium irregulare* enhanced systemic immune response of *A. thaliana* to *B. cinerea*, by inducing the expression of disease response genes (Ávila & Poveda, 2022). The composition of the supernatants was not evaluated though. Cell free supernatant of the fungus *Piriformospora indica* enhanced seed production and seed oil content of *Helianthus annus* plants (Bagde et al., 2011). In our study, the mechanisms employed in the CFSs to enhance plant growth were not investigated. However, as mentioned above, its possible that some of the identified proteins possibly played a role, especially for *B. amyloliquefaciens* EB2003A. It is also possible that other derivatives, such as metabolites played a part. Based on the findings of our study and those of other researchers, microbial CFSs are potential biostimulants that could enhance plant growth. Taken together, findings of this study suggest that *B. amyloliquefaciens* EB2003A and *L. helveticus* EL2006H, tolerate high levels of NaCl, and one the mechanisms they employ is the change in their exoproteome profiles, upregulating some proteins and downregulating others. The CFSs of the two strains investigated here enhance plant growth which makes them promising approaches for enhancing plant growth under stressed and ideal conditions, sustainably. The bioactivity of the CFS could be related in part, to some of the upregulated proteins although this is not guaranteed. More studies need to be done, especially under field conditions, that are focused on changes in plant physiology following application of the CFS investigated, to understand the actual mechanisms through which the CFSs enhance plant growth.

Chapter 8: Final conclusions and summary

Soil salinity is a major constraint to crop production worldwide. Plant growth promoting microorganisms (PGPM) and their derivatives, such as cell-free supernatants (CFSs), are an environmentally friendly and sustainable approach to enhancing crop production in both non-salinity stressed, and salinity affected areas. However, to be effective, under salt stressed conditions, PGPM strains should be able to tolerate the salty conditions and maintain their ability to enhance plant growth. Two PGPM strains, *Lactobacillus helveticus* EL2006H and *Bacillus amyloliquefaciens* EB2003A, which are members of the EVL Inc. commercial microbial consortium were tested for their ability to tolerate high levels of NaCl stress, as well as produce plant growth promoting substances in their growth media when exposed to NaCl stress.

Results of the salt tolerance study showed that the two strains were tolerant to high levels of NaCl, up to 1000 mM NaCl. A proteomic profiling of their CFSs, when exposed to 200 mM NaCl stress, showed that both strains down regulated and upregulate some proteins. The upregulated proteins were mostly those that play part in microbe tolerance to salt stress, through increasing tolerance to oxidative stress, increasing water and nutrient uptake, maintaining integrity of the cell wall, being involved in formation of spores, as well as repair and synthesis of nucleic acids and proteins. Some of the upregulated proteins were also reported to enhance plant growth, at different stages, both in stressed and normal conditions.

The strains' cell-free supernatants, when exposed to 200 mM NaCl were tested for their ability to enhance plant growth. Results showed that when exposed to 200 mM NaCl, *B. amyloliquefaciens* EB2003A CFS enhanced germination and root length for corn and soybean, under NaCl stressed and non-stressed conditions. Cell-free supernatant of *L. helveticus* EL2006H enhanced germination and root length of corn and soybean, as well as fresh weight, leaf greenness and photosynthetic rate of potato, all while the crop plants were exposed to NaCl. The effect of the CFS varied across the NaCl levels to which the plants were exposed, concentration of the CFS used as treatment, and plant species under study.

Taken together, findings of this project suggest that *B. amyloliquefaciens* EB2003A, and *L. helveticus* EL2006H CFSs can be used as a sustainable and environmentally friendly approach to enhance plant growth, under salt stressed and non-salinity stressed conditions. However, before commercialisation, more studies

ought to be carried out, especially under field conditions, since inconsistencies, again, especially under field conditions, is one of the major constraints for using microbial inoculant technology. Moreover, studying changes in plant tissue, following application of CFS could give a better picture of the mechanisms employed by the CFS to enhance plant growth. Future prospects should also consider isolating the active compounds from the CFS and have them tested, independently, for ability to enhance plant growth, under both stressed and non-stressed conditions, since these could be easier to manage and be required in lesser quantities as compared to the CFSs.

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APPENDIX

Supplementary Table 4.3: Proteins unique to the exoproteome of *B. amyloliquefaciens* EB2003A treated with 200 mM NaCl, according to fisher's exact test ($p \leq 0.05$)

#	Protein ID	Molecular weight
842.2	MULTISPECIES: tRNA (adenine(22)-N(1))-methyltransferase TrmK [Bacillus]	28 kDa
451.11	peptidase S8 [*Bacillus amyloliquefaciens*]	154 kDa
593.2	MULTISPECIES: PTS fructose transporter subunit IIC [*Bacillus amyloliquefaciens* group]	67 kDa
711.6	2',3'-cyclic-nucleotide 2'-phosphodiesterase [*Bacillus amyloliquefaciens*]	155 kDa
624.3	subclass B1 metallo-beta-lactamase [*Bacillus amyloliquefaciens*]	28 kDa
803.2	MULTISPECIES: amidase [*Bacillus amyloliquefaciens* group]	76 kDa
720.2	thioredoxin [*Bacillus amyloliquefaciens*]	25 kDa
830.3	MULTISPECIES: peptidoglycan endopeptidase [*Bacillus*]	52 kDa
833.2	MULTISPECIES: phage portal protein [*Bacillus subtilis* group]	54 kDa
845.2	amino acid ABC transporter substrate-binding protein [*Bacillus amyloliquefaciens*]	29 kDa
848	MULTISPECIES: ABC transporter substrate-binding protein [*Bacillus*]	28 kDa
856	MULTISPECIES: amino acid ABC transporter permease [*Bacillus*]	26 kDa
857	MULTISPECIES: cell division protein FtsH [*Bacillus*]	71 kDa
858	MULTISPECIES: OxaA precursor [*Bacillus*]	29 kDa
861	MULTISPECIES: zinc ABC transporter substrate-binding protein [Bacillus subtilis group]	35 kDa

862	Cluster of MULTISPECIES: hypothetical protein [*Bacillus amyloliquefaciens* group] (WP_013351197.1)	31 kDa
862.1	MULTISPECIES: hypothetical protein [*Bacillus amyloliquefaciens* group]	31 kDa
863	Cluster of scaffold protein [*Bacillus amyloliquefaciens*] (WP_013351225.1)	25 kDa
863.1	scaffold protein [*Bacillus amyloliquefaciens*]	25 kDa
864	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	14 kDa
871	MULTISPECIES: rod shape-determining protein [*Bacillus subtilis* group]	36 kDa
873	penicillin-binding protein [*Bacillus amyloliquefaciens*]	70 kDa
875	MULTISPECIES: LytR family transcriptional regulator [*Bacillus subtilis group*]	37 kDa
876	DUF5082 domain-containing protein [*Bacillus amyloliquefaciens*]	17 kDa
877	MULTISPECIES: phosphoribosylformylglycinamidine cyclo-ligase [*Bacillus subtilis* group]	37 kDa
879	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	15 kDa
880	Cluster of hypothetical protein [*Bacillus amyloliquefaciens*] (WP_014471509.1)	15 kDa
880.1	hypothetical protein [*Bacillus amyloliquefaciens*]	15 kDa
880.3	hypothetical protein [*Bacillus amyloliquefaciens*]	16 kDa
881	Cluster of MULTISPECIES: phage portal protein [*Bacillus subtilis* group] (WP_014471720.1)	38 kDa
881.2	MULTISPECIES: phage portal protein [*Bacillus subtilis* group]	38 kDa
883	Cluster of phage portal protein, partial [*Bacillus amyloliquefaciens*] (WP_021493831.1)	140 kDa

886	Cluster of LytR family transcriptional regulator [*Bacillus amyloliquefaciens*] (WP_047475797.1)	35 kDa
887	MULTISPECIES: NarK/NasA family nitrate transporter [*Bacillus amyloliquefaciens* group]	43 kDa
890	Cluster of penicillin-binding protein [*Bacillus amyloliquefaciens*] (WP_071346857.1)	78 kDa
890.2	penicillin-binding protein [*Bacillus amyloliquefaciens*]	78 kDa
874	MULTISPECIES: imidazoleglycerol-phosphate dehydratase [*Bacillus subtilis* group]	21 kDa
883.2	phage portal protein, partial [*Bacillus amyloliquefaciens*]	140 kDa
588.2	proteinase inhibitor [*Bacillus amyloliquefaciens*]	17 kDa
859	MULTISPECIES: NupC/NupG family nucleoside CNT transporter [*Bacillus*]	42 kDa
860	MULTISPECIES: rRNA pseudouridine synthase [*Bacillus*]	28 kDa
868	MULTISPECIES: zinc metalloprotease HtpX [*Bacillus subtilis* group]	33 kDa
883.7	phage portal protein [*Bacillus amyloliquefaciens*]	181 kDa
885	Cluster of XRE family transcriptional regulator [*Bacillus amyloliquefaciens*] (WP_041481598.1)	9 kDa
885.1	XRE family transcriptional regulator [*Bacillus amyloliquefaciens*]	9 kDa
829.3	MULTISPECIES: carbamoyl-phosphate synthase (glutamine-hydrolyzing) large subunit [*Bacillus amyloliquefaciens* group]	118 kDa
854	MULTISPECIES: holin [*Bacillus subtilis* group]	16 kDa
870	MULTISPECIES: protein translocase subunit SecDF [*Bacillus subtilis* group]	82 kDa
883.1	phage portal protein [*Bacillus amyloliquefaciens*]	185 kDa
883.5	phage portal protein [*Bacillus amyloliquefaciens*]	185 kDa
883.8	phage portal protein [*Bacillus amyloliquefaciens*]	187 kDa

886.1	MULTISPECIES: LytR family transcriptional regulator [*Bacillus*]	34 kDa
891	Cluster of DUF4355 domain-containing protein [*Bacillus amyloliquefaciens*] (WP_071347902.1)	27 kDa
891.2	DUF4355 domain-containing protein [*Bacillus amyloliquefaciens*]	27 kDa
403.7	carboxylesterase/lipase family protein [*Bacillus amyloliquefaciens*]	46 kDa
414.4	N-acetyltransferase [*Bacillus amyloliquefaciens*]	21 kDa
576.2	MULTISPECIES: flavodoxin family protein [*Bacillus*]	20 kDa
851	MULTISPECIES: heme A synthase [*Bacillus*]	34 kDa
855	MULTISPECIES: divalent metal cation transporter [*Bacillus*]	42 kDa
865.2	hypothetical protein [*Bacillus amyloliquefaciens*]	39 kDa
867	MULTISPECIES: ABC transporter substrate-binding protein [*Bacillus subtilis* group]	64 kDa
869	MULTISPECIES: alanine:cation symporter family protein [*Bacillus subtilis* group]	52 kDa
884	MarR family transcriptional regulator [*Bacillus amyloliquefaciens*]	17 kDa
889	MFS transporter [*Bacillus amyloliquefaciens*]	57 kDa
810.2	MULTISPECIES: member of the processed secretome [*Bacillus*]	17 kDa
852	Cluster of MULTISPECIES: STAS domain-containing protein [*Bacillus*] (WP_003154749.1)	32 kDa
852.1	MULTISPECIES: STAS domain-containing protein [*Bacillus*]	32 kDa
881.1	MULTISPECIES: phage portal protein [*Bacillus*]	38 kDa
882.3	esterase family protein [*Bacillus amyloliquefaciens*]	28 kDa

Supplementary Table 4.4: Identified proteins unique to the exoproteome of *B. amyloliquefaciens* EB2003 A, exposed to 200 mM NaCl, according to a fold change equal to or more than 1.2.

#	Protein ID	Molecular weight
711.6	2',3'-cyclic-nucleotide 2'-phosphodiesterase [*Bacillus amyloliquefaciens*]	155 kDa
711.7	2',3'-cyclic-nucleotide 2'-phosphodiesterase [*Bacillus amyloliquefaciens*]	155 kDa
711.8	2',3'-cyclic-nucleotide 2'-phosphodiesterase [*Bacillus amyloliquefaciens*]	155 kDa
720.2	thioredoxin [*Bacillus amyloliquefaciens*]	25 kDa
794.3	peptidoglycan endopeptidase [*Bacillus amyloliquefaciens*]	45 kDa
803.2	MULTISPECIES: amidase [*Bacillus amyloliquefaciens* group]	76 kDa
810.2	MULTISPECIES: member of the processed secretome [*Bacillus*]	17 kDa
816.4	transposase [*Bacillus amyloliquefaciens*]	72 kDa
816.7	transposase [*Bacillus amyloliquefaciens*]	72 kDa
829.3	MULTISPECIES: carbamoyl-phosphate synthase (glutamine-hydrolyzing) large subunit [*Bacillus amyloliquefaciens* group]	118 kDa
830.3	MULTISPECIES: peptidoglycan endopeptidase [*Bacillus*]	52 kDa
831.3	signal peptidase I [*Bacillus amyloliquefaciens*]	22 kDa
833.2	MULTISPECIES: phage portal protein [*Bacillus subtilis* group]	54 kDa
842.2	MULTISPECIES: tRNA (adenine(22)-N(1))-methyltransferase TrmK [*Bacillus*]	28 kDa
845.2	amino acid ABC transporter substrate-binding protein [*Bacillus amyloliquefaciens*]	29 kDa
846	MULTISPECIES: ATP synthase subunit gamma [*Bacillus*]	32 kDa

847	MULTISPECIES: N utilization substance protein B [*Bacillus*]	15 kDa
848	MULTISPECIES: ABC transporter substrate-binding protein [*Bacillus*]	28 kDa
849	MULTISPECIES: flagellar motor switch protein FliG [*Bacillus*]	38 kDa
850	MULTISPECIES: 30S ribosomal protein S16 [*Bacillus*]	10 kDa
851	MULTISPECIES: heme A synthase [*Bacillus*]	34 kDa
852	Cluster of MULTISPECIES: STAS domain-containing protein [*Bacillus*] (WP_003154749.1)	32 kDa
852.1	MULTISPECIES: STAS domain-containing protein [*Bacillus*]	32 kDa
853	MULTISPECIES: ABC transporter permease [*Bacillus*]	34 kDa
854	MULTISPECIES: holin [*Bacillus subtilis* group]	16 kDa
855	MULTISPECIES: divalent metal cation transporter [*Bacillus*]	42 kDa
856	MULTISPECIES: amino acid ABC transporter permease [*Bacillus*]	26 kDa
857	MULTISPECIES: cell division protein FtsH [*Bacillus*]	71 kDa
858	MULTISPECIES: OxaA precursor [*Bacillus*]	29 kDa
859	MULTISPECIES: NupC/NupG family nucleoside CNT transporter [*Bacillus*]	42 kDa
860	MULTISPECIES: rRNA pseudouridine synthase [*Bacillus*]	28 kDa
861	MULTISPECIES: zinc ABC transporter substrate-binding protein [*Bacillus subtilis* group]	35 kDa
862	Cluster of MULTISPECIES: hypothetical protein [*Bacillus amyloliquefaciens* group] (WP_013351197.1)	31 kDa
862.1	MULTISPECIES: hypothetical protein [*Bacillus amyloliquefaciens* group]	31 kDa
862.2	recombinase RecT [*Bacillus amyloliquefaciens*]	31 kDa
863	Cluster of scaffold protein [*Bacillus amyloliquefaciens*] (WP_013351225.1)	25 kDa
863.1	scaffold protein [*Bacillus amyloliquefaciens*]	25 kDa

864	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	14 kDa
865	Cluster of MULTISPECIES: hypothetical protein [*Bacillus subtilis* group] (WP_013351259.1)	39 kDa
865.1	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	39 kDa
865.2	hypothetical protein [*Bacillus amyloliquefaciens*]	39 kDa
866	Cluster of MULTISPECIES: transposase [*Bacillus subtilis* group] (WP_013351308.1)	27 kDa
866.1	MULTISPECIES: transposase [*Bacillus subtilis* group]	27 kDa
867	MULTISPECIES: ABC transporter substrate-binding protein [*Bacillus subtilis* group]	64 kDa
868	MULTISPECIES: zinc metalloprotease HtpX [*Bacillus subtilis* group]	33 kDa
869	MULTISPECIES: alanine:cation symporter family protein [*Bacillus subtilis* group]	52 kDa
870	MULTISPECIES: protein translocase subunit SecDF [*Bacillus subtilis* group]	82 kDa
871	MULTISPECIES: rod shape-determining protein [*Bacillus* subtilis group]	36 kDa
872	MULTISPECIES: asparagine synthetase B [Bacillus *subtilis* group]	73 kDa
873	penicillin-binding protein [Bacillus amyloliquefaciens]	70 kDa
874	MULTISPECIES: imidazoleglycerol-phosphate dehydratase [*Bacillus subtilis* group]	21 kDa
875	MULTISPECIES: LytR family transcriptional regulator [Bacillus subtilis group]	37 kDa
876	DUF5082 domain-containing protein [*Bacillus amyloliquefaciens*]	17 kDa
877	MULTISPECIES: phosphoribosylformylglycinamidine cyclo-ligase [*Bacillus subtilis* group]	37 kDa
878	Cluster of MULTISPECIES: hypothetical protein [*Bacillus subtilis* group] (WP_014470758.1)	40 kDa

878.2	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	40 kDa
879	MULTISPECIES: hypothetical protein [*Bacillus subtilis* group]	15 kDa
880	Cluster of hypothetical protein [*Bacillus amyloliquefaciens*] (WP_014471509.1)	15 kDa
880.1	hypothetical protein [*Bacillus amyloliquefaciens*]	15 kDa
880.3	hypothetical protein [*Bacillus amyloliquefaciens*]	16 kDa
881	Cluster of MULTISPECIES: phage portal protein [*Bacillus subtilis* group] (WP_014471720.1)	38 kDa
881.1	MULTISPECIES: phage portal protein [Bacillus]	38 kDa
881.2	MULTISPECIES: phage portal protein [*Bacillus subtilis* group]	38 kDa
882	Cluster of MULTISPECIES: esterase family protein [*Bacillus amyloliquefaciens* group] (WP_016938189.1)	28 kDa
882.2	MULTISPECIES: esterase family protein [*Bacillus amyloliquefaciens* group]	28 kDa
882.3	esterase family protein [*Bacillus amyloliquefaciens*]	28 kDa
883	Cluster of phage portal protein, partial [*Bacillus amyloliquefaciens*] (WP_021493831.1)	140 kDa
883.1	phage portal protein [*Bacillus amyloliquefaciens*]	185 kDa
883.2	phage portal protein, partial [*Bacillus amyloliquefaciens*]	140 kDa
883.5	phage portal protein [*Bacillus amyloliquefaciens*]	185 kDa
883.7	phage portal protein [*Bacillus amyloliquefaciens*]	181 kDa
883.8	phage portal protein [*Bacillus amyloliquefaciens*]	187 kDa
884	MarR family transcriptional regulator [*Bacillus amyloliquefaciens*]	17 kDa
885	Cluster of XRE family transcriptional regulator [*Bacillus amyloliquefaciens*] (WP_041481598.1)	9 kDa
885.1	XRE family transcriptional regulator [*Bacillus amyloliquefaciens*]	9 kDa
886	Cluster of LytR family transcriptional regulator [*Bacillus amyloliquefaciens*] (WP_047475797.1)	35 kDa

886.1	MULTISPECIES: LytR family transcriptional regulator [*Bacillus*]	34 kDa
886.2	LytR family transcriptional regulator [*Bacillus amyloliquefaciens*]	35 kDa
887	MULTISPECIES: NarK/NasA family nitrate transporter [*Bacillus amyloliquefaciens* group]	43 kDa
888	BMP family ABC transporter substrate-binding protein [*Bacillus amyloliquefaciens*]	35 kDa
889	MFS transporter [Bacillus amyloliquefaciens]	57 kDa
890	Cluster of penicillin-binding protein [*Bacillus* amyloliquefaciens] (WP_071346857.1)	78 kDa
890.2	penicillin-binding protein [*Bacillus amyloliquefaciens*]	78 kDa
891	Cluster of DUF4355 domain-containing protein [*Bacillus amyloliquefaciens*] (WP_071347902.1)	27 kDa
891.2	DUF4355 domain-containing protein [*Bacillus amyloliquefaciens*]	27 kDa

Supplementary Table 4.5: Identified upregulated proteins in exoproteome of *L. helveticus* EL2006H exposed to 200 mM NaCl, according to fisher's exact Test ($p \leq 0.05$)

#	**Protein ID**	**Molecular weight**
14	Cluster of SLAP domain-containing protein [*Lactobacillus helveticus*] (WP_172997630.1)	55 kDa
39	Cluster of peptide ABC transporter substrate-binding protein [*Lactobacillus helveticus*] (WP_172997611.1)	59 kDa
43	surface protein [*Lactobacillus helveticus*]	35 kDa
78	hypothetical protein GFB61_02825 [*Lactobacillus helveticus*]	24 kDa
85	Cluster of Stk1 family PASTA domain-containing Ser/Thr kinase [*Lactobacillus helveticus*] (QPB51502.1)	75 kDa

130	fibronectin type III domain-containing protein [*Lactobacillus helveticus*]	52 kDa
127	Cluster of metal ABC transporter substrate-binding protein [*Lactobacillus helveticus*] (TLQ22781.1)	34 kDa