Plant growth-promoting bacterial and their effects on the early growth of maize (*Zea mays* L.) under concurrent drought and heat stress

By

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Declaration

I, ...Iviwe Notununu.....declare that the thesis/dissertation, which I hereby submit for the degree....Masters (MSc) Microbiology.....at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: .....  .....

DATE:...18 August 2021.....

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Dedication

I dedicate this dissertation to my amazing wife Athenkosi Sakwe, my son Alukhethi, my brothers Ayabulela and Amkhitha Notununu.

I dedicate this dissertation to all the girls and boys who have been systemically devalued because of the colour of the skin they wear. To the village and the Kasi (Township) kid, you too can do it.

Remember these words “It is the possibility of having a dream come true that makes life interesting." (Paulo Coelho)

Abstract

Under field conditions, drought and heat stress are more prevalent in combination than as isolated phenomena. Studies that were aimed at characterizing the consequences of both heat and drought stresses on plants reported significantly enhanced negative effects on the growth and productivity of crops for the combination of these stresses compared to each stress applied individually. The combined effects of heat and drought stresses have unique effects on various plant physiological and molecular response parameters suggesting that plants have different response mechanisms to the combination of these stresses compared to the individual effects of these stresses. The use of plant growth-promoting rhizobacteria is thought to be key to the adaptation and survival of plants to abiotic stresses. As such, the study aimed to evaluate drought and heat tolerant PGPR isolates at mitigating the effects of dual drought and heat stress on the early growth of maize, as well as elucidate the expression of drought and heat stress response genes which actively participate in PGPR-induced tolerance.

The study screened and identified drought and heat tolerant PGPR that are potential biofertilizer candidates with unique attributes of enhancing plant response and adaptation to climate change effects. The isolated PGPR could promote the growth of maize plants under dual drought and heat stress under in-vitro conditions and in a pot trial experiment. The potential PGPR were identified as relatives of Bacillus thuringiensis (11MN1), Bacillus pseudomyciodes (21MN1B), Lelliottia amnigena (33MP1), and Leclercia sp. (36MP8). Bacterial isolates that demonstrated the potential to induce tolerance to maize plants subjected to dual drought and heat stresses in vitro were evaluated for compatibility using the dual culturing technique. The isolates from the Bacillus group showed slight antagonism against L. amnigena and Leclecia sp. belonging to the Enterobacteriaceae group. However, there was no antagonism between the Bacillus spp. and likewise between the Enterobacteriaceae group. The pot-experiments showed that isolates L. amnigena 33MP1, Leclercia sp. 36MP8 and the co-inoculation (11MN1, 21MN1, 33MP1, 36MP8) showed the greatest potential for alleviating the detrimental effects of induced concurrent drought and heat stress. The relative quantitative real-time PCR conducted to understand the expression of selected stress response genes in PGPR-inoculated maize plants showed that induced tolerance was achieved by initiating “Induced Systemic Tolerance” and by modulating the CAT2 and DHN2 stress response genes.

This approach is an environmentally friendly option for commercial and smallholder farmers in South Africa with the potential to maximize increase in maize production, especially under adverse climate conditions. These PGPR isolates also have the potential to reduce the need for extensive irrigation of maize crops throughout their growth cycle.

Thesis outputs

Poster presentation

- Notununu I, Moleleki L, Adeleke R. The role of PGPR (plant growth promoting rhizobacteria) on maize (Zea mays L.) seedlings under drought and heat stress. BGM Research Day, University of Pretoria, Pretoria, South Africa, 31 October 2019.

Publication

- Accepted manuscript "**Effects of plant growth-promoting rhizobacteria on the molecular response of maize under drought and heat stress: A review" in pedosphere Manuscript ID pedos2021042007.R1**

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List of Abbreviations

IPCC: Intergovernmental Panel on Climate Change

PGPR: Plant growth-promoting rhizobacteria

iPGPR: Intracellular PGPR

ePGPR: Extracellular PGPR

ACC: 1-aminocyclopropane-1-carboxylic

ABA: Abscisic acid

EPS: Exopolysaccharide

IST: Induced systemic tolerance

ROS: Reactive oxygen species

IAA: Indole acetic acid

ZT: Zeatin

S-AdoMet:S-adenosylmethionine

ACS: 1-aminocyclopropane-1-carboxylate synthase

BD: 2,3-butandiol

O^{2-}: Superoxide anion radicals

H2O2: Hydrogen peroxide

RO: Alkoxy radicals

GR: Glutathione reductase

CAT: Catalase

SOD: Superoxide dismutase

MDHAR:Monodehydroascorbate reductase

POX:Guaiacol peroxidase

APX:Ascorbate peroxidase

DHAR:Dehydroascorbate reductase

GST:Glutathione-S- transferase

GPX:Glutathione peroxidase

JA:Jasmonic acid

GA: Gibberellic acids

HSFs: Heat stress transcription factors

HSPs: Heat shock proteins

LEA: Late embryogenesis abundant

SSH: Suppression subtractive hybridization

ESTs: Expressed sequence tags

GST7: Glutathione S-transferase

DBF1 gene: Transcription factor DRE-binding factor

rab17: Ras-related-protein

RBCS: Ribulose bisphosphate carboxylase small subunit

HKT:1Sodium transporter

NHX:1Na^+/H^+ antiporter

PIPs:Plasma membrane intrinsic protein

ERD1: 5Early response to dehydration 15

DD-PCR:Differential display polymerase chain reaction

2D-PAGE:2-D polyacrylamide gel electrophoresis

Cadhn:*Capsicum annuum*dehydrin

encoding ACC oxidase:*caACCO*

SAMS:1S-adenosylmethionine synthase 1

PEG: Polyethylene glycol

ARC-SCW:Agricultural research Council- Soil, Water and Climate

TSA: Tryptic soy agar

TSB: Tryptic soy broth

OD: Optical density

BLAST:Basic Local Alignment Search Tool

MS:Murashige and Skoog

RWC:Relative water content

PGP:Plant growth-promoting

DW: Dry weight

FW: Fresh weight

1. Chapter 1: Literature Review

1.1. Abstract

Drought and heat are major environmental stresses that continually influence the growth and development of plants. Under field conditions, drought and heat stresses occur more frequently in combination than alone. Their combined efforts reportedly have significantly greater detrimental effects on the growth and productivity of agriculturally important crops. Plant responses to abiotic stresses are quite complex and manifest in a range of developmental, molecular, and physiological modifications that lead to either stress sensitivity or tolerance/resistance. Maize (*Zea mays* L.) is known for its sensitivity to abiotic stresses, which often results in substantial losses in the crop's productivity. Hence, bioaugmentation, with microorganisms of agricultural importance, known as plant growth-promoting rhizobacteria is a promising and eco-friendly strategy to ensure long-term, sustainable maize production under adverse climatic conditions. Plant growth-promoting rhizobacteria have the potential to mitigate the adverse effects of drought and heat stress in plants. This is achieved through various mechanisms that have a direct or indirect effect on plant molecular and metabolic responses as well as plant physiology. This review aims to assess the current knowledge regarding the ability of PGPR to induce drought and heat stress tolerance in maize plants. Furthermore, we discuss genes that play a role in drought and heat stress response in maize and how PGPR influences their expression.

1.2. Introduction

Drought and heat are two critical environmental stress factors influenced by climate change (Barnabás *et al*., 2008). Both stresses continually affect the development and growth of plants (Kapoor *et al*., 2020; Manoj *et al*., 2018). Their resulting adverse effects play a significant role in the decline of agricultural productivity (Rizhsky *et al*., 2004; Savin and Nicolas, 1996). With the predicted increased frequency of water shortages and temperature extremes in future climates, extensive agricultural losses will only worsen. According to the Intergovernmental Panel on Climate Change (IPCC), on a global scale, surface air temperatures rose by 0.8 °C over the past century and are expected to be between 1.8 and 3.6 °C by the year 2100 (IPCC, 2007; IPCC, 2014). Precipitation levels are expected to vary greatly across the globe as experienced in semi-arid regions, where the average rainfall has declined severely, with an estimated 450 mm rainfall per year, which is well below the world's average of 860 mm per year (DWAF, 2004).

Some economically essential plants such as maize, rice, and wheat are acknowledged as sensitive crops to abiotic stresses, often leading to substantial losses (Bita and Gerats, 2013). Among these crops, maize is an essential staple grain crop in Latin America, Asia, as well as Sub-Saharan Africa and is primarily used for human consumption and animal feed production (Department of Agriculture, Forestry and Fishery, 2016). Maize requires 450 to 600 mm of water per season under normal circumstances, mainly acquired from soil water storages, and temperatures over 32 ºC are detrimental to maize growth (Department of Agriculture, Forestry and Fishery, 2016). Simulation results suggest that climate change scenarios involving the combination of increases in temperature with a reduction in rainfall resulted in greater average simulated maize yield reduction by 21, 33, and 50% under 1, 2, and 4 °C warmings, respectively (Tesfaye *et. al*., 2018). Lobell *et al.* (2011) showed that each degree rise in temperature above 30 °C resulted in a 1% reduction in maize grain yield under optimal growing conditions, and under drought stress, a 1.7% reduction was observed however, a reduction of up to 40% or more was observed under the combination of drought and heat stress. Similarly, Maseka *et al.* (2018) found that while drought stress reduced maize grain yield by 58%, the combination of drought and heat stress reduced maize grain yield by 77%, highlighting the detrimental effects of the combined efforts of these two stresses.

The response of plants to abiotic stresses is quite complex and is presented in a variety of developmental, physiological, and molecular modifications that initiate either stress tolerance/resistance or sensitivity (Harb *et al*. 2010). Detrimental effects on plants, such as disruption in cellular homeostasis, hindrance in growth and development, reduced yield, and plant death have been reported under heat and drought stress (Barnabás *et al*., 2008; Tiwar *et al*., 2019). To survive, plants must respond and adapt to such stresses. However, plants are challenged beyond their capacity to adapt (physically, biochemically, and molecularly) to current climate change-induced variations in temperature and precipitation patterns (Hussain *et al*., 2019). This is largely due to the lack of energy and resources required by plants to adapt to such abiotic stress conditions *e.g.* limited energy to produce stress response proteins such as late embryogenesis abundant proteins or heat shock proteins (Mittler. 2006).

The impact and mitigation of environmental stresses, predominantly drought, and heat, have been studied in isolation even though, under field conditions, these abiotic stresses exist more frequently in combination (Prasad and Staggenborg, 2008). The co-occurrence of both high temperatures and

drought has been shown to considerably increase the deleterious effects, such that the resulting consequence substantially surpasses the straightforward additive impact of the one stress (Prasad and Staggenborg, 2008). According to Mittle (2006), plant, metabolic and molecular responses to the combined effect of these stresses are unique and are impossible to extrapolate from the response induced by each of these different stresses applied individually. This is a result of the close association between temperature and plant water status thus making it impossible to distinguish whether abiotic stresses in the field are a result of heat or drought (Mittle, 2006).

There are many research studies aimed at developing technologies that promote sustainable agricultural practices, which favor increased crop productivity as well as enhanced resistance or tolerance against various stress factors (Ali *et al*., 2011; Ansary *et al*., 2012; D'Alessandro *et al*., 2014; Park *et al*., 2017; Sandhya *et al*., 2010; Zafar-ul-Hye *et al*., 2014). Beneficial plant-microbe interactions have been exploited and are considered promising focus areas for sustainable agricultural practices (Reddy *et al*., 2014). These beneficial soil microorganisms such as plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi produce large quantities of plant growth-promoting substances. They are also capable of directly and/or indirectly influencing the growth and morphology of plants thereby enhancing development and yield. Subsequently, these substances have been successfully applied as biofertilizers or phytostimulators in agriculture (Bano *et al*., 2013). Another remarkable feature is their capability to support plants under stressed environments. They can protect plants against deleterious effects of different environmental stresses to which crop plants are intermittently exposed such as drought and elevated temperatures (Chukwuneme *et al*., 2020; Hassan *et al*., 2020; Pereira *et al*., 2020). The use of PGPR, which is abundantly available in agriculture, offers an eco-friendly, inexpensive, and non-ethically restricted approach to solving the crisis revolving around drought and heat stress. Therefore, in this review, we provide an overview of the present knowledge about mechanisms utilized by PGPR at inducing tolerance in maize plants under drought and heat stress as well as their combined effects. We also highlight the maize drought and heat stress response genes affected by these abiotic stresses and how PGPR could alter the expression of such stress response genes.

1.3. Plant growth-promoting Rhizobacteria

The rhizosphere is a dynamic nutrient-rich habitat that is directly influenced by plant root exudates and harbors a vast variety of naturally occurring microorganisms (Guerrieri *et al*., 2020; Sayyed,

2019). Among these microorganisms, exists a group of bacteria known as plant growth-promoting rhizobacterium (PGPR) that augment plant growth and development (Sayyed, 2019). This group comprises different bacteria genera like *Bacillus, Serratia, Azospirillum, Arthobacter, Enterobacter, Burkholderia, Alcaligenes, Azotobacter, Pseudomonas, Klebsiella*, and many more. PGPR exerts several positive effects on plants, ranging from direct mechanisms aimed at plant nutrition and plant growth regulation to indirect effects related to biocontrol activity (Guerrieri *et al*., 2020). Another remarkable feature is their ability to induce plant tolerance under abiotic stress (Danish *et al*., 2020). As such, PGPR have been exploited and applied to seeds or crops to enhance or maximize plant growth and yield (Ahemad and Kibret, 2014).

Plant growth-promoting rhizobacteria meet the following criteria: (1) proficiency to colonize the plant root surface or rhizosphere, (2) potential to outcompete other microorganisms occupying the same physical space at the same time, and (3) potential to promote plant growth (Ahemad and Kibret, 2014). Furthermore, based on interaction, these PGPR can be separated into two categories, namely symbiotic and free-living (Singh, 2018). The former is known as intracellular PGPR (iPGPR) and inhabits root cells internally or the intercellular spaces present in the plant, while the latter is called extracellular PGPR (ePGPR) and exist outside plant part within the rhizoplane or rhizosphere (Hayat *et al*., 2010; Singh, 2018).

1.4. General PGPR mechanisms for mitigating drought and heat stress in maize

The biological and physicochemical properties of the soil are heavily altered during drought and heat stress, as a result, the soil becomes unsuitable for microbial and crop growth (Meseka *et al*., 2018; Tesfaye *et al*., 2018). However, the interactions that occur between PGPR and plants could alleviate such stresses. Several processes have been proposed for PGPR mitigated heat and drought stress tolerance in plants. These include modification in root morphology, phytohormonal levels, antioxidant defense, production of bacterial exopolysaccharides (EPS) and 1-aminocyclopropane-1-carboxylic (ACC) deaminase, and the accumulation of several compatible organic solutes like sugars, amino acids, and polyamines (Alia *et al*., 2011; García *et al*., 2017). Production of heat-shock proteins (HSPs), dehydrins, and volatile organic compounds (VOCs) also play a significant role in the acquisition of drought and heat tolerance (Kaushal *et al*., 2016).

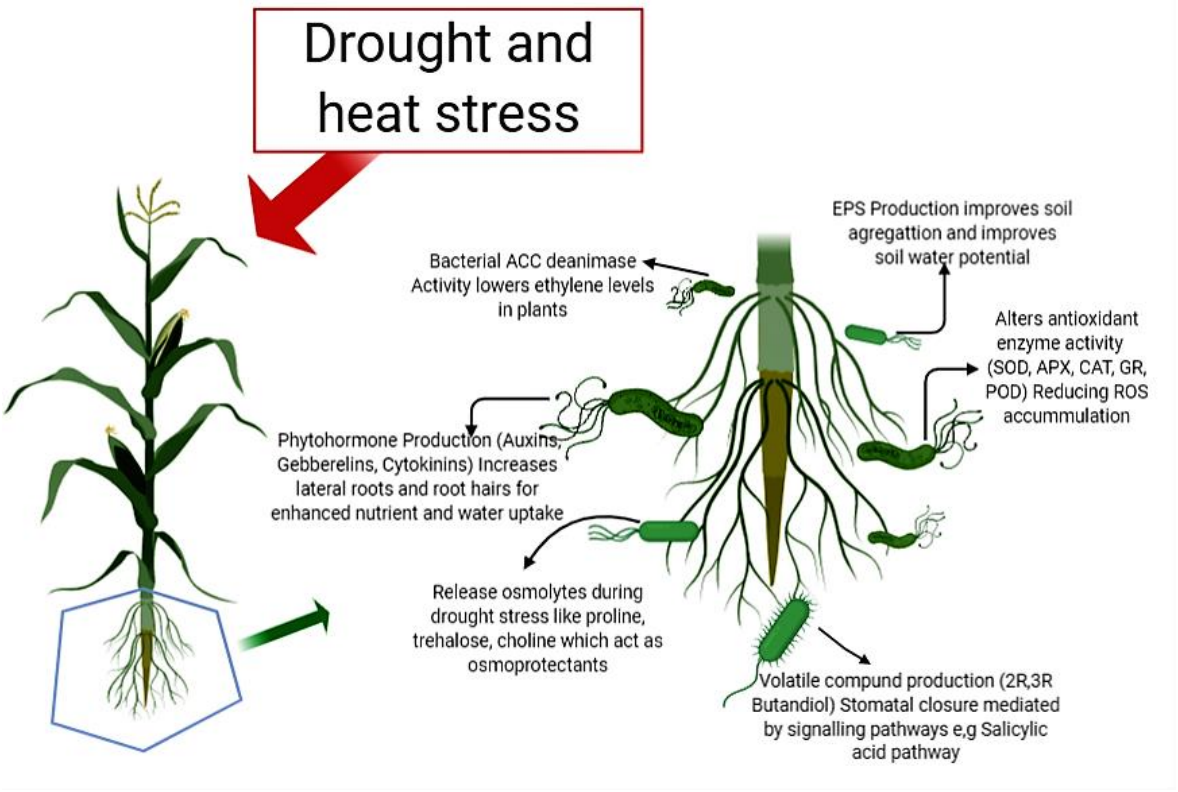

Figure 1: Mechanisms utilized by plant growth-promoting rhizobacteria (PGPR) at inducing resistance to plants subjected to drought stress.

Furthermore, PGPR can initiate chemical and physical changes in maize plants, which result in improved resilience to environmental stress through induced systemic tolerance (IST) ((Kannojia *et al.*, 2019). These defense mechanisms can cause substantial alterations in the plant's structural and functional characteristics that lead to abiotic resistance. These microorganisms can achieve this feat largely because in the same way plants suffer hydric stress or heat stress when the availability of water is low, or temperatures are high, bacteria can also be affected by this stress. However, bacteria can sense changes in soil, such as salt concentration, which is sensed by microorganisms as osmotic variations, or increased temperatures and respond accordingly (García et al., 2017). Therefore, plant growth-promoting rhizobacteria are quite specialized in their plant growth mechanisms thus are ideal targets for use in improving overall maize productivity.

1.5. Drought induced stress on maize plants: PGPR ameliorated stress

Drought induces water stress in maize plants primarily due to the limited supply of water to their roots and increases in transpiration rates (Lisar *et al.*, 2012). Drought triggers multiple effects that manifest at both the molecular and morphological level, and these can be noticed at all plant growth

stages when water deficit is experienced. Drought stress weakens the metabolic activity of maize, decreases biomass accumulation, and decreases the photosynthetic rate due to reduced chlorophyll content in leaves (Song *et al*., 2019). Moreover, stressed plants show decreased cell turgor and water potential in the extracellular matrix and cytosol, resulting in reduced cell size, leading to inhibited growth and failure to reproduce (Kapoor *et al*., 2020). Consequently, oxidative stress by the over-production of reactive oxygen species (ROS) causes wilting, further aggravating the adverse effects of drought stress (Anjum *et al*., 2017; Hussain *et al*., 2019) (Figure 2). Drought also affects stomatal closure and limits gaseous exchange and transpiration (Lisar *et al*., 2012). The unfavorable effects exhibited on nutrient transport, metabolism, and uptake result in decreased leave area and altered partitioning of nutrients among the different plant organs ((Kapoor *et al*., 2020; Hussain *et al*., 2019). Changes in cell wall elasticity disrupted homeostasis, and ion distribution in cells can be observed in water-stressed plants resulting in cell expansion and plant growth retardation. Plant growth-promoting rhizobacteria have unique properties that can help plants tolerate drought stress using various mechanisms as outlined below.

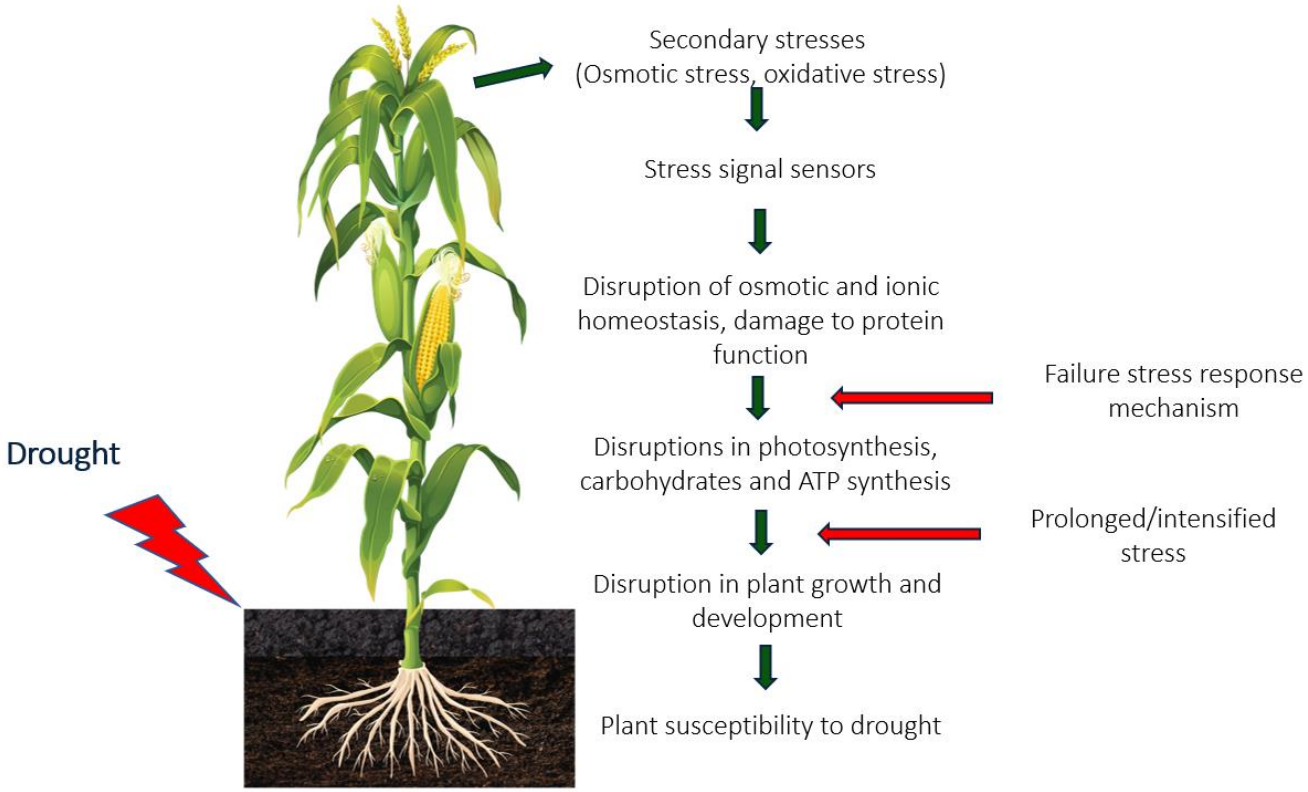

Figure 2: Typical overview of drought stress signaling pathways in drought sensitive plants.

1.5.1. Plant hormone production

Plant growth-promoting rhizobacteria have been shown to synthesize plant hormones or stimulate endogenous plant hormones that promote plant drought tolerance by inducing plant cell growth and division (Egamberdieva, 2013). The application of synthetic and natural plant growth factors has been demonstrated to initiate plant growth enhancement under environmental stresses (Ahmad *et al*., 2019; Vurukonda *et al*., 2016; Tayyab *et al*., 2020). These plant growth factors include gibberellic acid, uniconazole, brassinolide, 1-aminocyclopropane-1-carboxylic, jasmonic acid, ABA, indole acetic acid (IAA), salicylic acid, and zeatin (ZT) (Pavlović *et al*., 2018; Ullah *et al*., 2018). Ethylene and Abscisic acid are plant growth suppressing hormones, whereas auxins, salicylic acid, 1-aminocyclopropane-1-carboxylic acid, gibberellic acid, and ZT act as growth promoters, thus their ratio dictates plant growth responses, especially under stressed conditions (Weiss and Ori 2007). Since maize plants are intimately linked with PGPR that reside within plant tissues or the rhizosphere, microbial-derived phytohormones strongly influence the plant's physiological processes and metabolism under hostile environments. Indole acetic acid, a particularly well-studied phytohormone secreted by PGPR, was shown to play a role in numerous growth and developmental events such as cell division, elongation, and differentiation (Alqarawi *et al*., 2017; Asgher *et al*., 2015).

In one study, the co-inoculation of two IAA producing PGPR *Cupriavidus necator* 1C2 (B1) and *Pseudomonas fluorescens* S3X (B2) on maize plants under moderate water deficit mitigated the negative effects of drought and increased shoot biomass up to 89% (Pereira *et al*., 2020). Maize plants inoculated with *Pseudomonas fluorescens* strain 153 had increased auxin, abscisic acid, cytokinin, and gibberellin content in the leaves, as well as alleviated drought stress (Ansary *et al*., 2012). In contrast, compared to maize control plants, those inoculated with *Herbaspirillum. seropedicae*, strain Z-152 and *Azospirillum brasilense* strain SP-7 exhibited greater resistance to the negative effects of drought stress with lower levels of plant growth-suppressing hormones, namely ABA and ethylene (Curá *et al*., 2017). These studies indicate that the application of PGPR can and induce tolerance to water deficit stress conditions in maize by regulating the phytohormonal content.

1.5.2. ACC deaminase activity

The enzyme ACC deaminase is involved in mitigating the effects of drought stress in plants by regulating ethylene levels and is produced by PGPR (Zafar-ul-Hye *et al*., 2014). However, unlike the direct production of hormones by PGPR and uptake by plants, ACC deaminase activity indirectly lowers ethylene levels by breaking down ACC in plant tissues, thereby lightening the negative effects of ethylene (Pandey and Gupta, 2019). Ethylene regulation is dependent on biological and environmental stress factors. Its biosynthetic pathway includes the conversion of S-adenosylmethionine (S-AdoMet) to ACC (immediate precursor of ethylene) by the enzyme 1-aminocyclopropane-1-carboxylate synthase (ACS) (Hardoim *et al*., 2008). Plants subjected to stress conditions have their root and shoot growth reduced due to the hormone ethylene endogenously regulating plant homeostasis. Plant growth-promoting rhizobacteria can sequester and degrade ACC into α-ketobutyrate and ammonia using ACC deaminase, providing plants with N2 and energy (Pandey and Gupta, 2019).

Such an incidence was observed in maize plants inoculated with drought-tolerant and ACC deaminase producing (0.903 and 0.899μmol aKB mg^{-1} h^{-1} respectively) *Streptomyces pseudovenezuelae* and *Arthrobacter arilaitensis* strains. These PGPR increased plant growth and reduced the undesirable effect of drought stress when used as bioinoculants on maize plants (Chukwuneme *et al*., 2020). Co-inoculated plants significantly increased maize shoot weights by 81.9% at field capacity, 44.9% at moderate water application, and 139.5% at zero water application, while lower shoot weights were observed in un-inoculated plants at the same water regimes. Combination of four strains (*P. fluorescens* P1, *P. fluorescens* P3, *P. fluorescens* P8, *P. fluorescens* P14) with ACC deaminase activity among other PGP traits, significantly increased the yield traits of sweet corn such as ear and (44%) and canned seed yield (27%) over control under limited availability of irrigation water (Zarei *et al*., 2020). Correspondingly, a combination of ACC-deaminase containing PGPR, *A. xylosoxidans,* and biochar (0.75%) significantly increased, maize growth and productivity under drought stress. Maize plants exhibited enhanced grain yield plant-1, photosynthetic rate, stomatal conductance, chlorophyll a, total chlorophyll, and carotenoids contents up to 200, 213, 113, 152, 148, and 284%, respectively over control under severe drought stress (Danish *et al*., 2020).

1.5.3. Volatile compound production

Under various environmental stresses, plants produce volatile organic compounds (VOCs) which serve as signals that prime and prompt systematic response within the plant and surrounding ones (Ahemad and Kibret, 2014). In addition, VOCs have been shown to quench reactive oxygen species (ROS), stabilize cell membranes, and may play a key role in regulating plant growth, development, and senescence by interacting with plant hormones (Brilli *et al*., 2019). However, there is little information on how PGPR VOCs improve drought stress tolerance, specifically in maize plants. Nevertheless, *Bacillus mojavensis*, a close relative of *B. subtilis* and an endophytic bacterium of maize, was shown to produce VOCs acetoin and 2,3-butanediol, which were also linked to plant growth-promoting attributes (Bacon and Hinton, 2002; Rath *et al*., 2018). *Enterobacter aerogenes* could produce 2,3-butanediol (BD) and rendered maize plants more resistant to biotic stresses (D'Alessandro *et al*., 2014). Similarly, VOCs produced by *P. pseudoalcaligenes* promoted the growth of plants and induce resistance to drought stress in maize. Increased shoot (118.2%) and root (133.3%) length were observed in plants exposed to the VOCs of *P. pseudoalcaligenes* compared to the control plants under drought stress (Yasmin *et al*., 2020). Moreover, the induced systemic resistance was induced by the VOCs via complex mechanisms.

1.5.4. Osmolyte Production

Osmolytes or compatible solutes reportedly take part in inferring tolerance to plants subjected to drought. Under water-stressed conditions, plants adjust their metabolic processes resulting in the accumulation of compatible solutes, namely polyamines, polyhydric alcohols, sugars, betaines, quaternary ammonium compounds, other amino acids, and proline (Ahemad and Kibret, 2014). Sandya *et al*. (2010) reported increased relative water content, leaf water potential, and plant biomass due to the accumulation of soluble sugars, free amino acids, and proline in maize plants when they were primed with *Pseudomonas putida* GAP-P45. Plant growth-promoting rhizobacteria inoculated plants under drought-stressed conditions result in increased concentration of proline due to upregulation of proline biosynthesis pathways, thereby protecting proteins and membranes from stress, thus maintaining cell turgor (Sandhya *et al*., 2010). Plant inoculation with proline producing PGPR supplements the existing proline concentration within a plant (Ansary *et al*., 2012). Gusain *et al*. (2015) concluded that accumulation of proline in plants inoculated with PGPR indicates plant tolerance to drought stress. Maize seedlings exposed to VOC producing *Pseudomonas pseudoalcaligenes* exhibited an increase by 135% in roots and 102% in

shoots in proline contents compared to control plants under drought stress, however a decrease in total amino acid content by 82.9% was observed compared to control plants. This led to an increase in the dry weight of roots and shoots by 84.2% and 140%, respectively, compared to control under drought-stressed (Yasmin *et al*., 2020). *Azospirillum* (Az19) could produce trehalose (soluble sugar) (4.4 ug mg-1 FW) and was 388% greater than that produced by the reference strain (García *et al.*, 2017). Moreover, maize inoculation with Az19 improved the plant response to stress increasing 11.6 times the proline content of roots compared with uninoculated controls. These studies demonstrate a positive association between osmotic stress-tolerance and trehalose. It is hypothesized that minute amounts of trehalose are transported to maize roots and signal the plant stress-resistant pathways (Rodriguez *et al*., 2009). Correspondingly, among three PGPR strains, namely *Klebsiella variicola* F2 *Raoultella planticola* YL2 and *Pseudomonas fluorescens* YX2, YL2 substantially enhanced the accumulation of choline (1.2 to 1.7 μmol g^{-1} DW)) and GB (1.6 to 2.5 μmol g^{-1} DW) better than the other strains and in turn improved leaf relative water content and dry weight under drought stress (Gou *et al*., 2015). This shows that PGPR-produced osmolytes can not only affect direct action on cell turgor maintenance in maize plants under drought stress but can also indirectly affect plant drought tolerance by acting as signaling molecules.

1.5.5. Exopolysaccharide (EPS) production

Rhizobacteria can produce EPS which are thought to participate in mitigating the effects of drought stress. Bacteria secrete EPS in high quantities, especially under drought stress situations, to protect themselves from inhospitable conditions since they are directly dependent on water availability for protein and polysaccharide production (Vurukonda *et al*., 2016). Exopolysaccharides are released into the soil as slime and capsular materials that are subsequently absorbed by clay surfaces using mechanisms such as anion adsorption, cation bridges, Van der Waals forces, and hydrogen bonding (Sandhya *et al*., 2009). This leads to the formation of a defensive rhizosheath around soil aggregates that provides an environment capable of holding more water and decreasing drying up time, as a result, the plant roots are protected from desiccation for longer periods (Khan and Bano, 2019). Exopolysaccharides effectively improve surface attachment of bacteria, biofilm formation, microbial aggregation, and plant-microbe interactions. Besides increasing water potential around plants, EPS also improves water permeability, thereby increasing nutrient uptake by microbes and plants (Naseem *et al*., 2018).

Naseem and Bano (2014) found that EPS-producing bacteria *Alcaligenes faecalis*, *Pseudomonas aeruginosa*, and *Proteus penneri* combined with their respective EPS extracts improved maize plant biomass, soil moisture content, leaf area as well as shoot and root length. The strain *Bacillus velezensis* D3 with ACC-deaminase (152 μmol kg^{-1} $h^{-1)}$ and exopolysaccharides activity (83.0 μg ml^{-1}) was significantly better in colonizing maize roots and resulted in improved in root length (88%) and shoot dry weight (152.4%) (Nadeem *et al*., 2020). Similarly, application of two ACC-deaminase Containing PGPR strains *Pseudomonas syringae* S1 and *Pseudomonas fluorescens* S2, and a full dose of recommended fertilizers under dual drought and salinity stress resulted in the increased number of cobs $plant^{-1}$ (19.05%), plant height (58.14%), number of grains per cob (28.29%), and grain yield (55.14%) over control (Zafar-ul-Hye *et al.*, 2014).

1.5.6. Scavenging of ROS and alteration of antioxidant enzyme activity

Exposure Exposure of plants to environmental stresses such as extreme temperatures and drought can lead to oxidative stress that has detrimental secondary effects on plant cells due to accumulating reactive oxygen species (ROS) (Vurukonda *et al*., 2016). Reactive oxygen species are partially reduced or activated forms of atmospheric oxygen (O_2), which are by-products of aerobic metabolism due to the impairment of the electron transport system (Choudhury *et al*., 2017). Normally, ROS is controlled by a delicate balance between production and scavenging via a highly efficient scavenging mechanism (Hasanuzzaman *et al*., 2019; Tripathi *et al*., 2020). Examples of ROS include hydroxyl radicals (OH), superoxide anion radicals (O2−), hydrogen peroxide (H_2O_2), and alkoxy radicals (RO) (Mittler, 2017). Uncontrolled ROS can damage normal cell functions by causing oxidative damage resulting in DNA nicking, impaired production of amino acids, photosynthetic pigments, and increased lipid peroxidation (Farooq *et al*., 2009; Raja *et al*., 2017). Organelles that are primary targets for ROS production under drought conditions include mitochondria, chloroplast, and peroxisomes (Vurukonda *et al*., 2016).

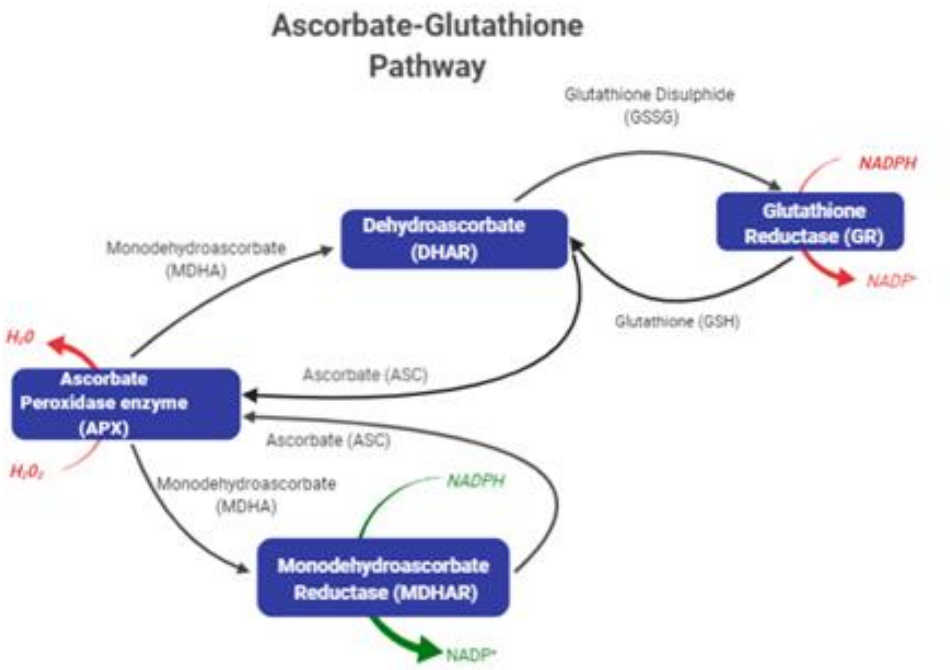

Figure 3: The Ascorbate–Glutathione Pathway, Reactive Oxygen Species (ROS) scavenging by antioxidant enzymes (Zechman, 2014).

To overcome these effects, plants possess an antioxidant defense system which is made up of both enzymatic and non-enzymatic components in plant cells (Das *et al*., 2016). Scavenging of ROS by both components of the antioxidant defense system alleviates oxidative damage in plant tissues. The non-enzymatic components include α-tocopherol, reduced glutathione, β-carotenes, and ascorbic acid (Farooq *et al*., 2009). Whereas the enzymatic components include glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), monodehydroascorbate reductase (MDHAR), guaiacol peroxidase (POX), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione-S- transferase (GST) and glutathione peroxidase (GPX) (Figure 3) (Sen, 2012).

Plant growth-promoting bacteria have been shown to produce antioxidants such as phenolic acids and soluble sugars as well as induce or suppress antioxidant defense enzymes in favor of plant endurance under water deficit conditions. For instance, Sandhya *et al*. (2010) inoculated maize plants subjected to drought stress with five *Pseudomonas* spp. (*P. putida, P. stutzeri, P. entomophila, P. montelli,* and *P. syringae*) and reported reduced antioxidant enzyme activity in

inoculated plants compared to the controls (uninoculated). Moreover, plants resulted in reduced oxidative stress and improved plant health. Another study also showed that maize plants that developed tolerance against drought stress when inoculated with *Bacillus* spp. resulted in decreased antioxidant enzyme activity (APX and GPX) compared to uninoculated control (Vardharajula *et al*., 2011). In another experiment, maize plants developed resistance to drought stress when inoculated with *Bacillus* spp. due to reduced activity of antioxidant enzymes APX and GPX while maintaining plant health (Vardharajula *et al*., 2011). Likewise, Khan and Bano (2019) showed that the combined treatment of *Planomicrobium chinense, Bacillus cereus*, and salicylic acid significantly decreased (46%, 63%, and 38%) the activities of antioxidant enzymes catalase, POD, and APOX in the sensitive maize genotype respectively. This translated to a maximum increase of 59 and 75% in the shoot and root dry weight of maize respectively. In contrast, *Pseudomonas* application on maize transplant plants under severe water stress increased the CAT activity by 50% compared to well-watered plants, alleviating the adverse effects of water stress on physiological (RWC increased by 8%) characteristics of maize (Rezazadeh *et al*., 2019). In contrast, inoculation of basil (*Ocimum basilicum L.)* plants under water stress with *Pseudomonades* sp. significantly enhanced the activity of the CAT enzymes, but the highest activity was observed for GPX and APX in plants inoculated with a combination of three species (*Bacillus lentus*, *Azospirillum brasilens*, and *Pseudomonades* sp.) (Heidari and Golpayegani, 2012). This highlights that induced plant-drought tolerance by altering antioxidant enzymes, can result either from the activation or suppression of their activity. Studies such as these prove alteration of antioxidant activity in plants by PGPR can be beneficial for enhancing drought tolerance of plants under stressed conditions.

1.6. Effects of PGPR on heat stressed maize plants

The water status of plants is of great significance under varying climate conditions. Commonly, plants maintain a high cell turgor irrespective of temperature changes when little moisture is available by stabilizing water content in their tissues; however, the continued increase in temperatures proves fatal under water deficit conditions (Machado and Paulsen, 2001). Under field conditions worldwide, heat generally occurs in conjunction with water stress (MT *et al*., 2020).

Figure 4: General plant response to the effects of heat stress

Generally, the loss of water under heat stress is greater during the day, primarily due to elevated transpiration rates that ultimately impair vital physiological activities in plants, and maize is no exception. Instant decreases in leaf tissue water content, altered root conductance, extensively enhanced respiration, and opening of stomata have been observed under heat stress (Figure 4) (Manoj *et al.*, 2018). Net photosynthesis is severely hindered at leaf temperatures over 38°C, and the severity is greater when the temperatures are increased rapidly instead of gradually (Vinayan *et al.*, 2019). Moreover, high temperatures lead to water deficit and high evaporation that disturbs cellular homeostasis. The consequent increased turnover of enzymes leads to extensive hindrances in plant development and growth, and possibly plant death (Rizhsky *et al.*, 2002). Even so, heat stress effects on maize can be reduced with the application of PGPR. There is however limited knowledge available on PGPR mitigated heat stress in maize plants.

1.6.1. ACC deaminase activity

The role of ethylene in plant response to abiotic stresses is widely documented. In general, the ethylene levels in plants in response to heat stress are increased, reducing the growth rate as an immediate response to stress (Sharma *et al.*, 2019). However, the intensity and duration of the

stress can negatively influence the intended effects of ethylene (Savada *et al*., 2017). An initial small peak in ethylene levels initiates a protective response to the plant however, after some time, a second larger peak reportedly induces abscission, chlorosis, and senescence, all of which are detrimental for plant survival (Cicchino *et al*., 2013; Glick *et al*., 2005). Reduction in ethylene levels allows the plant to be more resistant to a wide variety of abiotic stresses, including heat (Chatterjee *et al*., 2017).

Studies focusing on PGPR mitigated heat stress in maize plants are scarcely available. Even so, ACC deaminase-producing soil microorganisms can reduce harmful ethylene levels through the sequestration and hydrolysis of plant-produced ACC, resulting in decreased levels in the plant (Dubois *et al*., 2018). Bacterial isolates from the agro-climatic zone producing the enzyme ACC deaminase have been shown to display heat tolerance. One study showed that most of the ACC deaminase-producing isolates that demonstrated tolerance towards high temperature (45 °C) were *Bacillus* spp. (Misra *et al*., 2017). Mukhtar *et al*. (2020) showed that heat stress enhanced EPS production and cleavage of ACC into a-ketobutyrate and ammonia due to ACC-deaminase producing *Bacillus cereus*. Could survive temperatures of up to 60 °C. The tolerance and ACC deaminase-producing ability displayed by these PGPR show great promise in alleviating heat stress in maize plants. The alleviation of heat stress using ACC deaminase producing PGPR has been shown on plants such as wheat, which is also considered a grain plant (Meena *et al*., 2015).

1.6.2. Alteration of antioxidant enzymes and scavenging of reactive oxygen species

Exposure to high temperatures induces the production of highly toxic reactive oxygen species (Sarkar *et al*., 2018). Reactive oxygen species disturb the cellular homeostasis by damaging different cellular, sub-cellular membranes, and macromolecules (Foyer and Noctor, 2011). These molecules also disturb the photosynthetic machinery (PSI and PSII) and the stability of cell membranes by triggering electrolyte leakage and autocatalytic lipid peroxidation (Tiwari *et al*., 2019). Like other abiotic stress factors, these ROS molecules also act as signals and activate the antioxidative defense mechanism to help the plant cope with high-temperature stress, protecting cells from oxidative injury (Mittler *et al*., 2012).

Microbial inoculations reduce injury to cell membranes and alter the activity of several antioxidant enzymes such as APX, CAT, and SOD under elevated temperatures. Plants usually increase the activity of these antioxidant enzymes to alleviate oxidative damage in plant tissues

resulting from ROS however, heat stress in maize plants reportedly reduces antioxidant activity (Manoj *et al*., 2018). The application of PGPR can decrease ROS accumulation, thus reducing the water deficit effect on the activity of these enzymes (Farooq *et al*., 2009). One way is by augmenting the plants' phenolic content which constitutes the non-enzymatic antioxidants (Vardharajula *et al*., 2011). Plants can accumulate phenolic compounds as a response to heat stress, and these can scavenge ROS (Soengas *et al*., 2018). Phenolic compounds help inhibit the action of oxidizing enzymes by forming complexes with metals, which catalyze oxygenation reactions (Król *et al*., 2014). Heat stress has been shown to enhance the exogenous production of antioxidants in microorganisms. *Bacillus* isolates (HT1, HT3, and HT4) and *Pseudooceanicola marinus* HT2 subjected to heat stress (56 °C) exhibited increased levels of flavonoids and polyphenols in all strains and glutathione (GSH) in HT2 (Hassan *et al*., 2020). The microbes also displayed improved CAT, SOD, GPX, and POX activity under heat stress compared to the control. As such, exogenously produced PGPR antioxidants have the potential to reduce oxidative damage in heat-stressed maize.

Currently, there are no documented studies that report the effects of PGPR on the antioxidant enzymes of maize subjected to heat stress. However, numerous studies have been conducted under drought stress. Moreover, the exogenous application of antioxidants in maize under heat stress has been shown to mitigate the detrimental effects of heat stress on maize. Exogenous application of ascorbic acid in late maize by seed priming and as a foliar increased the activity of SOD, CAT, and POX and was translated to improved grain yields (Mgha-1) by 24.7-24.9% in 2007 and 18.5-24.0% in 2008 (Ahmad *et al*., 2017). Likewise, exogenously applied ascorbic acid improved SOD, CAT, POD activity by 6, 47, and 11% in NP maize and by 3, 42, and 11% in LP maize respectively. This translated to a grain yield increase (Mg ha^{-1}) of 3% in NP and 24% LP (Iqbal *et al*., 2020). This highlights the potential of PGPR-produced antioxidants in ameliorating heat stress effects in maize plants.

1.6.3. Production of phytohormones

Phytohormones act as chemical messengers coordinating various signal transduction pathways between cells during the abiotic-stress response (Sabagh *et al*., 2020). These include abscisic acid (ABA), gibberellins (GAs), ethylene, brassinosteroids, salicylic acid (SA), jasmonates (JAs) cytokinins (CKs), and auxins (IAA) (Wani *et al*., 2016). Among these, IAA is widely common,

and the most studied auxin produced by plants and bacteria. IAA plays a role in root development, germination of seeds, apical dominance, cell division, and differentiation (Hakim *et al*., 2021). It also influences processes like biosynthesis of metabolites, photosynthesis and plays a role in stress resistance (Maheshwari *et al*., 2015). Heat stress reportedly suppresses the biosynthesis of phytohormones such as IAA and GA by downregulating genes involved in their biosynthesis, thus hampering their functions (Sharma *et al*., 2018; Wu *et al*., 2016).

Even so, the exogenous application of phytohormones has been shown to alleviate the destructive effects of heat stress on maize. For instance, exogenously applying SA resulted in significantly enhanced plant growth parameters such as grain yield (27%), growth rate (13%), photosynthetic pigment (17%), relative water content (16%), membrane stability index (26%) compared to the control in late-planted spring maize (Iqbal *et al*., 2020). Agreeably, Ahmad *et al*. (2017) also observed improved morphological, biochemical, physiological, and yield-related traits such as grain yield (19.3-21.2%) and grain quality of late spring sown maize in 2007 and 2008 when SA was exogenously applied through seed priming. Moreover, in both years, higher peroxidase (8.7-60.3%), catalase (39.4- 74.3%), and superoxide dismutase (21.0-31.1%) were reported in plants primed with SA than the control (Ahmad *et al*., 2017). Exogenous application of ABA in heat-stressed maize induced the production of heat shock proteins and strengthened the tolerance of PSII to heat stress (Maestri *et al*. 2002; TAO *et al*., 2016).

Reports suggest that more than 80% of microbes inhabiting the rhizosphere can synthesize and release phytohormones (Ole´nska *et al*., 2020). Like drought stress, PGPR can produce high levels of phytohormones under heat stress and show great potential in alleviating its detrimental effects in maize plants. *Pseudomonas putida* AKMP7 could produce IAA and GA under both ambient (28°C) and high temperature (50°C) (Ali *et al*., 2011). A total of 61.9 and 32mg mg^{-1} of IAA were produced under ambient and heat stress respectively. With GA, 0.43 and 0.18mg mg^{-1} of GA were produced under ambient and heat stress respectively. Likewise, *Bacillus cereus* SA1 could tolerate temperatures of up to 45°C and produced significant amounts of IAA (2.4µg ml^{-1}) and GA (1.3µg ml^{-1}) (Khan *et al*., 2020). Similarly, in a study conducted by Park *et al*. (2017), high levels of jasmonic acid (JA), gibberellic acids (GA4, GA7, and GA12), and IAA were recorded in plants inoculated with *Bacillus aryabhattai* SRB02 under heat stress. Such physiological changes are also conventional markers for integrated stress response (ISR), consequently suggesting that plant

resistance to heat stress-mediated by bacteria is associated with ISR induction (Dimkpa *et al*., 2009). Few studies have been conducted on the amelioration of heat stress in maize using IAA producing PGPR. However, IAA-producing microbes have been shown to mitigate heat stress effects on other plants such as wheat and tomato seedlings **(**Abd El-Daim *et al*., 2014; Ali *et al*., 2011; Khan *et al*., 2020; Mukhtar *et al*., 2020). The ameliorating effects of IAA producing PGPR on plants show promise, and these effects could potentially be observed under heat stress maize plants as well.

1.7. Effects of drought, heat and their combined efforts on maize stress response genes

Temperatures above the normal optimum and water below the required amount are interpreted as heat and drought stress by all living organisms. These two abiotic stresses are more prevalent in combination than as isolated phenomena under field conditions (Prasad and Staggenborg, 2008). In addition, the combined efforts of heat and drought stress have unique effects on various plant physiological, developmental, growth, yield, and genetic parameters suggesting that plants have different response mechanisms to combined stresses than their individual stress (Jagtap *et al.,* 1998). Research has however not been conducted extensively on how stress combination affects crop productivity, as compared to singly isolated effects, especially in maize. Even so, the few studies that were aimed at characterizing the consequences of both heat and drought stress on plants reported significantly enhanced negative effects on the growth and productivity of crops for the combined efforts of these stresses compared to each stress applied in isolation (Jian and Haung, 2001; Prasad and Staggenborg, 2008). Alterations in plant molecular mechanisms such as transcript expression, accumulation of lipids, and photosynthesis have been reported under the combined stresses of drought and heat (Rizhysky *et al*., 2004).

To elucidate the molecular mechanisms of drought and heat stress in plants, various studies have been conducted. Genes regarded as regulators, influencing heat and drought resistance of plants have been identified and are well-studied. Regulatory proteins encoded by these genes include heat stress transcription factors (HSFs), heat shock proteins (HSPs), dehydrins, late embryogenesis abundant (LEA) proteins, MYC and MYB transcription factors, and transporter proteins among many (Min *et al*., 2016). Typical regulation of HSPs in plants during heat stress is illustrated in Figure 5.

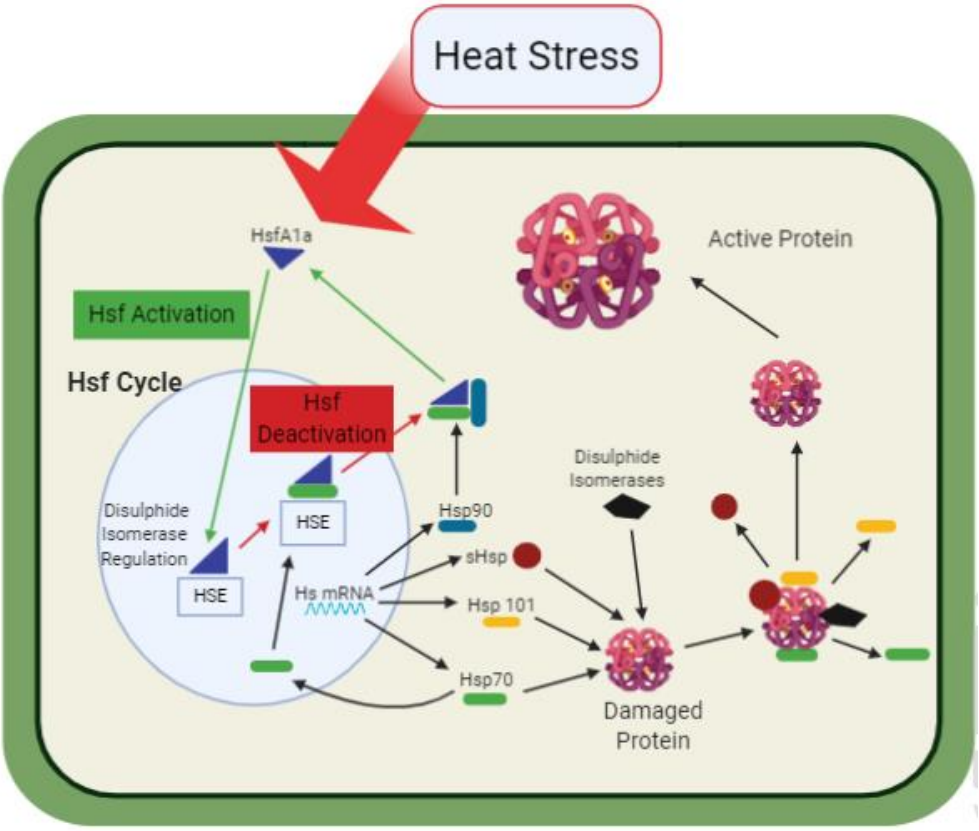

Figure 5: Typical illustration on how heat stress transcription factors (HSFs) influences the expression of heat shock proteins (HSPs) during heat stress response (Baniwal *et al.*, 2004).

During drought stress alone, suppression subtractive hybridization (SSH) conducted on maize plants showed a significant upregulation of 113 expressed sequence tags (ESTs) in roots and 67 ESTs in the leaves. Fifty-seven of the ESTs were common for both roots and leaves (Zheng *et al.*, 2004). Moreover, as time progressed (2, 6 hours), the induced transcripts in the roots and leaves increased compared to initial exposures of drought stress. For instance, the glutathione S-transferase (GST7) gene involved in the detoxification of ROS was expressed 10-fold more at 6 hours than the unstressed roots tissues compared to earlier stages of stress. Similarly, glutathione reductase transcripts showed increased expression in both roots (1.7- and 2-fold) and leaves (1.7 and 2.2-fold) of maize plants after 10 and 12 days of drought, respectively (Aslam *et al.*, 2015). The transcription factor DRE-binding factor (DBF1 gene) showed a 7-fold increase after 2 hours, whereas Ras-related-protein (rab17) its target gene, showed an 18-fold increase after 6 hours in drought-stressed maize (Zheng *et al.*, 2004). However, after 24 and 72 hours of drought stress, the expression of rab17 had accumulated 24-fold and 56-fold respectively compared to unstressed

maize plants (Zheng *et al.,* 2004). RAB17 is a group 3 LEA protein linked to induction of drought resistance and prevention of cells from shrinking due to water loss (Aslam *et al*., 2015). The expression of EMB564 and MLG3, which are group 1 and 2 LEA proteins respectively, have previously been described under water deficit conditions (Aslam *et al*., 2015). Using RT-qPCR, the transcript level of *ZmbZIP4* was rapidly and strongly induced by polyethylene glycol (PEG) (11-fold after 2 hrs) and heat (10-fold after 6 hrs) treatments (Ma *et al*., 2018). The transcription factor gene ZmbZIP4 is a positive regulator of plant abiotic stress responses and is involved in root development in maize. In maize, the ZmDBF3 member of the DREB transcription factor, which acts as a regulatory factor involved in multiple stress response pathways, was induced by drought (22-fold after 1 hr) and high temperature (34-fold higher after 5 hours) (Zhou *et al*., 2016).

One study applied comparative transcriptomics analysis to understand the differential expression of genes (DEGs) in maize ears at the V9 stage, kernels, and ear leaf at the 5DAP (days after pollination) under drought stress conditions. The study found that more expressed transcripts were identified in the ear and kernel compared to the ear leaf. A total of 1136 genes were upregulated, and 689 genes were downregulated in the ear, 2357 were upregulated and 1402 were downregulated in the ear leaf, and 2627 were upregulated and 3565 were downregulated in the kernel (Wang *et al*., 2019). When comparing the DEGs in different tissues, the kernel shared 662 DEGs with the ear and 1014 DEGs with the ear leaf, while only 141 DEGs were shared in the ear and ear leaf. Some of which are involved in the ABA-, NAC-mediate signaling pathway, osmotic protective substance synthesis, and protein folding response.

Transcriptional analysis has shown that low and high molecular weight HSP have increased transcript expressions in response to drought and heat stress in maize (Hu *et al*., 2010). Using MALDI-TOF mass spectrometry, it was observed that sHSP26, sHSP17.4, and sHSP17.2 were highly expressed in maize leaves in reaction to drought stress (Hu *et al*., 2010). Lund *et al*. (1998) also reported a maize sHSP22, which showed a dramatic increase in expression under heat stress and a decline after mitigation of the stress. This indicates that maize mitochondrial HSP22 may have a protective role against heat stress. However, no changes in the expression levels of the higher molecular weight HSP70 under heat stress were observed (Lund *et al*., 1998). The protein was greatly expressed under heat-stressed and non-stressed conditions, probably for constitutive folding of proteins or to afford pre-emptive protection for stressful events (Lund *et al*., 1998).

On the other hand, the analysis of the plant transcriptome under combined heat and drought stress depicts novel defence reaction patterns that include partial combination multiple multigene defence pathways (Rizhsky *et al*., 2002; Rizhsky *et al*., 2004; Zhao *et al*., 2016). Furthermore, the characterisation of plant physiological response under heat, drought stress or their combined effects shows that the combination of stresses has multiple unique aspects. For instance, the simultaneous occurrence of low photosynthesis with high respiration, high leaf temperatures and closing of stomata (Mittler, 2006). There exists a strong correlation between the physiological analysis and transcriptome profiling of plants subjected to drought or heat stress individually or in combination; however, there is a gap on how maize plants respond to the combination of drought and heat stress at transcriptomic level.

Even so, Zhao *et al*. (2016) found that inducing drought stress or the combination of heat and drought stress in maize downregulated RAB17 protein, glutathione S-transferase GST6, ABA-responsive protein, MTN3, aquaporin PIP2-6, and dehydrin proteins. Furthermore, heat stress and combined stress conditions upregulated 36 proteins, of which 20 were heat shock proteins (HSPs), and these included 14 small HSPs (sHSPs). Drought stress only slightly affected these proteins. The study also found that under the combined stress, ABA stress and ripening-inducible-like protein, ethylene-responsive protein, and ABA stress ripening protein 2 were highly expressed, however under heat stress, these proteins were downregulated (Zhao *et al*., 2016). Similarly, Rizhsky *et al.* (2002) found that 8 HSPs were upregulated in combined drought and heat stress, 4 of the same HSPs were upregulated under heat stress, and only 1 HSP was upregulated under drought stress in tobacco plants. Hussain *et al*. (2019) showed an increased regulation of SOD by combined drought and heat stress compared with their individual effects in two maize cultivars (Xida 889 and Xida 319). However, CAT and APX regulation in Xida 319 and Xida 889 was significantly decreased under stress conditions compared to control conditions. Moreover, a severe decline of CAT, POD, and APX occurred under heat stress, followed by drought and drought + heat combine stress. This places emphasis on the fact that plant molecular and metabolic responses to the combined effects of heat and drought stress are unique from the response observed from each of the stresses is applied individually.

Plants such as *Arabidopsis*, subjected to transcriptomic DNA array showed that 262 transcripts were upregulated under heat stress and 1,075 transcripts were upregulated under drought stress,

with only 29 transcripts overlapping under these conditions (Rizhsky *et al*., 2004). Correspondingly, for down-regulated transcripts, 496 and 279 transcripts were reported under drought and heat stress, respectively, with an overlap of 48 transcripts (Rizhsky *et al*., 2004). Transcripts specific for a combination of drought and heat stress belonged to several different groups including HSPs, proteases, starch degrading enzymes and lipid biosynthesis enzymes, transcripts encoding signal transduction proteins, and transcripts encoding membrane channels (Rizhsky *et al*., 2004). In a transcriptomic cDNA array study conducted by Rizhsky *et al*. (2002) on tobacco plants, transcripts upregulated under drought stress, such as those encoding glycolate oxidase, catalase, and dehydrin, and transcripts upregulated under heat stress, such as ascorbate peroxidase and thioredoxin peroxidase, were downregulated under the combination of heat and drought stress. In contrast, some transcripts were specifically upregulated during a combination of heat and drought stress, namely glutathione peroxidase, alternative oxidase, pathogenesis-related proteins, phenylalanine, ammonia-lyase, an ethylene response transcriptional co-activator, and a WRKY transcription factor (Rizhsky *et al*., 2002). This places emphasis on the fact that plant molecular and metabolic responses to the combined effects of heat and drought stress are unique from the response observed from each of the stresses is applied individually.

In wheat plants, HSP70, molecular chaperones, and other high and low molecular weight HSPs were upregulated during drought and combined drought and heat stress, relative to the control and heat stress alone (Grigorova *et al*., 2011). The molecular chaperone, HSP70, was expressed more than eight-fold in plants under drought and ten-fold in combined heat and drought-stressed plants, compared to their respective controls (Grigorova *et al*., 2011). Similarly, the expression pattern of all 21 *Arabidopsis* HSFs during a combination of drought and heat stress was different from that observed during drought or heat stress individually (Rizhsky *et al*., 2004). In contrast, Zhou *et al*. (2017) concluded that the combined stress does not always increase plant damage as previously thought. Moreover, the study found that the main responses prominent in tomato plants under combined drought and heat stress were caused by drought stress. Furthermore, in one study, the combined efforts of salinity and heat stress demonstrated extraordinary induction of resistance to salinity stress in tomatoes which contradicts the expected negative outcome of stresses applied in combination (Rivero *et al*., 2014). This places emphasis on the fact that plant species' responses to drought and/or heat stress vary widely and are different from the response of plants to each of these stresses applied individually.

1.8. Effects of inoculation with PGPR on the regulation of plant stress response genes in maize subjected to drought and heat stress

Plant growth-promoting rhizobacteria used to induce mitigating effects to plants under environmental stress have been shown to induce ISR (Dimkpa *et al*., 2009). This suggests that the primed physiological state of an inoculated plant could be one explanation for increased tolerance against abiotic stresses (Dimkpa *et al*., 2009). The priming phenomenon has not been fully elucidated at the molecular level however, a strong relationship with the accumulation of inactive signaling proteins activated and transduced under stress has been shown. This is especially true upon future exposure of the plant to the same stress (Park *et al*., 2017). This enables PGPR to alter the transcriptional expression of plant genes, thereby inducing tolerance to abiotic stress.

Inoculation of maize grown under drought conditions with *Herbaspirillum seropedicae* Z-152 and *Azospirillum brasilense* SP-7 led to decreased expression of ZmVP14 gene, which is implicated in the biosynthesis of abscisic acid while inferring resistance to maize plants under drought stress (Curá *et al*., 2017). Also, maize plants inoculated with *Serratia liquefaciens* KM4 showed tolerance to osmotic stress induced by salinity, and this was associated with the increased expression of stress-related genes such as SOD, CAT, APX, ribulose bisphosphate carboxylase small subunit (RBCS), Ribulose bisphosphate carboxylase large chain (RBCL), H+-translocating inorganic pyrophosphatase (H+-PPase), sodium transporter (HKT1), and Na+/H+ antiporter **(**NHX1), and downregulation of 9-cis-epoxycarotenoid dioxygenase (NCED) the primary gene in the biosynthesis of ABA (El-Esawi *et al*., 2018). The response of plants to drought and salinity are usually comparable due to the osmotic effect which is the initial phase of salinity stress. Another study showed that *Bacillus megaterium* which could improve drought tolerance in clover plants also improved osmotic stress in maize plants. Moreover, the expression of plasma membrane intrinsic protein (PIPs) genes, which included ZmPIP1;1, ZmPIP2;1 and ZmPIP2;5 were reduced on inoculated leaves than in uninoculated ones. The opposite was observed for ZmPIP1;2 expression (Marulanda *et al*., 2010). These studies show that PGPR can induce abiotic tolerance by modifying the transcriptional expression of maize stress response genes. Even so, it must be noted that there are few published studies on the topic, especially on maize plants.

To highlight the potential of PGPR on inducing plant tolerance under drought and heat stress by influencing plant stress response genes, we show studies also on wheat, *Arabidopsis*, pepper,

soybean, and sugarcane. *Paenibacillus polymyxa* B2 could increase *Arabidopsis thaliana* plant resistance to drought stress (Timmusk and Wagner, 1999). Furthermore, the expression of the early response to dehydration 15 (ERD15), a drought response gene, was elevated in treated plants relative to untreated control plants (Timmusk and Wagner, 1999). In pepper plants inoculated with *Bacillus licheniformis* K11, under water deficit conditions, differential display polymerase chain reaction (DD-PCR) and 2-D polyacrylamide gel electrophoresis (2D-PAGE), showed six differentially expressed stress proteins (Lim and Kim, 2013). Among these, *Capsicum annuum* dehydrin (Cadhn), sHSP, and *Capsicum annuum* pathogenesis-related protein 10 (CaPR-10) showed an increase greater than 1.5-fold in inoculated plants relative to controls (Lim and Kim, 2013). In *Bacillus* sp.TW4 inoculated pepper plants subjected to osmotic stress, abiotic stress-inducible genes *caLTPI* (encoding a lipid transfer protein), and *caACCO* (encoding ACC oxidase) associated with ethylene metabolism were down-regulated (Sziderics *et al.*, 2007). The study suggested that this might have been attributed to the ACC deaminase activity of *Bacillus* sp. TW4. Using qPCR to detect stress-related gene expression in the leaves of wheat, transcripts of S-adenosylmethionine synthase 1 (SAMS1), ascorbate peroxidase 1 (APX1), and HSP17.8 were upregulated when plants were inoculated with *A. brasilense* NO40 and *Bacillus amyloliquefaciens* 5113, which in turn mitigated the negative effects of drought stress (Kasim *et al*., 2013). *Bacillus aryabhattai* SRB02 was shown to significantly promote the growth of soybean and greatly ameliorate the effects of heat stress (Park *et al*., 2017). At the transcriptional level, qPCR analysis of inoculated plants subjected to heat stress reportedly had a significantly greater accumulation of *GmIAA16* and *GmIAA9* transcripts. These genes both encode the Aux/IAA transcriptional repressor proteins involved in auxin signaling (Park *et al*., 2017). Moreover, two soybean cytokinin-dehydrogenase genes *GMCKX04* and *GmCKX07* that encode cytokinin dehydrogenase proteins, which are involved in the degradation of the cytokinin phytohormone in plants, showed an increase in transcripts in plants inoculated with *B. aryabhattai* SRB02 under both high temperature and normal conditions (Park *et al*., 2017).

Pseudomonas chlororaphis O6 could ameliorate drought stress effects in *A. thaliana* plants by altering the drought signaling response genes (Cho *et al*., 2013). Through microarray analysis, upregulation of VSP1 and pdf-1.2, jasmonic acid-marker genes, HEL, PR-1, and the ethylene-response gene and salicylic acid regulated gene was observed in inoculated plants (Cho *et al*., 2013). Using Illumina sequencing, the inoculation of sugarcane subjected to water deficit

conditions with *Gluconacetobacter diazotrophicus* PAL5 activated the ABA-dependent signaling genes resulting in drought-tolerant plants (Vargas *et al*., 2014). Such transcriptional changes are also typical indicators for ISR, suggesting that there is a strong correlation between ISR and PGPR-mediated plant tolerance to abiotic stresses. These studies demonstrate that PGPR infers their beneficial effects on maize and other important crops primarily at a molecular level.

1.9. Study Rationale

Maize is known for its sensitivity to stresses which often results in substantial losses for crop production under climate-induced abiotic stresses (Bita and Gerats, 2013). Among agricultural crops, maize (*Zea mays* L.) is an important staple grain crop in South Africa and is primarily used for human consumption and animal feed production (Department of Agriculture, Forestry and Fishery, 2016). The cereal has been the largest contributor to the gross value of field crops in South Africa for the past seasons (48 %). Maize needs 450 to 600 mm of water per season, mainly acquired from the soil moisture reserves, and the critical temperature detrimentally affecting yield is approximately 32 ºC. With more than 90% of the South African climate either arid or semi-arid with characteristic drought and hot temperature, climate change threatens to worsen yields in crop production. The 2015/16 rainy season showed that the weather condition could exceed the optimal maize growth conditions. The incident was among the worst in 30 years in South Africa, with below-average maize production due to elevated temperatures and drought (Department of Agriculture, Forestry and Fishery, 2016). In the country, maximum temperatures often exceed 32 ºC in the summer and reach 38 ºC in some areas of the far north. The country's highest recorded temperatures in 2017/2018 were close to 48 ºC and have occurred in both the Northern Cape and Mpumalanga whereas, over many of the maize producing regions of the Free State and North West provinces, the onset of the rains occurred late and the totals for October to January were 25-50% below normal in 2018 (Agricultural research council - Institute for Soil weather and Climate, 2018). As a result, there has been an urgency to find technologies that would induce drought and heat stress tolerance in agricultural plants.

Beneficial plant-microbe interactions have been exploited and promote promising and environmentally friendly strategies in sustainable agricultural practices (Sharma *et al*., 2014). These beneficial soil microorganisms called PGPR, have been shown to produce plant growth-promoting substances in large quantities that indirectly influence the growth and morphology of

plants (Guerrieri *et al*., 2020). Furthermore, they have been applied as biocontrol agents, biofertilizers and/or phytostimulators in agriculture or as degrading microorganisms in phytoremediation applications (Pereira *et al*., 2020; Tyagi and Singh, 2014). Another remarkable feature is their capability to support plants under stressed environments. They can protect plants against deleterious effects of different environmental stresses to which crop plants are intermittently exposed, such as drought and elevated temperatures (Bano *et al*., 2013). Moreover, PGPR can alter the transcriptional expression of plant genes, thereby inducing plant tolerance to abiotic stress (El-Esawi *et al*., 2018).

With advances in techniques for monitoring responses at the molecular and gene regulation level, it is possible to more precisely determine how PGPR induces drought and heat tolerance in maize plants to alter the expression of maize stress response genes (Eastburn *et al.*, 2011). With molecular techniques such as metatranscriptomics, metabolomics and metaproteomics, it is possible to successfully quantify the expression levels of these stress response genes. The antioxidant enzymes, heat shock transcription factors (HSFs), high and low molecular weight heat shock proteins (HSPs), late embryogenesis abundant (LEA) proteins and Dehydrins are thought to be central in plants acquiring tolerance to drought and heat stress (Farooq *et al*., 2009; MT *et al*., 2020; Vardharajula *et al*., 2011). As such, it is of interest that we also understand how these stress response genes are altered under PGPR mitigated combined drought and heat stress as these abiotic stresses occur more frequently in combination.

The amassed knowledge over the past decades has helped to understand key features regarding the mode of action of plant-PGPR interactions inferring abiotic stress tolerance in plants, but major knowledge gaps remain. Drought and heat stress effects on plants, as well as how PGPR ameliorated drought stress affects physiological, growth, developmental, yield, and genetic parameters on maize are well documented. However, the combined effects of drought and heat stress on maize and how PGPR induce abiotic tolerance to these stresses is extensively lacking.

Moreover, the focus on laboratory and greenhouse tests has been on the single occurrence of these stresses, which does not necessarily correlate to field conditions where plants are more regularly exposed to a combination of these stresses. As such, PGPR fail to induce any positive response in the field primarily because combinatory stress of drought and heat is distinctly unique from the response of plants to these stresses applied individually. As such, studies aimed at mitigating

abiotic stresses using PGPR should be reassessed. During early test phases, the effect of combinatory stresses, primarily drought and heat stress on plant metabolism and stress response under such climatic conditions should be considered. Therefore, the current project aims to assess the ability of PGPR at inducing stress tolerance to a combination of drought and heat in maize (*Zea mays* L.) plants. In addition, to unravel innate maize drought and heat stress response genes which actively participate in microbial induced drought and heat stress tolerance.

1.10. Aims and Objectives

1.10.1. Aim

The current study aims to screen and identify bacterial isolates with plant growth-promoting attributes and tolerance to drought and heat stress. Furthermore, evaluate the ability of selected drought and heat tolerant PGPR isolates at mitigating the effects of dual drought and heat stress on the early growth of maize, as well as unravel innate maize drought and heat stress response genes which actively participate in PGPR-induced tolerance.

1.10.2. Objectives

- To screen for bacterial isolates with exceptional plant growth-promoting traits and tolerance to drought and heat stress.
- To evaluate drought and heat tolerant PGPR isolates at inducing tolerance in maize plants exposed to concurrent drought and heat stress *in vitro*.
- To evaluate whether the selected drought and heat tolerant PGPR isolates can be used in combination.
- To assess the phenotypic responses (physiological/physical development) of PGPR-inoculated maize under dual drought and heat stress under greenhouse conditions.
- To elucidate the expression of selected stress response genes in PGPR-inoculated maize under combined drought and heat stress.

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2. Chapter 2: Assessing the plant growth-promoting capabilities of potential PGPR and their tolerance to drought and heat stress

2.1. Abstract

Drought and heat stress are the most critical environmental stresses among the abiotic stresses that affect plant growth, crop yield, and cultivation throughout the world. Plant growth-promoting bacteria are promising candidates for the alleviation of such abiotic stresses in crops. Various drought and heat tolerant PGPR have been successfully applied or tested for plant growth promotion under these abiotic stresses. As such, the current study aimed to screen bacterial isolates for plant growth promoting properties and tolerance to both drought and heat stress. From the 104 isolates obtained, 61 isolates demonstrated superior plant growth-promoting capabilities (Indole acetic acid production, nitrogen fixation, phosphorus solubilization). From the 61, a total of 12 isolates exhibited potential tolerance to both drought and heat stress. All isolates exhibited IAA production that ranged between 10.56- 28.77 ug/ml. However, of the 12 isolates, nine demonstrated nitrogen-fixing capabilities and, three showed phosphorus solubilizing potential. The drought and heat tolerant PGPR were identified as relatives of *Bacillus thuringiensis* (7 isolates), *Bacillus pseudomyciodes*, *Lelliottia amnigena*, *Acinetobacter* sp., *Leclercia* sp (2 isolates). This study reports for the first time the tolerance of *Lelliottia amnigena* and *Leclercia* sp. to both drought (osmotic) and heat stress *in vitro*. These bacterial isolates are promising candidates for alleviating heat and drought stress in plants while enhancing plant growth.

2.2. Introduction

Drought and heat stress are the most critical environmental stresses among the abiotic stresses affecting plant growth, crop yield, and cultivation throughout the world (Rizhsky *et al*., 2004; Savin and Nicolas, 1996). As a result, these abiotic stresses have become a problem in global food security. Furthermore, the current climate change trends may increase the occurrence and intensity of drought and heat stress worldwide (Khan *et al*., 2019). The effects of heat stress occur throughout the plant's growth stages but they are more pronounced in grain crops during anthesis and grain filling stages (Ali *et al*., 2011). Moreover, due to the rising temperatures worldwide, the geographical distribution, and maize growing season may be altered, resulting in the optimum temperature for the start of the season and crop maturity to be reached much sooner (Ali *et al*., 2011). The effects of drought stress are also evident at whatever plant growth stage the water deficit occurs (Farooq *et al*., 2009). Plants under drought stress have exhibited disturbances in

plant water relations, severe reductions in water-use efficiency, germination, seedling establishment, limited total nutrient uptake, and photosynthesis (Farooq *et al*., 2009). Therefore, we need to develop low-cost and eco-friendly methods that can easily be adopted by farmers.

To improve plant tolerance to drought and heat stress, strategies such as traditional plant breeding, use of fertilizers, and genetic engineering, have been extensively studied for decades. However, the use of plant growth-promoting bacteria, which are abundantly available in agricultural soils offers an eco-friendly, inexpensive, and non-ethically restricted approach to solving the crisis revolving around drought and heat stress (Niu *et al*., 2018). Plant growth-promoting bacteria associate with plants in the rhizosphere, endophytically within plant roots or on the surface. This association can directly or indirectly facilitate plant growth under abiotic stress conditions (Niu *et al*., 2018). PGPR-induced drought and heat tolerance in plants results from various mechanisms such as the production of phytohormones, 1-aminocyclopropane-1-carboxylic (ACC) deaminase, volatile compounds, and exopolysaccharide (EPS); by altering root morphology; inhibiting ethylene synthesis; regulating endogenous abscisic acid (ABA) levels, antioxidant defense. Various drought and heat tolerant PGPR such as *Bacillus, Pseudomonas, Burkholderi*a, *Acetobacter, Rhizobium,* and *Azospirillum*, have been successfully tested or applied for enhancing plant growth under these abiotic stresses (Dimkpa *et al*., 2009, Meena *et al*., 2015).

Even so, these abiotic stresses not only affect plants but also affect microbes. To outlive and to prevail despite the intense competition among microorganisms in the rhizosphere, regularly some PGPR actuate survival induced mechanisms that induce abiotic resistance. Inoculation of plants with tolerant PGPR results in improved plant response to abiotic stress (ATEŞ and Kivanc, 2020). Thus, selecting more resistant PGPR is crucial when designing new inoculants for use in drought-stricken climates or zones with high temperatures. García *et al.* (2017) showed that the bacterial cells of *Azospirillum* accumulate compatible solutes such as glutamate, trehalose, glycine betaine, and proline to cope with osmotic stress under water deficit conditions, preventing the degenerative processes and improving cell growth under drought stress. The trehalose poly sugar can protect biologically produced compounds and molecules from breakdown during water-induced osmotic stresses (Khan *et al*. 2016). Some microbes use the cellulase enzyme that dissolves the cellulose cell wall of plant roots to gain entrance into the cell wall interior (apoplast) as well as the vascular bundle (xylem), where they live and undergo regular metabolic functions (Enebe *et al*., 2018).

Some of these organisms are excellent in the secretion of exopolysaccharide substances (EPS) and forming biofilms, which induces bacterial cell stability and protection from desiccation during stress (El-Akhdar *et al*., 2020). Organisms respond to rapid increases in temperature by inducing the synthesis of specific polypeptides, known as heat shock proteins (HSP). For instance, *Pseudomonas aeruginosa* AMK-P6 isolated from a semiarid region displayed thermotolerance and produced HSP when exposed to high temperature (Ali *et al*., 2014). These then highlight the importance of screening PGPR not only for abilities to promote plant growth but also for resistance to specific abiotic stresses for use as abiotic stress mitigators in plants. These abiotic tolerant PGPR could thus outperform others and thrive under drought or the heat-stressed environment in sufficient numbers to deliver beneficial effects on plants. Polyethylene glycol (PEG), a non-toxic, non-metabolized and non-absorbable, osmotic agent, is commonly used to tempt osmotic stress in microbes by decreasing the nutrient solution's water potential, thus restricting water availability to the plant roots (Akinyosoye *et al*., 2015; Ahmad *et al*., 2018). Therefore, PEG solutions are more appropriate for inducing osmotic stress in microbes and for assessing their potential to tolerate drought stress in nutrient solutions. (Ali *et al*., 2014). Similarly, incubation of isolates in nutrient media exposed to high temperatures is widely used to induce heat stress in microbes (Praveen Kumar *et al*., 2014).

In this study, bacterial isolates, isolated from the rhizosphere soil of land used to cultivate maize located in South Africa, the Mpumalanga province, were used. The study investigated the metabolic characteristics of these bacterial isolates related to PGP attributes including, the production of indole acetic acid (IAA), the solubilization of inorganic phosphate, nitrogen fixation, and the ability to tolerate high temperature and drought (osmotic stress).

2.3. Materials and methods

2.3.1. Preparation of bacterial cultures

In a previous study, soil samples were collected from the rhizosphere of maize in Sheepmoor village, Msukaligwa municipality located in Gert Sibande District Municipality, Mpumalanga province, South Africa, coordinate 26'' 45'18'' S, 30' 13'55'' E (Figure 6). Bacterial isolates obtained were screened for plant growth-promoting attributes in a previous study (Tsipinana, 2019). Isolates found to exhibit potential PGPR abilities were screened for drought and heat

tolerance. The PGPR isolates that exhibited better growth and tolerance to drought and heat stress were subsequently identified by 16 sRNA gene sequencing.

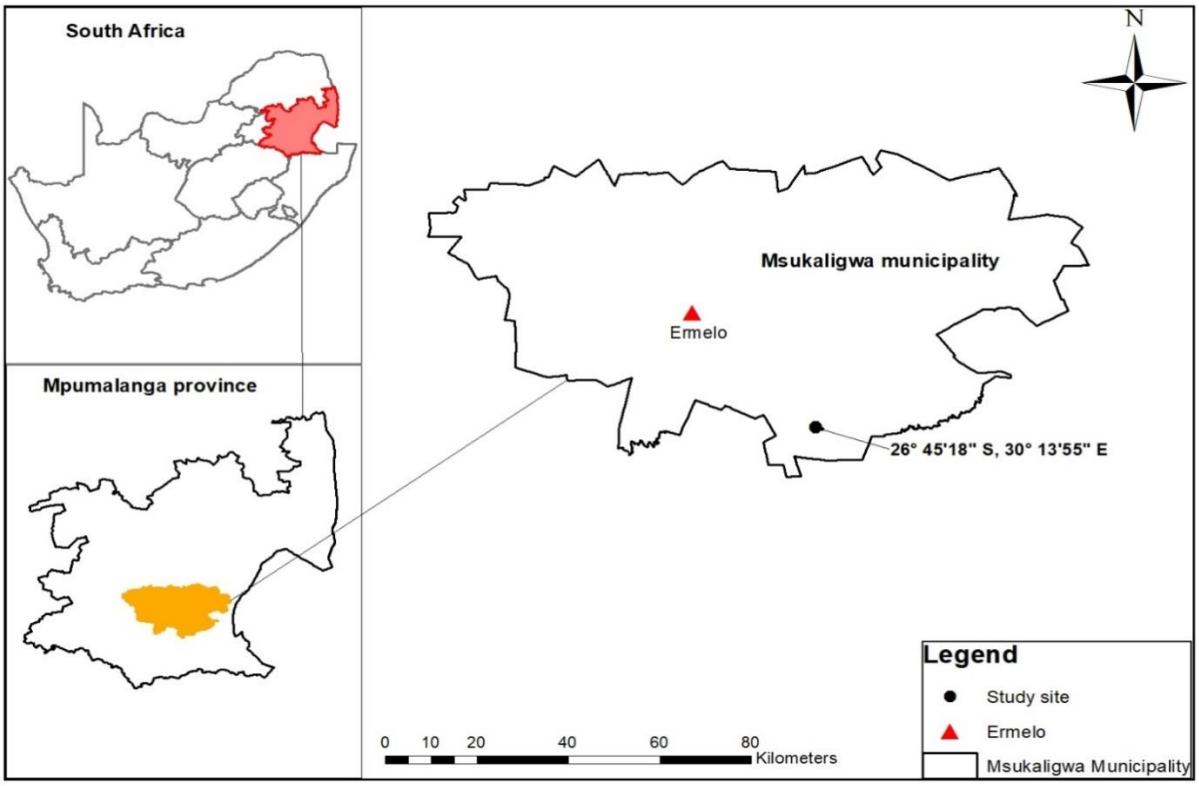

Figure 6: The study site in Mpumalanga Province of South Africa

Bacterial isolates with plant growth-promoting capabilities were kept in 50% glycerol at -80 ºC. These were thawed and cultured into tryptic soy agar (TSA) using the streak plate method to obtain single colonies. A single colony of the test isolates was used to inoculate 10 ml tryptic soy broth (TSB) and grown for 6 h at 32 ºC on a shaker incubator rotating at 120 rpm (Ali *et al*., 2014; Praveen Kumar *et al*., 2014). The optical density was measured at 600 nm using a spectrophotometer. These cultures were used to inoculate fresh TSB for testing their ability to grow under drought and heat-stressed conditions.

2.3.1. Microbial screening for drought stress tolerance

Drought-tolerant microbes were screened by making a 40% polyethylene glycol (PEG) 6000 solution in TSB, resulting in osmotic potential of approximately -2.70 MPa (Busse and Bottomly, 1989; Ali *et al*., 2014). Afterward, an appropriate volume of the initial inoculum was added to the modified growth media to give a uniform cell density with an OD of 0.05 and a final volume of

10mL. Tubes were incubated at 32 °C in a shaker at 120 rpm for 24 h, and the growth was estimated by measuring the optical density at 600 nm using a spectrophotometer. Bacterial isolates were considered stress-tolerant if an OD greater than 0.400 was recorded. All tests were conducted in four replicates.

2.3.2. Microbial screening for heat stress tolerance

Heat-tolerant isolates were screened by adding an appropriate volume of the initial inoculum to fresh TSB giving uniform cell density with an OD of 0.05 and a final volume of 10mL (Praveen Kumar *et al.,* 2014). Tubes were then incubated at 42 °C in a shaker at 120 rpm for 24 h, and the bacterial growth was estimated by measuring the optical density at 600 nm using a spectrophotometer. Bacterial isolates were considered stress-tolerant if an OD greater than 0.400 was recorded. All tests were conducted in 4 replicates.

2.3.3. Molecular characterization of both drought and heat stress tolerant isolates

Only bacterial isolates that demonstrated both drought and heat stress tolerance were identified. Bacterial genomic DNA was isolated using the microwave method and subjected to Polymerase Chain Reaction (PCR) for amplification of 16S rDNA gene using universal forward 341F and 907R. The PCR cycling conditions were as follows: After denaturation at 95°C for 5 min, 35 rounds of temperature cycling (95 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min) were followed by incubation at 72°C for 7 min. The PCR product purity was confirmed by electrophoresis on 1 % agarose gel (Figure 8). The PCR products were sent for Sanger sequencing (Inqaba biotec, South Africa). Obtained sequencings were edited using Bioedit sequence alignment editor (Hall *et al*., 2011) and compared to known sequences using NCBI's nucleotide Basic Local Alignment Search Tool (BLAST) (Work, 2015).

2.4. Results

2.4.1. Screening for plant growth-promoting attributes

A total of 104 bacterial isolates were obtained from these soils and screened for plant growth-promoting attributes at the Agricultural Research Council- Soil, Water, and Climate (ARC-SCW). From these isolates, 53 (51 %) demonstrated phosphorus solubilizing capabilities, and a total of 51 (49 %) isolates exhibited nitrogen-fixing attributes (Figure 7). All isolates were able to produce indole acetic acid (IAA). However, these plant growth-promoting characteristics varied between the isolates. Therefore, the bacterial isolates that demonstrated the least capabilities for performing

these functions were removed from further screening. As such, from the 104 isolates obtained, a total of 61 bacterial isolates displayed superior plant growth-promoting capabilities. The 61 bacterial isolates were subsequently screened for the ability to grow adequately under reduced osmotic pressure and elevated temperatures.

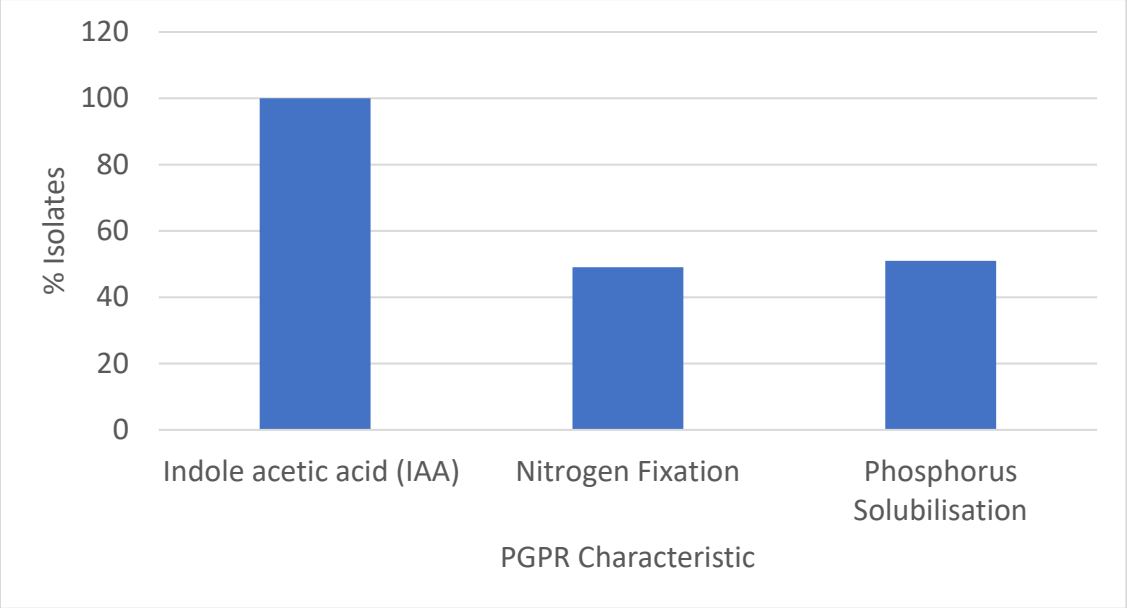

Figure 7: Percentage of bacterial isolates exhibiting each of the evaluated plant growth-promoting characteristics.

2.4.2. Microbial screening for heat stress tolerance

Out of the 61 isolates screened with plant growth-promoting (PGP), more than 90 % demonstrated efficient growth at a temperature of 42 °C *i.e.* an OD greater than 0.40 was recorded. A total of six (less than 10 %) isolates, namely 27MP1, 28MP1W, 23MP3, 21MP2Y, 21MP2, and 19MP4W, had an OD measurement that was below 0.40 (Supplementary Table 1). The results suggest that the microbial population in Sheepmoor village, located in Mpumalanga is well adapted to high temperatures.

2.4.3. Microbial screening for drought stress tolerance

From the 61 isolates with PGP attributes, 12 isolates achieved an OD greater than 0.40 with 14MN3B, 36MP8, 34MP2, 33MP1, 11MN1, 11MN2, 32MN1B, 31MN1B, 14MN5A, 21MN1B, 21MN2B, 11MN3, therefore, showing tolerance to 40 % PEG 6000 (Table 1 and Supplementary Table 1). Surprisingly, more of the bacterial isolates that were proficient in nitrogen fixation

showed greater tolerance to 40 % PEG 6000 than the isolates that were efficient in phosphorus solubilization. A total of nine of the 12 isolates exhibited N-fixing attributes, whereas only 3 exhibited P-solubilizing capabilities. All 12 of these bacterial isolates also displayed tolerance to heat stress, making a total of 12 isolates that exhibited the potential to tolerate both drought and heat stress *i.e.* when the bacterial isolates were cultured in TSB with 40 % PEG 6000 and when cultured at 42 °C, an OD of greater than 0.40 was observed.

Table 1: PGPR with high tolerance to drought and Heat stress and their respective plant growth-promoting properties.

Isolate ID	N-Fixation	P solubilisation	IAA Conc ug/ml)	Heat Tolerance (42 ^{0}C stress) (OD)	Drought Tolerance (40 % PEG 6000) (OD)
32MN1B	**	--	20.75	1.1	0.5
14MN3B	**	--	13.77	1.05	1.15
14MN5A	**	--	16.85	1.12	0.49
21MN2B	**	--	11.04	1.02	0.45
11MN3	**	--	28.77	0.93	0.42
21MN1A	**	--	14.99	0.94	0.45
11MN1	**	--	12.78	1.07	0.53
11MN2	**	--	13.19	1.14	0.5
31MN1B	**	--	10.46	0.66	0.5
33MP1	--	**	15.81	0.87	0.53
34MP2	--	**	13.77	0.86	0.48
36MP8	--	**	13.54	0.64	0.96

**Indicate positivity for characteristic

2.4.4. Molecular characterization of both drought and heat stress tolerant isolates

Isolates 14MN3B, 36MP8, 34MP2, 33MP1, 11MN1, 11MN2, 32MN1B, 31MN1B, 14MN5A, 34MP3W, 21MN1A, 21MN2B and 11MN3 demonstrated capabilities to tolerate both drought and heat stress. As such, these isolates were further characterized using the 16S rDNA gene for identification. PCR products were successfully obtained (Figure 8).

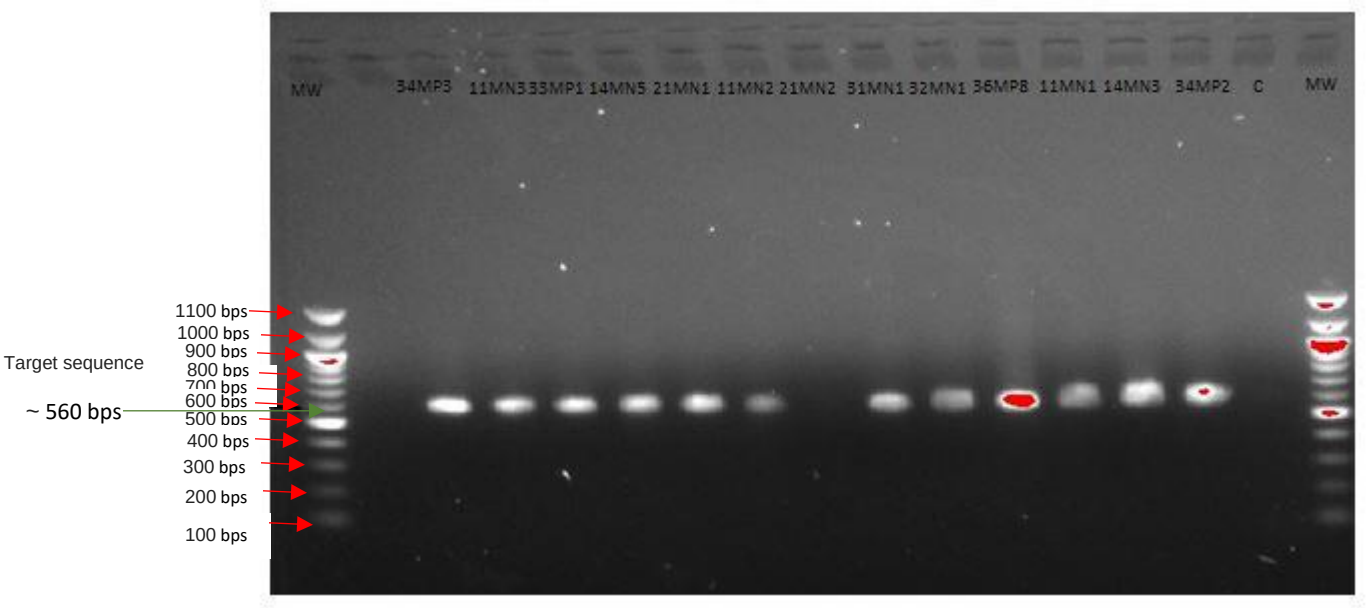

Figure 8: Pure PCR product of the 16S rDNA gene confirmed on 1% agarose.

Isolates 11MN1, 11MN2, 11MN3, 14MN5A, 21MN2, 31MN1, and 32MN1B had 100 % sequence similarity with *Bacillus thuringiensis* strain C15 according to NCBI'S nucleotide BLAST. Isolate 14MN3A had a 99 % sequence similarity with *Acinetobacter* sp. DSM30007. Isolate 21MN1A had a 100 % sequence similarity with *Bacillus pseudomycoides* strain MF-68. Isolate 33MP1 showed 99 % sequence similarity with *Lelliottia amnigena* strain NCTC12124 a type of *Enterobacteriaceae* and isolates 34MP2 and 36MP8 showed 99 and 100 % sequence similarity with *Leclercia* sp. strain T3196-2 respectively that also belongs to the *Enterobacteriaceae* group.

Table 2*:*Gene sequence similarity (%) of test isolates to known isolates

Isolate ID	**Sequence analysis results**	**% of Similarity**

36MP8	*Leclercia_sp._strain_T3196-2*	100
34MP2	*Leclercia_sp._strain_T3196-2*	99
33MP1	*Lelliottia_amnigena_strain_NCTC12124*	99
32MN1B	*Bacillus_thuringiensis _strain_C15*	100
31MN1	*Bacillus_thuringiensis _strain_C15*	100
21MN2	*Bacillus_thuringiensis _strain_C15*	100
21MN1A	*Bacillus_pseudomycoides_strain_MF-68*	100
14MN5A	*Bacillus_thuringiensis _strain_C15*	100
14MN3B	*Acinetobacter_sp*._DSM30007	99
11MN3	*Bacillus_thuringiensis*_strain _15	100
11MN2	*Bacillus_thuringiensis* _strain_C15	100
11MN1	*Bacillus_thuringiensis* _strain_C15	100

Isolates 32MN1B, 14MN5A, 21MN2B, 11MN3, 11MN1, 31MN1B, and 11MN2 had 100 % sequence similarity according to Percent Identity Matrix generated using Clustal Omega (Clustal2.1), EMBL-EBI's tool for multiple sequence alignment. This was also confirmed by using MEGA (Molecular Evolutionary Genetics Analysis) version 5.2.2 pairwise distance matrix (Sievers and Higgins, 2014; Tamura *et al.*, 20011). Similarly, 34MP2 and 36MP8 also exhibited 100 % sequence similarity.

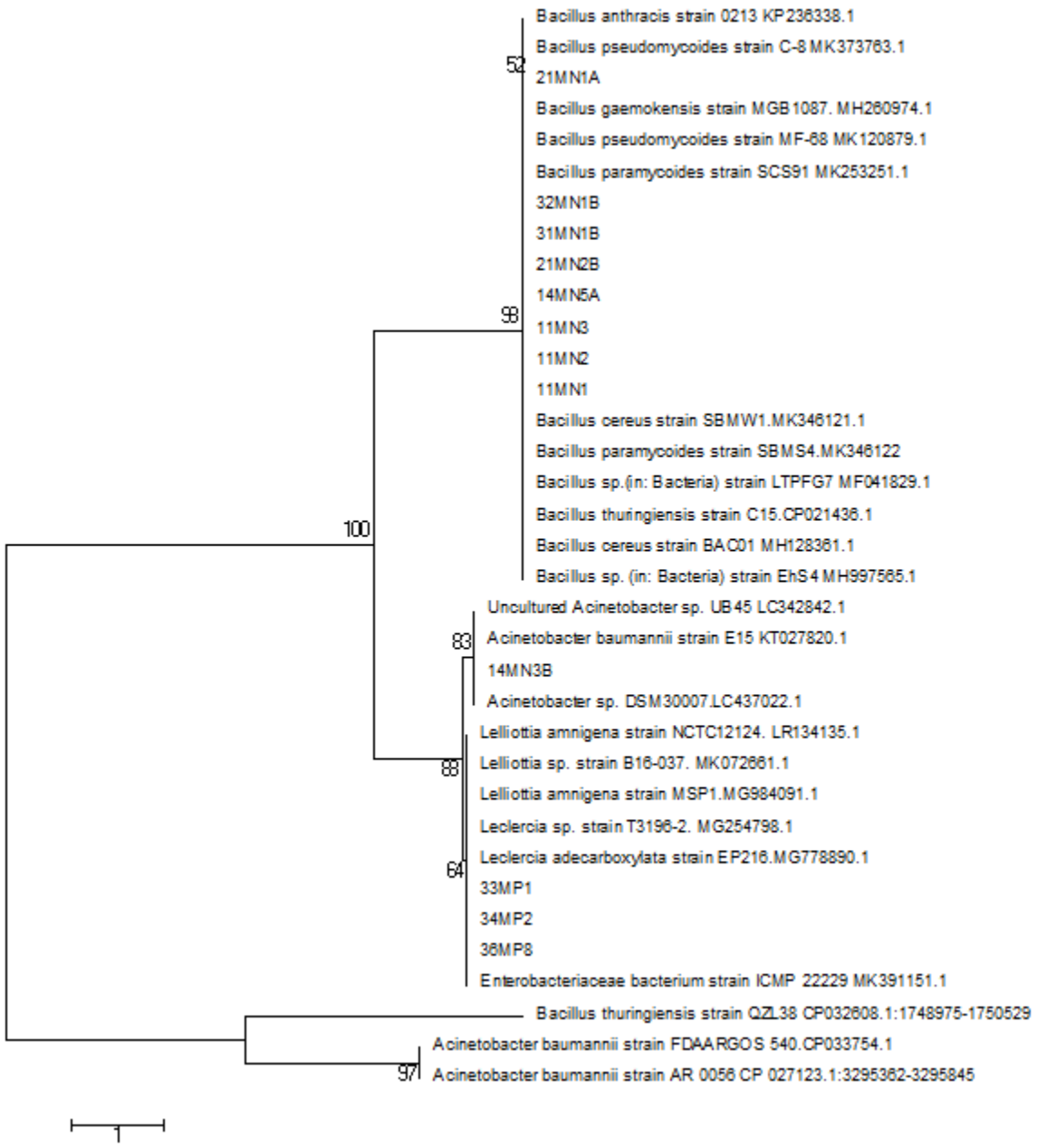

Figure 9: Evolutionary relationships of taxa using the Neighbour-Joining method based on the 16S rRNA gene. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method.

Isolates 32MN1B, 14MN5A, 21MN2B, 11MN3, 11MN1, 31MN1B, and 11MN2 grouped when the phylogenetic tree showing evolutionary relationships of taxa using the Neighbour-Joining method based on the 16S rRNA gene (Figure 9). This further confirms the results obtained using Clustal Omega and MEGA suggesting a shared evolutionary background. Furthermore, these isolates show a close evolutionary relationship with *Bacillus cereus*. Isolate 21MN1A showed a similar evolutionary background to *Bacillus pseudomycoides.* Isolate 14MN3A is closely related to *Acinetobacter Baumanni*. Isolates 34MP2, 33MP1 and 36MP8 clustered together suggesting a shared evolutionary background, like that of known *Enterobacteriaceae, Leclercia adecarboxylata*, and *Lelliotta* sp.

2.5. Discussion

The combined effects of drought and heat stress on plants, especially maize, are quite prevalent under field conditions. However, how PGPR induce abiotic tolerance to these stresses is extensively lacking, as laboratory and greenhouse tests have been focused on the single occurrence of these stresses. As such, PGPR fail to induce any positive response in the field primarily because currently available PGPR lack the genetic disposition to survive under such conditions. Therefore, studies aimed at mitigating dual abiotic stresses through the use of PGPR should be reassessed, including the selection, screening, and application of stress-tolerant microorganisms to multiple abiotic stresses. The current study investigated the plant growth promoting attributes of potential PGPR including, the production of indole acetic acid (IAA), the solubilization of inorganic phosphate, nitrogen fixation, and the ability to tolerate high temperature and drought (osmotic stress).

The study found 12 bacterial isolates with high plant growth-promoting capabilities demonstrated the potential to tolerate drought and heat stress. Phylogenetic analysis of the 16S rRNA gene sequence revealed that they belonged to four genera: *Bacillus, Lelliottia, Acinetobacter, Leclercia.* The *Bacillus* genera was the most represented with seven isolates sharing similarities with *Bacillus thuringensis*, and the other with *B. pseudomycoides*. *Bacillus* sp. are widespread in agricultural soils and many have been widely studied for their enhancement of plant growth. For instance, *Bacillus* strains that were isolated from healthy tomato roots among them *B. Pseudomycoides* strain NBRC 101232, displayed the potential to promote plant growth and control bacterial wilt caused by *R. solanacearum* in tomatoes (Yanti *et al*., 2017). *Bacillus*

pseudomycoides have been shown to possess the ability to solubilize potassium which enhances potassium uptake in plants by increasing potassium availability resulting in enhanced plant height by 45.15 % (D'Amato *et al.*, 2019). The *Bacillus* spp. identified in the study exhibited nitrogen fixing abilities. Nitrogen-fixing microorganisms can fix atmospheric nitrogen, and several studies have evaluated their capacity to fix N_2 and possibly reduce or replace N-fertilizers when associated with various plants (Fukami *et al*., 2018). *Acinetobacter* sp. also displayed the ability to fix nitrogen. Previous studies have shown the species to possess plant growth-promoting traits such as siderophore production, mineral solubilization, and nitrogen fixation *in-vitro*. Moreover, *Acinetobacter* strain RSC7 was able to solubilize 27.10 µg/ml phosphate with 1.5% tricalcium phosphate and produced an estimated 20.89 µg/ml IAA (Prittesh *et al*., 2017).

The *Enterobacteriaceae, Leclercia* sp. (34MP2 and 36MP8), and *Lelliotta amnigena* (36MP8) tested positive for phosphorus solubilization. Phosphorus binds to metal ions in the soil, thus exists in an insoluble form that is not easily accessible to plants. Plant growth-promoting rhizobacteria convert the insoluble phosphorus into a form that is readily available to plants (Yadav *et al*., 2016). Several mechanisms are involved in the solubilization of insoluble P however, the main one is through the production of organic acid (Park *et al*., 2009). Isolate 33MP1, 34MP2 and 36MP8 may produce such organic acids thereby making more phosphorus available for plant growth. These microbe have presented PGP attributes associated with plant growth promotion and protection of plants against phytopathogenic microorganisms. *Leclercia* sp. strain QAU-66 could solubilize phosphorus and significantly enhanced the shoot and root lengths of *Phaseolus vulgaris* (Naveed *et al*., 2014). ACC deaminase producing *Leclercia adecarboxylata* could alleviate drought stress by improving root elongation (2.2-fold), shoot dry weight (1.5-fold), root dry weight (1.4-fold), NPK uptake, and possibly decreasing ethylene in plants (Danish *et al.*, 2020). *L. adecarboxylata* and *L. amnigena* have been shown to commonly display multiple plant growth-promoting (PGP) traits such as ACC deaminase activity, and the production of phytohormones (IAA) as well as phosphorus solubilization (ATEŞ and Kivanc, 2020; Fazzolari *et al*., 1990; El-Akhdar *et al.*, 2020).

All these isolates demonstrated the ability to produce IAA. Indole acetic acid is one of the critical auxins which are the key growth regulators that are involved nearly in all aspects of plant growth and development. In recent years, several studies have provided undisputed evidence for the

involvement of auxins in response to abiotic stress (Khan *et al.*, 2019). Thus, these isolates have great potential for promoting plant growth and alleviating drought and heat stress. Overall, these strains demonstrated capabilities to promote plant growth and protected plants against phytopathogenic microorganisms. Moreover, these studies provide unflinching evidence for the involvement of these bacterial isolates in promoting plant growth. The benefits inferred to plants by PGPR are reportedly not only attributed to its one plant growth-promoting characteristic, but multiple plant growth-promoting mechanisms are involved. The effect of these bacteria is better explained by the "Multiple Mechanism Theory" formulated by Bashan and Levanony (1990), which assumes that several factors participate in the successful association of these PGPR with plants (Zeffa *et al*., 2019). Baig *et al*. (2012) noted that bacterial strains with dual PGP traits were more effective than strains with single traits under soil conditions (pot trial) in increasing root weight and root elongation, dry shoot weight, and grain yield. In the current study, the bacterial isolates possessed more than one PGP trait, thus showing the potential to promote plant growth even under adverse conditions.

Lastly, it has been shown that inoculation of plants with tolerant PGPR help improve plant response to abiotic stress (García *et al*., 2009). Therefore, it is crucial to select more resistant PGPR for application in drought-stricken climates or zones with high temperatures. The 12 isolates could adequately grow under artificially induced drought and heat stress in the current study. Bacteria possess innate capabilities to adapt to stressful conditions that allow them to continue inferring their beneficial effects on plants even under stressed conditions. For instance, Wu *et al*. (2011) studied *B. thuringiensis* under heat stress (42 ^{o}C) and found that as a survival strategy under long-term heat stress, the strain up-regulated enzymes (BDH1, GuaB, and PepA) down-regulate metabolic enzymes to reduce metabolic burden, increase the synthesis and accumulation of poly(3-hydroxybutyrate) (PHB). Correspondingly, *Azospirillum* has been shown to accumulate compatible solutes such as trehalose glycine betaine, glutamate, and proline, to cope with osmotic stress under water deficit conditions, preventing the degenerative processes and improving cell growth under adverse osmotic conditions (García *et al*., 2009; Khan *et al*. 2016). These sugars are responsible for osmotic adjustment, the detoxification of ROS, and stabilize the quaternary structure of proteins under water deficit conditions (Khan *et al.*, 2019). A thermotolerant *Pseudomonas aeruginosa* strain AMK-P6 isolated from a semiarid region, exhibited the induction of HSP when exposed to high temperature (Ali *et al.*, 2009). As such, the

ability to survive drought and heat stress promises that these isolates could infer beneficial effects in plants subjected to the same abiotic stresses.

2.6. Conclusion

The use of PGPR for the mitigation of drought and heat stress is widely accepted. However, most fail to induce any positive response in the field primarily because of inability to survive such conditions. Therefore, the current study reassessed the approach by focusing on selection, screening and application of microganisms that exhibit stress-tolerance to multiple abiotic stresses. The study screened and identified potential PGPR isolates with superior multiple plant growth-promoting characteristics, as well as tolerance to drought and heat stress. The isolates were identified as *Bacillus thuringiensis*, *Bacillus pseudomyciodes*, *Lelliottia amnigena*, *Acinetobacter* sp. and *Leclercia* sp. The ability of these isolates to tolerate intense osmotic and high temperatures stress implies that they have the potential to survive under these conditions in agricultural soils and mitigate the adverse effects of drought and heat stress in plants while promoting plant growth. Thus, these PGPR isolates can potentially be used as essential components of biofertilizers formulated for adapting to climate change effects. As such, further investigations were conducted to evaluate the capabilities of these isolates at mitigating the combined effects of drought and heat stress in maize plants *in-vitro* and under greenhouse trials.

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3. Chapter 3. Drought- and heat-tolerant bacteria and their plant growth promoting attributes on maize seedling *in vitro*

3.1.Abstract

Under field conditions, drought and heat stresses occur more frequently in combination than alone. Their combination reportedly has a significant detrimental effect on the growth and productivity of agriculturally important crops such as Maize (*Zea mays* L.). Biofertilization with microorganisms of agricultural importance, namely plant growth-promoting rhizobacteria, is a promising and eco-friendly strategy to ensure sustainable, long-term maize production under adverse climatic conditions. In the current study, four PGPR isolates, *Bacillus thuringiensis* (11MN1), *Acinetobacter* sp. (14MN3B), *Bacillus pseudomycoides* (21MN1B), *Lelliottia amnigena* (33MP1), and *Leclercia* sp. (36MP8), were evaluated for their potential to alleviate adverse effects induced by the combination of these stresses in the early growth of maize seedlings *in vitro*. Isolate 11MN1, 21MN1B, and 36MP8 statistically ($P < 0.05$) improved the wet weight of maize seedlings by 48.52, 220.59, and 107.35%, respectively compared to the control, under concurrent drought and heat stress. The moisture content was significantly ($P < 0.05$) improved by 43.75, 258.70, 103.6, and 95.48% for maize seedlings inoculated with 11MN1, 21MN1B, 33MP1, and 36MP8 respectively. Under the combined stress, all evaluated isolates improved the total dry weight of maize seedlings, except for 14MN3B that exhibited pathogenic effects. However, only isolate 33MP1 (53.53%) statistically ($P < 0.05$) improved the dry weight compared to the control. The study also found that isolates from the *Bacillus* group seem to show slight antagonism against *L. amnigena* and *Leclecia* sp. belonging to the *Enterobacteriaceae* group. However, there was no antagonism between the *Bacillus* spp. and likewise between the *Enterobacteriaceae* group. Overall, 11MN1, 21MN1B, 33MP1, and 36MP8 show great potential for alleviating the adverse effects inferred by the combination of these stresses in maize seedlings. These bacterial isolates could improve the adaptability of maize seedlings in their early growth stage to concurrent drought and heat stress and potentially increase maize yield.

3.2.Introduction

Maize (*Zea mays* L.) is an essential staple grain crop in Latin America, Asia, and Sub-Saharan Africa, which is primarily used for human consumption and animal feed production (Department of Agriculture, Forestry and Fishery, 2016). More than 73% of the maize-growing areas are in developing countries, where maize is a vital staple food crop for millions of people (Shiferaw *et*

al., 2011; Tesfaye *et al*., 2018). As such, an increase in the demand for maize is expected to increase in these regions due to an increase in both food consumption and feed requirements driven by population growth and economic development (Shiferaw *et al*., 2011). Climate change is threatening to worsen these existing challenges and further undermines the efforts made to reduce poverty and enhance food security within these regions (Tesfaye *et al*., 2017). Furthermore, the temperatures over Africa are expected to rise more rapidly than the global average temperatures and are estimated to exceed 2 °C by 2050 (Niang *et al*., 2014; Tesfaye *et al*., 2018). Currently, some areas have a temperature that has already exceeded the threshold for the production and growth of maize (Cairns *et al*., 2013; Nelimor *et al*., 2019). Moreover, rainfall under future climate change scenarios in Sub-Saharan Africa will either stop early or occur later than usual. As such, the production of maize in sub-Saharan Africa faces a threat (Lobell *et al*., 2011).

Maize is highly vulnerable to drought and heat stress during the reproductive stages (Badu-Apraku *et al*., 2017; Cairns *et al*., 2013; Nelimor *et al*., 2019). Under field conditions, high air temperatures often occur concurrently with water deficit and are far more limiting to plant growth than the presence of these individual stresses alone (Farooq *et al.*, 2012; Lipiec *et al*., 2013). Simulation results suggest that climate change scenarios concerning the combination of reduced rainfall and increasing temperatures resulted in increased average simulated maize yield reduction by 21, 33, and 50% under 1, 2, and 4 °C warmings, respectively (Tesfaye *et al.*, 2018). Lobell *et al*. (2011) showed that a rise by 1 °C above 30 °C resulted in a 1% decrease in maize grain yield under optimal growing conditions, and under drought stress, a 1.7% reduction was observed. However, a decrease of up to 40% or more was observed under the combination of drought and heat stress. Similarly, Maseka *et al*. (2018) found that while drought stress reduced maize grain yield by 58%, the combination of drought and heat stress reduced maize grain yield by 77%, highlighting the detrimental effects of the combined efforts of these combined two stresses.

To cope with these stresses, the agricultural community will need climate-smart solutions. There are many research studies aimed at developing technologies that promote sustainable agricultural practices, which favor increased crop productivity as well as enhanced resistance or tolerance against various stress factors (Alia *et al*., 2011; Ansary *et al*., 2012; D'Alessandro *et al*., 2014; Park *et al*., 2017; Sandhya *et al*., 2010; Zafar-ul-Hye *et al*., 2014). For instance, inoculation of plants growing under these extreme conditions with PGPR could improve the plants tolerability of

the stresses by directly or indirectly influencing their growth and morphology, thus enhancing their development and yield (Cohen *et al*., 2015; Kang *et al*., 2014). These microorganisms can achieve this feat because they experience hydric or heat stress when the water availability is low, and temperatures are high in a similar fashion to plants. However, bacteria can sense such changes in soil, such as salt concentration, sensed as osmotic variations, or increased temperatures and respond accordingly (García *et al*., 2017).

The various mechanisms utilized by PGPR that help plants gain resistance to drought and heat stresses include modification in antioxidant defense, phytohormonal levels, production of exopolysaccharides (EPS), heat shock proteins (HSPs), dehydrins, volatile organic compounds (VOCs), and metabolic adjustments (Kaushal *et al*., 2016). Metabolic adjustments include the accumulation of several compatible organic solutes like polyamines, amino acids, and sugars. For instance, *Azospirillum* has reportedly been shown to accumulate compatible solutes such as trehalose glutamate, glycine betaine, and proline to cope with osmotic stress (García *et al*., 2017). Microbes produce osmolytes extracellularly, which could be assimilated by plants and used for alleviation of certain abiotic stresses (Rodríguez-Salazar *et al*., 2009). Under heat stress, inoculation with *Pseudomonas putida* strain AKMP7 reduced membrane injury and the activity of several antioxidant enzymes in plants, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Ali *et al*., 2011). Moreover, levels of cellular metabolites like amino acids, proline, proteins sugars, starch, and chlorophyll, were also improved compared to untreated plants (Ali *et al*., 2011).

The selection, screening, and application of drought and heat-tolerant PGPRs in agriculture present an opportunity to overcome limits in productivity under drought and heat-struck lands (Kaushal *et al*., 2016). For instance, priming of seeds with PGPR improved the vigor, growth, and maize yield (ur Rehman *et al*., 2015). Wang *et al*. (2015) showed that switchgrass seeds inoculated with *Pseudomonas* PsJN *in vitro* had higher biomass. Similarly, under *in vitro* conditions, *Luteibacter rhizovicinus* MIMR1 with multiple PGP characteristics (IAA production, siderophore production, and phosphorus solubilization) significantly influenced the root growth of barley seedlings (Guglielmetti *et al*., 2013). Likewise, Gonzalez *et al*. (2015) showed that the *in vitro* inoculation of jojoba microshoots with *Azospirillum brasilense* rendered them less sensitive to NaCl indicated by their rooting ability. A thermo-tolerant (tolerant to temperatures up to 50 C) and saline-tolerant

(up to 3–4%) *Ochrobactrum cytisi* IPA7.2 promoted the growth of 15-day-old potato microplants *in vitro*, as seen with increases in the number of leaves (by 7%), the length of shoots (by 34%), and the number of roots (by 16%) (Burygin *et al*., 2019). These studies emphasize screening the early effect of PGPR on plants under various abiotic stresses. Moreover, co-inoculation of plants with a bacterial mixture/consortium has been shown to provide greater benefits to plant growth than inoculation with a single bacterial strain (Thomloudi *et al*., 2019; Molina-Romero *et al*., 2017). Studies have continually observed synergistic effects by co-inoculation of mixtures of bacteria strains (Prasad and Babu, 2017). Thus, the compatibility of bacterial strains becomes crucial for formulating bioinoculants that promote plant growth.

In vitro culture techniques have the advantage of more defined conditions, controlled conditions, homogeneity of stress application, and defined nutrient media, thus minimizing environmental variations (Akinyosoye *et al*., 2015; Niu and Kolter, 2018; Viscardi *et al*., 2016). They also have the added advantage of low cost, ease of handling, being less laborious, and permits the removal of less effective PGPR at the earliest time (Rauf *et al*., 2008). Polyethylene glycol (PEG), a solution of high molecular weight, is commonly used to induce drought/osmotic stress in plants and microbes *in vitro* (Ahmad *et al*., 2018; Sakthivelu *et al*., 2008). Unlike sodium chloride and mannitol that are also used to induce osmotic stress, PEG of high molecular weight (6000 or above) are non-toxic to plants and microbes (Akinyosoye *et al*., 2015). They cannot enter cell pores, thus have been shown to have similar effects to that observed in the field, similar to soil drying (Ahmad *et al*., 2018). To induce heat stress *in vitro*, incubation under elevated temperatures detrimentally affecting the plant is often used (Cheikh and Jones, 1994; Cheikh and Jones, 1995; Dupuis and Dumas, 1990).

According to the literature search conducted, there are no reported studies that investigated the amelioration of dual drought and heat stress on the early growth of maize using PGPR. As such, the study aimed to use PGPR isolates previously shown to have plant growth-promoting attributes and tolerance to drought/osmotic and heat stress at inducing plant tolerance to the combinatory stress of drought and heat stress under *in vitro* conditions. The PGPR isolates used were 11MN1 (*Bacillus thuringiensis*), 14MN1 (*Acinetobacter* sp.), 21MN1B (*Bacillus pseudomycoides*), 33MP1 (*lelliottia amnigena*), 36MP8 (*Leclercia* sp.). Furthermore, bacterial isolates with the potential to promote plant growth under these conditions were investigated for their compatibility.

3.3.Materials and Methods

3.3.1. Preparation of Polyethylene glycol (PEG) 6000 infused agar

The study used the procedures described by Verslues and Bray (2004) and van der Weele *et al*. (2000). Briefly, MS (Murashige and Skoog, 1962) medium with 25 % strength was used and supplemented with 5 g/L sucrose. For solid MS agar, 8 g/L of bacteriological agar was added. The PEG overlay solution was prepared as above but without bacteriological agar. Polyethylene glycol 6000 was added to the PEG overlay solution after autoclaving while the media solution was still hot to a final concentration of 20 %. The pH value of the MS agar media and PEG overlay was adjusted to 5.8 before autoclaving.

Under sterile conditions, 40 ml of MS agar was pipetted into magenta boxes and allowed to solidify. Afterward, a total volume of 60 ml PEG overlay solution was pipetted onto the top of each solidified MS agar. This achieved the desired water potential of approximately -1.2 MPa whereby the volume ratio of MS agar to PEG overlay solution volume was always 2:3. The media was kept overnight at room temperature to allow the PEG solution to absorb into the media. For extended media storage, the magenta boxes were covered in plastic to prevent drying and stored at 4 °C. Excess solution of the PEG overlay was removed before use, taking caution not to lose the agar floating on top of the solution.

3.3.2. Surface sterilization and germination of maize seeds

Maize seeds showing no disease symptom were used for the current experiment. Maize seeds were surface sterilized using 70 % ethanol and agitated using a shaker for 15 min at 120 rpm. Then the seeds were disinfected with 3.5 % Sodium Hypochlorite (Jik) for another 15 min. The seeds were then rinsed three times using sterile distilled water to reduce microbial load at the early stages of germination (Akinyosoye *et al*., 2014). The maize seeds were germinated between two filter papers in Petri dishes filled with 10 ml sterile distilled water. A total of 10 seeds were placed per petri dish facing up. The Petri dishes containing surface-sterilized seeds were incubated in the dark at 27 °C. After an incubation of 7 days, germinated maize seeds with a root of 1-2 cm were chosen and used in the subsequent steps.

3.3.3. Inoculation of bacteria on maize seedlings

The germinated maize seedlings were inoculated with the bacterial strains following the method described by Niu and Kolter, (2018). Each of the 5 bacterial strains was streaked on tryptic soy

agar (TSA) plates and incubated for 24 h at 32 °C. Afterward, a single colony of the bacterial isolates was inoculated in 10 ml of tryptic soy broth (TSB) and incubated in a shaker rotating at 120 rpm at 32 °C overnight. Following incubation, the bacterial cells were placed on ice and then collected in 2.0 ml tubes by centrifuging at 2,940 × g for 10 min. The cells were resuspended in 0.85% NaCl saline solution. The bacterial concentration was determined using the dilution series and direct colony count method. Subsequently, the bacterial cell suspensions were diluted to ~108 CFU/ml. Approximately 10 surface-sterilized and germinated maize seeds with primary roots of 1-2 cm were soaked in 30 ml of the bacterial suspensions in Petri dishes at room temperature for 1 h. To ensure the roots were submerged in the suspensions, the germinated seedlings were moved using sterile forceps. Another 10 surface-sterilized and germinated seeds were soaked in 0.85% NaCl saline solution for 1 h and used as a control. Following the 1 h, the maize seedlings were transferred onto the MS agar infused with PEG 6000 in magenta boxes using sterile forceps. The germinated seedlings were gently pressed onto the media using forceps inserting the primary roots into the agar. The magenta boxes were incubated in a growth room with 16 h/8 h light and dark, at 32 °C/28 °C day and night temperatures at a relative humidity of 50 % for 14 days.

3.3.4. Dual culturing method or cross streak method to test microbial compatibility

Bacterial isolates that demonstrated the potential to induce tolerance to maize plants subjected to dual drought and heat stress were evaluated for compatibility. Single isolates previously streaked onto tryptic soy agar (TSA) media were used for the experiment. The dual culture technique was used. The primary isolate was streaked first and incubated at 32 °C for 24 h, then the second microbial isolate was streaked perpendicularly, growing outward from the emerged colonies of the initial streak and incubated for another 24 h 32 °C (Prasad and Babu, 2017). Subsequently, photographic data of the agar plates were obtained. Visible clearings at intersections of the paired microbial isolates were regarded as inhibition zones.

3.3.5. Statistical analyses

To check whether the measured plant growth parameters were statistically different between treatments, the two-tailed Student's *t*-test (unequal variances) at $P < 0.05$ confidence level was conducted using R studio. Each treatment was replicated four times, and the data were expressed as mean ± SD. Different letters were used to depict treatments with statistical differences, whereas the same letters indicated that there were no statistical differences.

3.4.Results

3.4.1. Effects of PGPR on maize under drought and heat stress*in vitro*

An *in vitro* experiment in magenta boxes was conducted in a growth room with controlled conditions mimicking the combination of drought and heat stress. The effects of drought and heat tolerant PGPR strains on the growth of maize seedlings were investigated. Previously, a total of 12 isolates exhibited tolerance to both drought and heat stress and exhibited PGP traits. Among the 12, five isolates were chosen for the *in vitro* evaluations. The isolates used were*Bacillus thuringiensis* (11MN1), *Bacillus Pseudomycoides* (21MN1B), *Lelliottia amnigena* (33MP1), *Acinetobacter* sp. (14MN3B), and *Leclercia* sp. (36MP8), chosen based on the superior ability to tolerate drought and heat stress and because of the genetic differences.

All isolates except for 14MN3B improved the total wet weight of maize seedlings *in vitro* under the combined drought and heat stress (Figure 10). Plants inoculated with 14MN3B had a statistically lower wet weight compared to the control. Isolate 11MN1, 21MN1B, and 36MP8 statistically improved the fresh weight of maize seedlings by 48.52, 220.59, 107.35 % respectively, compared to the control (Figure 10). Isolate 33MP1 also increased maize fresh weight but the increase was not statistical. Maize seedlings inoculated with isolate 21MN1B demonstrated the highest improved fresh weight.

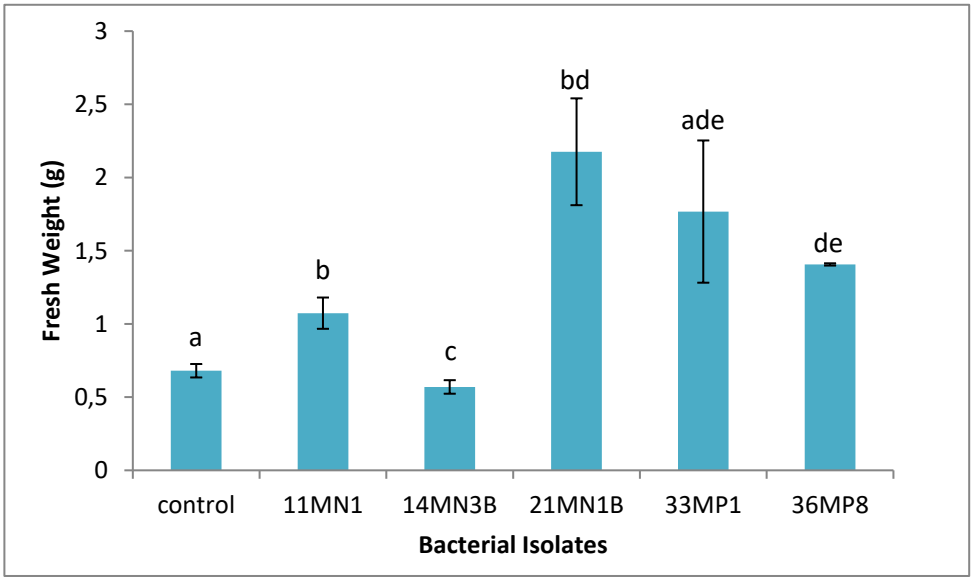

Figure 10: Influence of selected bacterial isolates with PGPR characteristics on maize wet weight *in vitro* under simulated drought and heat stress conditions. Drought was simulated by adding 20 % PEG 6000 and heat stress was simulated by incubation plants at 32/28 ºC day and night temperatures. Student T test; p <.05; different letters indicate significant differences.

All tested isolates, except 14MN3B, improved the total dry weight of maize seedlings under the combination of drought and stress (Figure 11). However, only isolate 33MP1 (53.53%)statistically improved maize dry weight compared to the control. The total dry weight of maize seedlings inoculated with 14MN3B was statistically lower than the control.

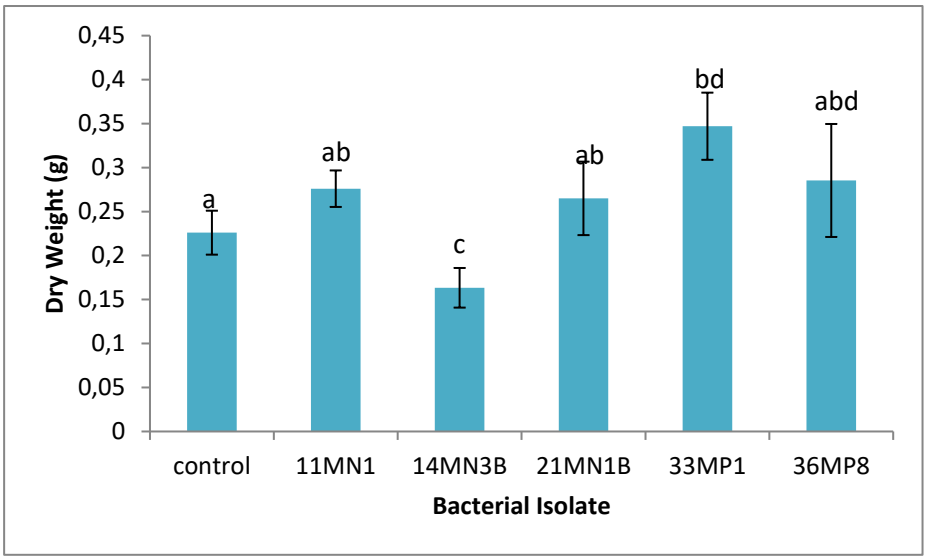

Figure 11: Influence of selected bacterial isolates with PGPR characteristics on maize dry weight *in vitro* under simulated drought and heat stress conditions. Drought was simulated by adding 20 % PEG 6000 and heat stress was simulated by incubation plants at 32/28 ºC day and night temperatures. Student T test; p <.05; different letters indicate significant differences.

Similarly, the moisture content was improved for maize plants inoculated with 11MN1, 21MN1B, 33MP1, and 36MP8 compared to the control (Figure 13). Maize moisture content was significantly improved by 43.75, 258.70, 103.6, and 95.48 % for 11MN1, 21MN1B, 33MP1, and 36MP8 respectively compared to the control plants. Maize seedlings inoculated with isolate 21MN1B

resulted in the highest moisture content. In contrast, isolate 14MN3B failed to improve moisture content compared to control under the tested growth conditions.

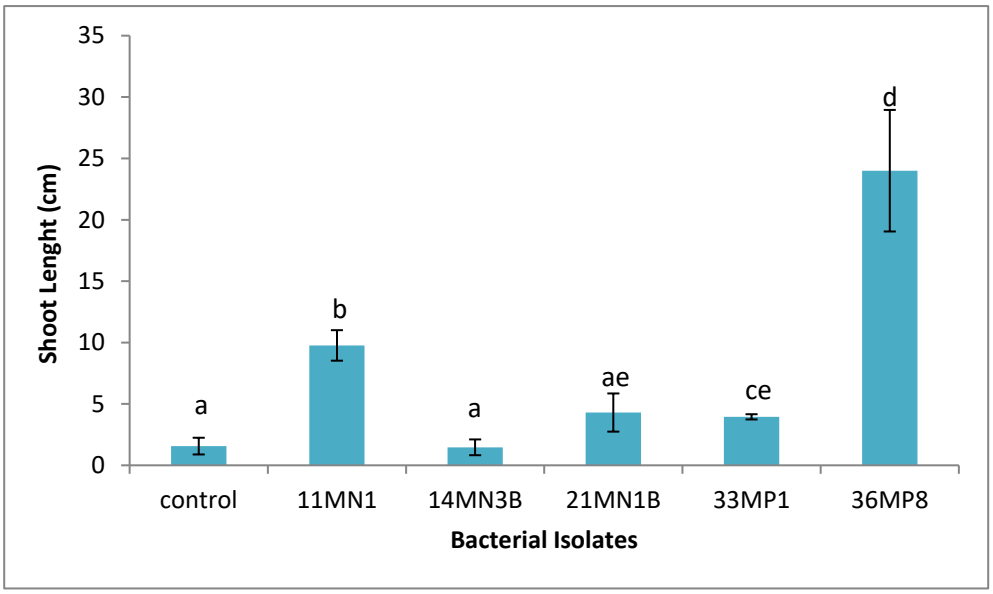

Figure 12: Influence of selected bacterial isolates with PGPR characteristics on maize shoot length *in vitro* under simulated drought and heat stress conditions. Drought was simulated by adding 20 % PEG 6000 and heat stress was simulated by incubation plants at 32/28 ºC day and night temperatures. Student T test; p <.05; different letters indicate significant differences.

Bacterial inoculation with isolate 11MN1, 33MP1 and 36MP8 significantly improved the shoot length of the maize seedlings (Figure 12). Maize seedlings inoculated with 11MN1, 33MP1 and 36MP8 had an average shoot length of 9.77, 3.95, and 24 cm respectively, whereas the control had an average shoot length of 1.57 cm. Isolate 21MN1 improved the shoot length of maize compared to the control however, there were no significant differences. Plants inoculated with 14MN3B did not improve maize shoot length under the combination of drought and heat stress. Moreover, visual depictions indicate the control and plants inoculated with 14MN1B resulted in plant death after the 14-day growth period, as observed by the brown colouring of the shoots (Figure 14).

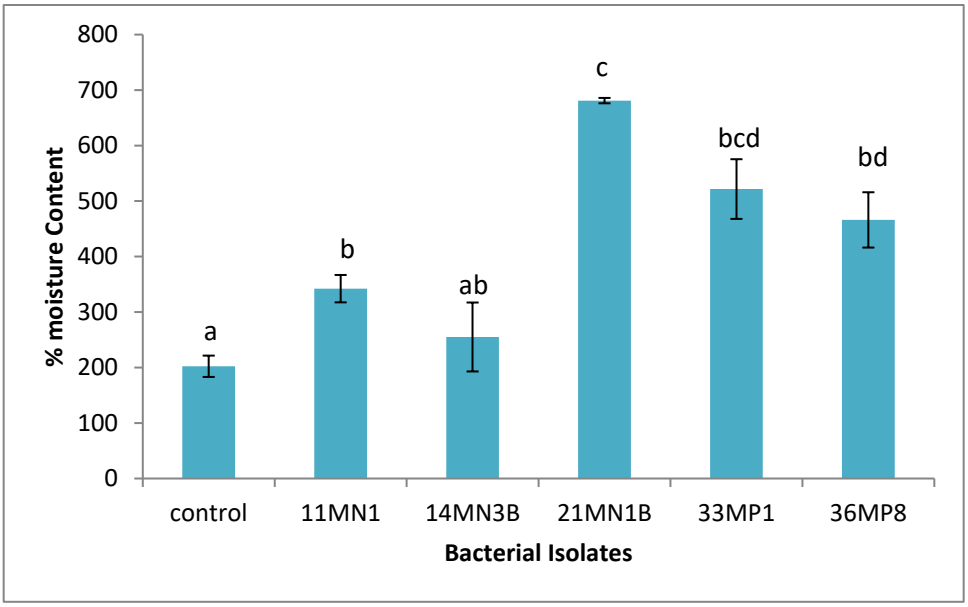

Figure 13: Influence of selected bacterial isolates with PGPR characteristics on maize wet weight *in vitro* under simulated combined drought and heat stress conditions. Drought was simulated by adding 20 % PEG 6000 and heat stress was simulated by incubation plants at 32/28 ^{o}C day and night temperatures. Student T test; p <.05; different letters indicate significant differences.

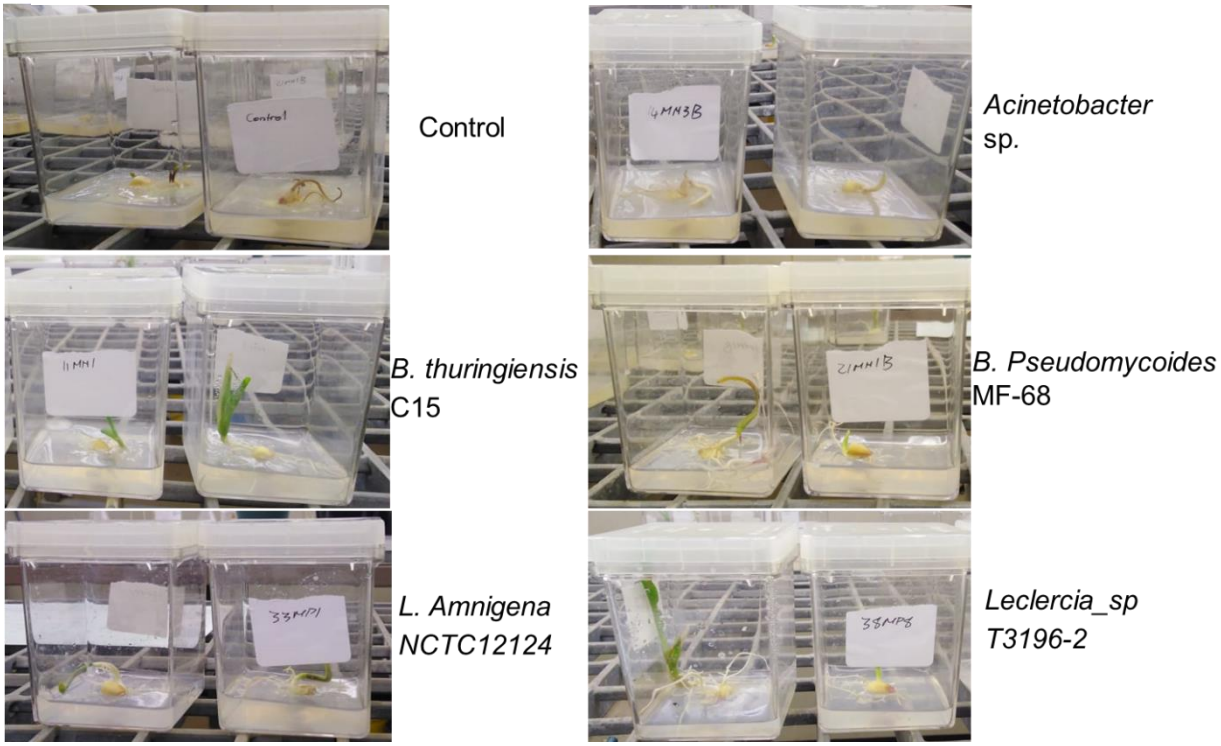

Figure 14: Visual depictions of inoculated plants with selected bacterial isolates under combined drought and heat stress.

3.4.2. Compatibility of bacterial strains using the dual culture technique

Bacterial isolates that exhibited the potential to alleviate the combination of drought and heat stress on maize plants were also tested for compatibility. This was done, to see whether these PGPR isolates could potentially be co-inoculated or formulated as one biofertilizer. The dual culturing approach was the method of choice in testing the compatibility of the strains that potentially mitigated the negative effect of drought and heat stress on maize plants

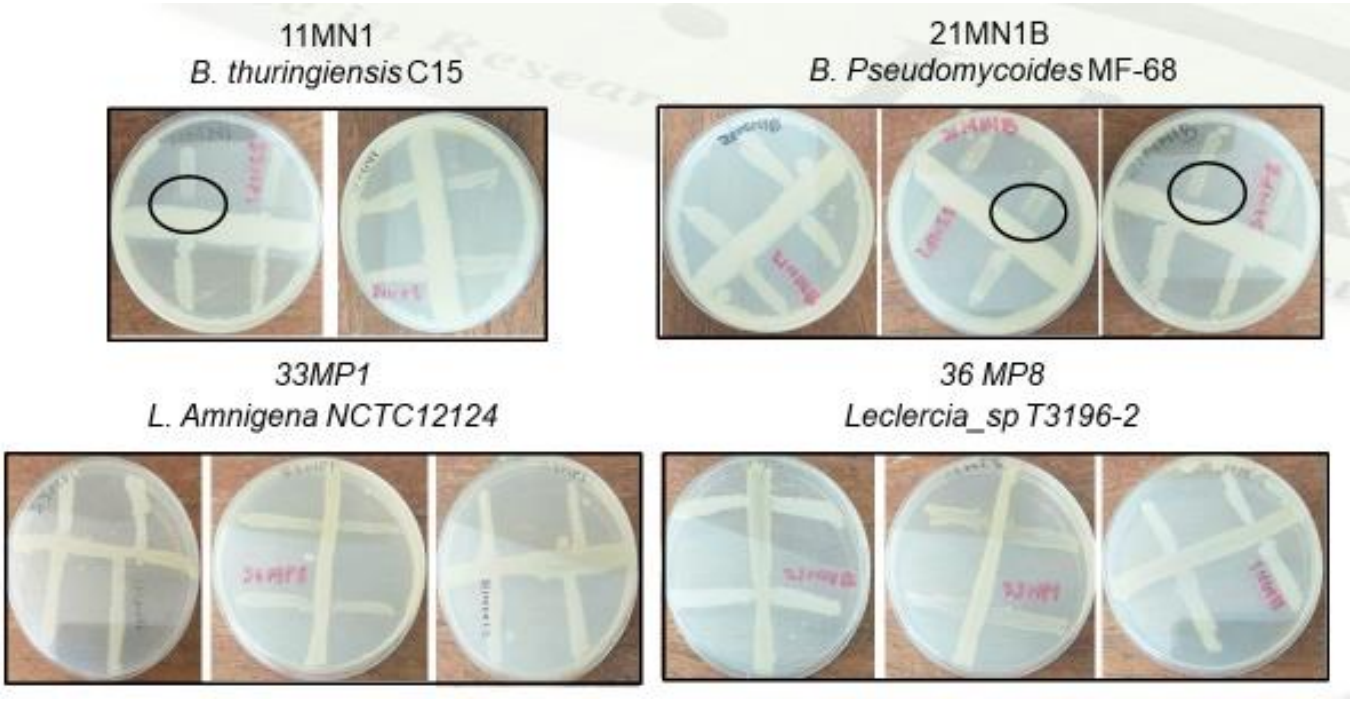

Figure 15: Compatibility of the bacterial isolates tested on TSA plates using the cross-streak dual culturing method.

Figure 15 depicts the dual culturing of the potential PGPR isolates using the cross-streak method. This method provides a visual observation of whether one isolate can inhibit another isolate or whether the two isolates are compatible *i.e.* no antagonism. Isolate 11MN1 (*B. thuringiensis*) showed slight inhibition against 33MP1 (*L. amnigena*), whereas 21MN1B (*B. pseudomycoides*) showed slight inhibition against 33MP1 (*L. amnigena*) and 36MP8 (*Leclercia* sp.). No antagonism was observed between 11MN1 and 21MN1B. Isolate 33MP1 and 36MP8 were not antagonistic against the other isolates (Figure 15). Overall, isolates from the *Bacillus* group seem to exhibit

slight antagonism against *L. amnigena* and *Leclercia* sp. belonging to the *Enterobacteriaceae* group.

3.5.Discussion

Numerous studies have investigated the use of plant growth-promoting rhizobacteria (PGPRs) to mitigate the adverse effects of abiotic stresses on crops (Sandhya *et al*., 2010; Zafar-ul-Hye *et al*., 2014). According to the literature search conducted on the current study there has not been any reports of PGPR exhibiting characteristics of inducing plant tolerance to a combination of abiotic stresses to date, especially drought and heat stress which are commonly prevalent under field conditions. As such, in this study, we report on bacterial isolates with PGPR characteristics and tolerance to drought and heat at mitigating the deleterious effects of concurrent drought and heat stress. The isolates 11MN1, 14MN3B, 21MN1B, 33MP1, and 36mp8 were previously identified as relatives of *B. thuringiensis, B. pseudomycoides, Acinetobacter sp., L. amnigena*, and *Leclercia* sp. Respectively.

Based on the results, isolate 11MN1 (*B. thuringiensis*) significantly improved the plant growth parameters of maize under drought and heat stress *in vitro*. Isolate 11MN1 significantly enhanced the total wet weight, shoot length, and % moisture content of maize seedlings under dual drought and heat stress. Isolate 11MN1 displayed nitrogen-fixing capabilities and IAA production (12.78 ug/ml) in previous investigations. Likewise, isolate 21MN1B (*B. pseudomycoides*) also exhibiting N-fixing capabilities and IAA production (11.04 ug/ml) and significantly improved the total wet weight and % moisture content of maize seedling subjected to drought and heat stress. *Bacillus thuringiensis and B. pseudomycoides* has been extensively studied as a PGPR. Both species are Gram positive, rod-shaped, and spore-forming bacterium belonging to the *B. cereus* species-group. These abilities of *B. thuringiensis and B. pseudomycoides* to promote plant growth and alleviate drought stress has been shown in previous studies. For instance, an IAA producing *B. thuringiensis* induced drought tolerance in *Lavandula dentata* plants and improved the plant's metabolic activities, physiology, and nutrition (Armada *et al*., 2014). Moreover, shoot proline accumulation was enhanced in inoculated plants compared to control plants and translated to improved plant growth parameters. Similarly, in *Salvia officinalis*, inoculation with *B. thuringiensis* improved the root development by 43 % over control plants under drought stress conditions (Armada *et al*., 2014). Also, in wheat seedlings under drought stress, priming with *B.*

thuringiensis AZP2 increased plant biomass, and a five-fold higher survival under severe drought was reported. (Timmusk *et al*., 2014). *Bacillus thuringiensis* improved wheat growth under heat stress (Ashraf *et al*., 2019). The treated seedlings had enhanced shoot and root fresh weight with higher antioxidant enzyme activity, protein content, and proline (Ashraf *et al*., 2019). Stefan *et al*., (2013) previously showed a *B. mycoides* S7 strain able to produce IAA (4.85 ug/ml) to improve yield of *Phaseolus coccineus* L by 88.5 % compared to control. The genus has previously been shown to exhibit phosphate solubilization and ACC deaminase production (Sezen *et al*., 2016). ACC deaminase activity indirectly lowers the level of ethylene by hydrolysing 1-aminocyclopropane-1-carboxylic acid (ACC) in plant tissues, lightening the negative effects of ethylene thereby promoting plant growth under stressed conditions (Sezen *et al*., 2016). One study on maize plants, showed that *B. mycoides* and consortium of two bacteria had an effective role in phytoextraction of cadmium-contaminated soils thus emphasizing on the potential for stress alleviation (Malekzadeh *et al*., 2012). These studies show the potential of similar strains at enhancing nutrient uptake and inducing plant growth under abiotic conditions.

Isolate 33MP1 (*L. amnigena*) and 36MP8 (*Leclercia sp.*) both displayed phosphorus solubilisation and IAA production (15.81 ug/ml and 13.54 ug/ml respectively). *Leclercia adecarboxylata* and *L. amnigena* belong to the *Enterobacteriaceae* family, previously called *Escherichia adecarboxylata* and *Enterobacter amnigenus* respectively. Both species are widely distributed in nature, inhabiting the rhizosphere, seeds, food, plants, water, and various other environmental sources. *Leclercia* sp. significantly improved the total wet weight, shoot length and % moisture content of maize seedlings under the combined effects of drought and heat stress. Likewise, 33MP1 *(L. amnigena.)*, had significant differences in total dry weight, shoot length and percentage moisture content of maize seedlings under the same conditions. The species have been shown to demonstrate PGPR properties in previous studies, and our results are consistent with previous studies. For instance, *Leclercia* sp. QAU-66, isolated from the rhizosphere of *Vigna mungo* demonstrated P solubilisation (solubility index, SI = 3.3) (Naveed *et al*., 2014). A 10 % increase in the root and shoot length of *Phaseolus vulgaris* was observed when treated with strain QAU-66. *Leclercia* sp. O has also been shown to have an IAA production (180 μg/mL), N fixation as well as the production of siderophores (Melo *et al*., 2016). One study showed that *Leclercia adecarboxylata* MO1 with IAA production (9.815 μg/ml) and showed the presence of ACC (1-Aminocyclopropane-1-Carboxylate) deaminase acdS gene and showed tolerance against salinity

stress, could alleviate salinity stress in tomato (*Solanum lycopersicum L.*). Moreover, ABA levels were significantly lower in MO1-inoculated (30.04 and 30.28%) relative to the control plants under salinity stress as well as normal condition, respectively (Kang *et al*., 2019). *Lelliottia amnigena* strain RZME03 and Strain RZME13 isolated from the root of maize have been shown to possess plant growth-promoting capabilities such as the production of IAA (0.1and 0.073 mg/L respectively), solubilisation of phosphate and ACC deaminase activity (2.082 and 1.700 mg/L respectively) (Menéndez *et al*., 2016). Therefore, the benefits of *L. amnigena* association with maize plants has been reported previously. Furthermore, *Leclercia adecarboxylata* ACC-deaminase producing PGPR improved photosynthetic rate, transpiration rate, stomatal conductance, total chlorophyll content, and grain yield of wheat under drought stress (Danish *et al*., 2019). In the current study, ACC deaminase activity of the isolates was not determined. However, this could potentially mean that 33MP1 and 36MP8 may possess this characteristic. The ACC deaminase activity has widely been demonstrated to be active in alleviating biotic stresses (Menéndez *et al*., 2016). Even so, *Lelliottia amnigena* has been shown to be human opportunistic pathogen that has been found in endophthalmitis and was shown to cause soft rot symptoms on potatoes and bulb decay on onion (Abd-Elhafeez *et al*., 2018, Menéndez *et al*., 2009). Thus, may be unideal as a PGPR for susceptible plants but could open up avenues as in the biological control of weed.

Unlike the other isolates 14MN3B identified as *Enterobacter* sp. showed pathogenicity, causing rot on maize plants under the combination of drought and heat stress. This has not been previously reported before. Generally, *Acinetobacter* sp. reportedly has beneficial effects on maize plants. This was the case in *Acinetobacter* sp. S3r2 inoculated maize plants, where a significant increase in the dry biomass and total N content was observed throughout the study (Kuan *et al*., 2016). However, this is the first-time plant inoculated with *Acinetobacter* sp. reported under the combinatory effects of drought and heat stress.

The study also evaluated the compatibility of the bacterial isolates that showed potential of inducing tolerance to maize plants and promoting growth (Figure 15). Plant inoculation with a bacterial mixture/consortium has been shown to provide more enhanced effects to plant growth than single bacterial strain inoculation (Thomloudi *et al*., 2019; Molina-Romero *et al*., 2017). Thus, the compatibility of strains is crucial for formulating bioinoculants that promote plant

growth because of the synergistic effects continually observed (Prasad and Babu, 2017). In the current study, it was observed that isolates from the *Bacillus* group seem to exhibit slight antagonism against *L. amnigena* and *Leclercia* sp. belonging to the *Enterobacteriaceae* group. However, no antagonism was observed between the *Bacillus* spp. and likewise between the *Enterobacteriaceae* group. *Bacillus* spp. are widely known for antimicrobial activity, with *B. thuringiensis* widely used commercially as an insecticide. By contrast, *B. mycoides* is scarcely available commercially. Nevertheless, *B. mycoides* strain BacJ was shown to elicit systemic resistance in sugar beet, which was related with increased activity of Peroxidase and could reduce *cercospora leaf spot (cercospora beticola sacc.)* of sugar beet by 38-91 % in both glasshouse and field experiments (Bargabus *et al*., 2002). Indigenous *Bacillus spp* isolates identified as *B. pseudomycoides* were reported to have increased seedling height with the effectivity between 20.23 to 61.13% and could decrease *R. solanacearum* incidence level-up to 100%in tomato plants (Yanti *et al*., 2018). This suggests that 11MN1 and 21MN1B could have the potential to protect plants against phytopathogens. In contrast, 33MP1 and 36MP8 exhibited no antimicrobial properties against each other and the other isolates. Similar with what was observed in the current study against the other bacterial isolates, *Leclercia* sp. O was reported without any antimicrobial activity (Melo *et al*., 2016). In addition, *L. adecarboxylata* LSE-1 strain was found to be compatible with *Bradyrhizobium* sp. LSBR-3 without producing zone of clearance on the tryptone soy agar plate assay, suggesting that *Leclercia* spp. are generally compatible with other PGPR isolates and can work synergistically to improve plant growth (Kumawat *et al*., 2019). However, Enterobacter sp. SA187 isolated from *Indigofera argentea*with the closest relative *Leclercia adecarboxylata* LMG 2803 was shown to possess numerous genes played roles in the defense against oxidative stress, survival, and antimicrobial microbial production, suggesting a possible role in antimicrobial defence (Andrés-Barrao *et al*., 2009). Even so, some studies have shown that co-cultures of antagonist microbes can provide still greater plant growth than single inoculations. This was the case with *Trichoderma asperellum* GDFS1009 and *Bacillus amyloliquefaciens* (Karuppiah *et al*., 2019). *Bacillus amyloliquefaciens* displayed antagonism against *Trichoderma asperellum*, however seeds treated with the co-culture significantly enhanced the plant growth and protection against phytopathogenic fungi (Karuppiah *et al*., 2019). Therefore, with the antagonism displayed by the *Bacillus* spp. against *Enterobacteriaceae* group, it is possible their co-inoculation may result in enhanced plant growth under the dual effects of drought and heat stress.

Also, 11MN1 and 21MN1B demonstrated antimicrobial properties against 33MP1 (*L. amnigena*) and 36MP8 (*Leclercia* sp.), suggesting the production of antimicrobial properties.

3.6.Conclusion

Drought and heat are the major environmental stresses that continually influence the growth and development of plants. Under field conditions, drought and heat stresses occur more frequently in combination than alone. In this study we report four PGPR isolates namely *B. thuringiensis, B. pseudomycoides, L. amnigena,* and *Leclercia sp.* with the potential to alleviate the adverse effects induced by the combination of these stresses in maize seedlings. These bacterial isolates improved the growth of maize under concurrent drought and heat stress *in vitro*. The application of these drought and heat-tolerant PGPRs in agriculture present an opportunity to overcome limits in productivity under drought and heat-struck lands. *Acinetobacter* sp. exhibited pathogenic effects in maize plants subjected to drought and heat stress, contrary to the plant growth-promoting characteristics observed in the previous chapter thus, making this the first report of such a characteristic on maize.

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4. Chapter 4: Drought and heat tolerant plant growth-promoting rhizobacteria: Effects on the early growth of maize subjected to dual drought and heat stress

4.1.Abstract

Maize (*Zea mays* L.) is a major crop for human consumption and animal feed in Sub-Saharan Africa. Moreover, it is highly susceptible to the stress combinations of drought and heat stress than these individual stresses alone. Previously, *B. thuringiensis* 11MN1, *B. pseudomycoides* 21MN1B, *L. amnigena*, 33MP1, and *Leclercia* sp. 36MP8 demonstrated the potential to alleviate the dual effects of drought and heat stress under *in vitro* conditions. As such, to better understand the effects of these potential PGPR under soil conditions, a greenhouse experiment was conducted simulating dual drought and heat stress conditions. The results show that under simulated concurrent drought and heat stress, isolate 33MP1, 36MP8 and the combination of all isolates (11MN1, 21MN1, 33MP1, 36MP8) significantly improved shoot dry weight and the leaf relative water content (RWC) of maize seedlings. Moreover, the mixture/co-inoculum performed the best, significantly improving root dry biomass as well. Maize plants inoculated with 11MN1 and 21MN1B only exhibited improvement in RWC. No significant improvements were observed in all treatments under no stress conditions. Overall, isolates *L. amnigena* 33MP1, *Leclercia sp.* 36MP8 and the co-inoculation of all the isolates show potential for conferring tolerance in maize plants under dual drought and heat stress.

4.2.Introduction

Maize (*Zea mays* L.) is a major crop for human consumption and animal feed in Sub-Saharan Africa. Maize is known for its susceptibility to drought stress and high temperatures. However, the combination of these stresses has been recognized as a greater threat to plants. It has been shown that a 1 °C temperature increment above 30 °C leads to reduced yield by 1% under an optimal rain-fed condition and a 1.7% reduction under water deficit conditions. However, under the dual effects of drought and heat stress, a decrease of up to 40% has been observed (Maseka *et al*., 2018; Lobell *et al*., 2011). Based on the observed changes in the worldwide climatic patterns, an estimated loss in the world maize production of 15–20% each year is expected, with these stresses becoming a major threat to maize yields (Chen *et al*., 2012, Lobell *et al*., 2011).

The interaction of drought and heat stress is known to alter plant metabolism differently compared to single stress (Pandey *et al*., 2015; Rizhsky *et al*., 2004; Shahsavandi *et al*., 2016). For example,

the accumulation of sucrose instead of proline was observed in *Arabidopsis thaliana* plants under combined drought and heat stress (Rizhsky *et al*., 2004). Moreover, enhanced transpiration, typical of plants under heat stress in an attempt to cool the leaf surface, is aggravated under the dual effects of drought and heat stress leading to more water loss and increased salt uptake (Rizhsky *et al*., 2002; Mittler, 2006). Furthermore, the concurrent effects of drought and heat stress on osmolyte accumulation, enzymatic and non-enzymatic antioxidants, nutrients uptake, and photosynthetic components of maize are more detrimental than the singular stresses (Hussain *et al*., 2019). The accumulation of ROS and rate of lipid peroxidation are reportedly higher, and these inhibit the photosynthetic efficiency thus restricting the growth of maize (Hussain *et al*., 2019). Thus, it is important that we find alternative climate smart solutions to address these issues.

Beneficial plant-microbe interactions have been exploited and are considered promising strategies in sustainable agricultural practices (Reddy *et al*., 2014). Plant growth-promoting rhizobacteria have been successfully applied as biofertilizers or phytostimulators in agriculture (El-Akhdar *et al*., 2020). Another remarkable feature is their capability to support plants under stressed environments. They can ameliorate the deleterious effects of abiotic stresses to which crop plants are often exposed, such as drought and elevated temperatures (Bano *et al*., 2013). These beneficial soil microorganisms can produce plant growth-promoting (PGP) substances in large quantities, which in turn indirectly influence the growth and morphology of the plants. For instance, maize seeds primed with EPS-producing bacterial strains could improve root and shoot length, soil moisture contents, leaf area, and plant biomass (Naseem and Bano, 2014). Meena *et al*. (2015) demonstrated that *Pseudomonas aeruginosa* strain 2CpS1 mitigated the detrimental effects of temperature stress in wheat plants leading to increased relative water content, chlorophyll content, root length, plant height, and reduced cell membrane injury. Wheat seeds inoculated with *Azospirillum brasilense* NO40 or *Bacillus amyloliquefaciens* UCMB5113 reduced heat stress and decreased the expression of several stress-related genes that were upregulated in heat-stressed non-inoculated plants (Abd El-Daim *et al*., 2014). Sandhya *et al*. (2010) reported increased leaf water potential, relative water content, and plant biomass due to the accumulation of proline, free amino acids, and soluble sugars in maize plants when inoculated with PGPR *Pseudomonas putida* GAP-P45.

A great number of biofertilizers available in the South African market are imported (Raimi *et al*., 2017; Raimi and Adeleke, 2018; Raimi *et al*., 2021). This is of great concern because they frequently do not perform as intended due to various factors such as the ability to proliferate under concurrent abiotic stresses (African climate), they fail to outcompete soil endemic microbes, thus are unable to successfully colonize plant roots and enhance growth. Therefore, it is crucial to produce biofertilizers utilizing microbial isolates endemic to South Africa and well adapted to the environment, with drought and heat tolerance. In the previous chapters, 4 isolates namely *B. thuringiensis* 11MN1, *B. pseudomycoides* 21MN1B, *L. amnigena*, 33MP1, and *Leclercia* sp. 36MP8 demonstrated the potential to alleviate the dual effects of drought and heat stress under *in vitro* conditions. Moreover, it was observed that these isolates could potentially be used as mixture/co-culture inoculum. As such, to better understand the response of these potential PGPR under these conditions in soil, greenhouses are often used. Greenhouse-based assays provide an attractive compromise between costly, time-consuming field trials and laboratory assays, which do not typically evaluate entire plants (Both *et al*., 2015). A major advantage of greenhouse screening is that the environment can be controlled to minimize disease pressure and the effects of confounding biotic and abiotic factors i.e. weather, fluctuations in temperature, interference from phytopathogens, and pollution (Forero *et al*., 2019). Moreover, they allow for rapid growth conditions throughout the year and microbial and nutrient effects can more easily be distinguished, compared to field studies (Simko *et al*., 2007). They can also help identify putatively promising PGPR that can then undergo more extensive field evaluations in subsequent studies. As such, the study aims to evaluate the potential of these PGPR isolates at mitigating the dual effects of drought and heat stress under greenhouse conditions.

4.3.Materials and Methods

4.3.1. Preparation of bacterial inoculum

Each of the 4 bacterial strains that previously showed the potential to alleviate the detrimental effects of the combination of drought and heat stress on maize *in vitro* was streaked on TSA plates and incubated at 32 °C for 24 h. Afterward, a single colony of each strain was inoculated in 100 ml of tryptic soy broth (TSB) and shaken at 120 rpm at 32 °C overnight. The cells were collected in 50 ml tubes by centrifuging at 2,940 × g for 10 min at 4 °C. The cells were resuspended in 40 ml 0.85 % saline solution. Viable cells were estimated using the dilution series and direct colony count method. Serial dilutions were performed for each bacterial suspension then spread plated

onto TSA and incubated at 32 °C overnight. Colony-forming units were counted, and the concentration was determined.

Subsequently, the bacterial suspensions were diluted to ~10^8 CFU/ml with 0.85 % saline solution. For multiple strain/mixture inoculum, the bacterial suspensions were mixed in a 50 ml falcon tube in equal volumes (10 ml for each of the 4 strains). A volume of 40 ml of the bacterial suspensions was used for seed inoculation.

4.3.2. Inoculation of maize seeds

Seeds used for the study were the commercially available "Zama Star' obtained from Starke Ayres (Pretoria, South Africa). Maize seeds were checked for disease symptoms. Healthy seeds were chosen for use. Maize seeds were surface sterilized using 70 % ethanol and agitated using a shaker for 5 min at 120 rpm. Then the seeds were disinfected in 3.5 % Hypochlorite acid for another 5 min and rinsed 5 times using sterile distilled water to reduce microbial load at early stages of germination (Akinyosoye *et al*., 2014). A total of 30 maize surface-sterilized seeds were then inoculated with bacteria by soaking in 40 ml of ~10^8 CFU/ml concentration of each single isolate and their mixture. The seedlings were allowed to soak in the bacterial solution for 1 h. An additional 30 surface-sterilized seeds were soaked in 0.85 % saline solution for 1h and used as a control.

4.3.3. Determining the water holding capacity

As an initial step, the moisture content potting mix was determined. Three empty aluminum-based weighing boats were weighed. Afterward, they were filled with 50 g of potting mix and placed to dry in an oven at 80 °C. These were left to dry until a constant weight was achieved (48 h). The weighing boats with the oven-dried soil were weighed, and the moisture content was determined using the equation below. The average moisture content of the three replicates was calculated.

$$\text{Soil moisture content} = \frac{(weighing\ boat\ mass + Soil) - (weighing\ boat\ mass + oven\ dried\ soil)}{(weighing\ boat\ mass + oven\ dried\ soil) - weighing\ boat\ mass} \times 100$$

Next, plastic pots were filled with a potting mix that was to be used in the pot experiment. The potting mix was firmly pressed to pack the soil particles evenly. Afterward, water was poured over the soil surface quickly, and evenly allowing the water to create a pool over the soil surface. The water was poured until the container began to leak from the bottom. The pots were

immediately covered with plastic and tied to prevent evaporation and allow water loss only via drainage. These pots were kept in the shade for 48 h. The potting mix was then collected from the middle section of the container and weighed. The water holding capacity was then determined using the equation above.

4.3.4. Pot-plant experiment

Briefly, a total of 1.6 kg of heat treated (80 ℃ for 1 hr) potting mix (bark and peat-based) was weighed into pots and pre-irrigated with distilled water and allowed to drain for 2h. Seeds previously soaked in bacterial solutions were planted 5cm deep. Seven seeds were planted per pot. The treatments included seeds bacterized with isolate 11MN1, 21MN1B, 33MP1, 36MP8, and the mixture of all isolates. Non-bacterized seeds that were soaked in 0.85% saline solution served as the control treatment. Under no stress conditions, the seedlings were grown under greenhouse conditions at 23/25 °C for 32 days. The water holding capacity was maintained at 80 % by watering, when necessary, with distilled water. Under dual drought and heat-stressed conditions, the seedlings were grown for 32 days at 32/28 °C, with the water holding capacity maintained at 40 % by watering, when necessary, with distilled water. Pots were fertilized twice during the growth period with 50 ml Murashige and Skoog (MS) at planting, and 15 days after planting. Plant growth parameters were then assessed. Leaf samples for qPCR were also obtained and stored at -80 °C.

4.3.5. Phenotypic responses of maize

Plant height, shoot and root dry mass

Shoot and root samples were dried at 105 °C, to determine their dry biomass. These samples were dried with continuous weighing within appropriate intervals until a constant weight was obtained and recorded (Brower *et al*., 1998). Plant height of all maize seedlings was also determined (Wood and Roper, 2009). Height measurements were taken from the border of the soil to the top of the main plant stem.

4.3.6. Relative water content (RWC)

The RWC of leaves was determined by recording fresh weight, saturated/turgor weight, and dry weight (Arndt *et al*., 2015; Teulat *et al*., 2003). The first two fully formed leaves were sampled per plant mid-morning from 4x replicates per treatment. The leaves were placed in zip-lock plastic

bags and kept on cooler boxes filled with ice to minimize moisture loss. The leaf samples were weighed immediately to obtain fresh weight. The standing rehydration technique was used to determine the turgor weight. The leaf samples were transferred to new Ziplock bags filled with 100 ml of deionized water with the cut end petiole submerged. The Ziplock bags were carefully stored in the dark at 10°C for rehydration. The leaves were rehydrated for 2h, then wiped with paper towels to remove access moisture and weighed to determine the turgor weight. For dry weight, the same leaf samples were heated in an oven at 80°C until all water weight was lost then the dry weight was measured. The RWC was determined using the formula: RWC = $\frac{Fresh\ weight - dry\ weight}{tugor\ weight - dry\ weight}$ x 100.

4.3.7. Statistical analyses

To check whether the results were statistically different between treatments, the two-tailed Student's *t*-test (unequal variances) at $P < 0.05$ confidence level was conducted using R studio. Each treatment was replicated four times, and the data were expressed as mean ± SD. Different letters were used to depict treatments with statistical differences, whereas the same letters indicated that there were no statistical differences.

4.4.Results

Previously, a total four promising PGPR isolates, 11MN1 (*B. thuringiensis*), 21MN1B (*B. pseudomycoides*), 33MP1 (*L. amnigena*), and 36MP8 (*Leclercia sp.*) exhibited the potential to alleviate the adverse effects inferred by the combination of drought and heat stress in maize seedlings under *in vitro* conditions. Moreover, 11MN1 and 2MN1B demonstrated the ability to fix nitrogen, and 33MP1 and 36MP8 exhibited P-solubilisation. All four isolates showed the capability of producing IAA and displayed the potential to tolerate drought and heat stress. A greenhouse experiment was conducted to evaluate the effects of these potential drought and heat-tolerant PGPR isolates on the early growth of maize seedlings, under the dual effects of drought and heat stress, as well as no-stress.

4.4.1. Effect of drought and heat tolerant isolates on the early growth of maize subjected to the dual stress of drought and heat stress.

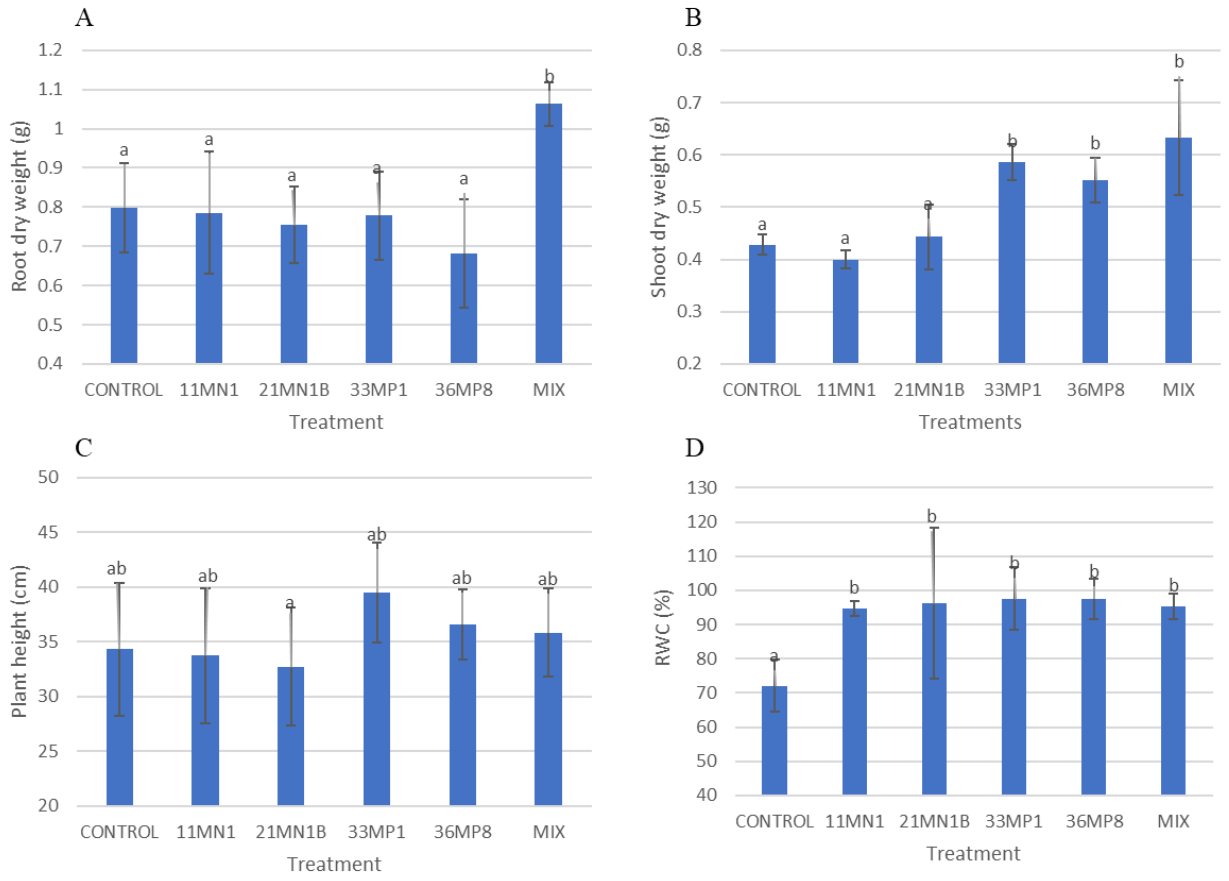


Figure 16:Effects of PGPR isolates on maize plant growth parameters cultivated under greenhouse conditions, with 40 % soil WHC and temperature of 32/28 °C. significant differences between treatments were conducted using the student's t-Test $p < 0.05$. Different letters distinguish between statistically different treatments whereas same letters suggest no statistical differences between treatments.

Under 40 % soil WHC and temperature of 32/28 °C, plants inoculated with the bacterial isolates showed an improvement in either one or more of the measured plant growth parameters compared to the control (Figure 16 and 17). According to the results, isolate 33MP1, 36MP8, and the combination of all isolates (11MN1, 21MN1, 33MP1, 36MP8) significantly improved shoot dry weight by 36.9, 28.7, and 47.9 % compared to control respectively (Figure 16B). Maize seedlings inoculated with the mixture gave the best plant response, which was followed by maize inoculated with isolate 36MP8, and then maize inoculated with isolate 33MP1. On the other hand, no significant increases were observed in the shoot-dry weight of maize seedlings inoculated with

11MN1 and 21MN1B (Figure 16B). The shoot-dry weight of maize inoculated with these bacterial isolates was on par with the control, with maize inoculated with 11MN1 performing the least.

The root dry weight was improved only by the mixture of all isolates by 33 % over the control (Figure 16A). No significant differences in root dry biomass were observed in maize inoculated with isolates 11MN1, 21MN1B, 33MP1, and 36MP8. Overall, maize inoculated with the single isolates shows less root dry biomass compared to control plants. Maize plants inoculated with 36MP8 exhibiting the least root dry biomass.

No significant differences were observed in plant height (Figure 16C). However, bacterial isolates 33MP1, 36MP8, and the mixture of all isolates improved plant height by 14.5, 6.1, 4.1 % compared to the control respectively. No increases in plant height were observed in maize plants inoculated with isolate 11MN1 and 21MN1B. This growth trend is like that observed in the shoot-dry biomass. Maize inoculated with 33MP1 shows the most improved plant height whereas, those inoculated with 21MN1B exhibited the least. Furthermore, the isolates tested significantly improved leaf relative water content (RWC). Isolates 11MN1, 21MN1, 33MP1, 36MP8, and the mixture increased maize leaf relative water content by 31.2, 33.3, 35.4, 35.1, and 32.0 % compared to control respectively under these stressed conditions (Figure 16D). The improvements on the RWC on the relative water content of maize were relatively the same for all treatments.


Control
11MN1


Control
21MN1


Control
33MP1

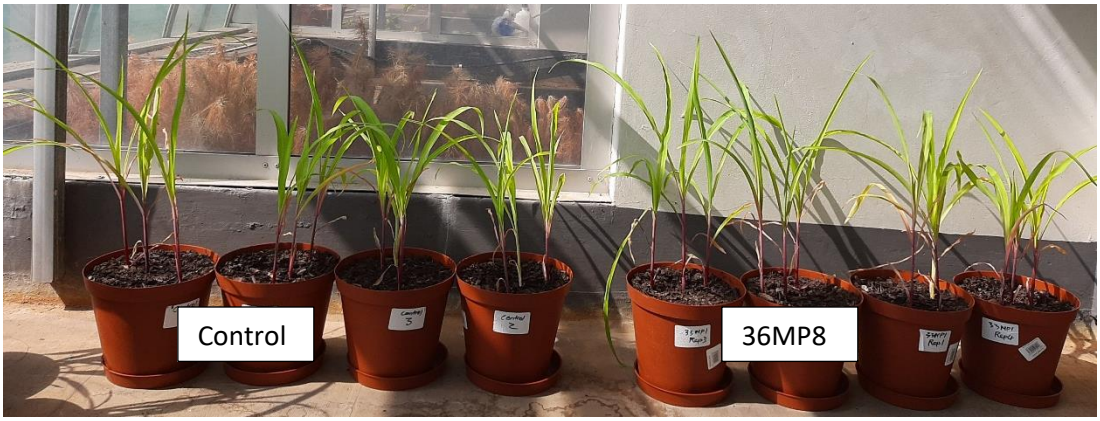

Control
36MP8

Figure 17: Visual depiction of bacterized maize seeds grown under the dual stress of drought (40 % WHC) and heat (32/28 °C) stress after 32 days.

4.4.2. Effect of drought and heat tolerant isolates on the early growth of maize subjected under nostress

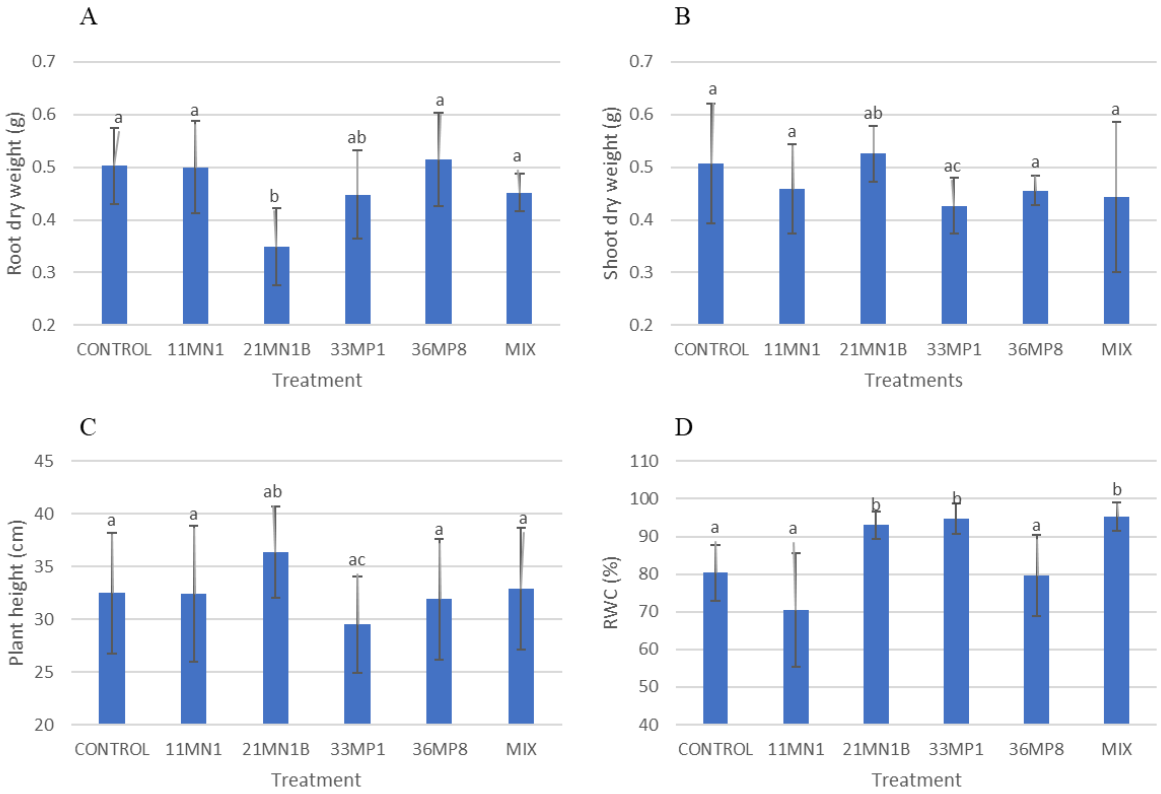
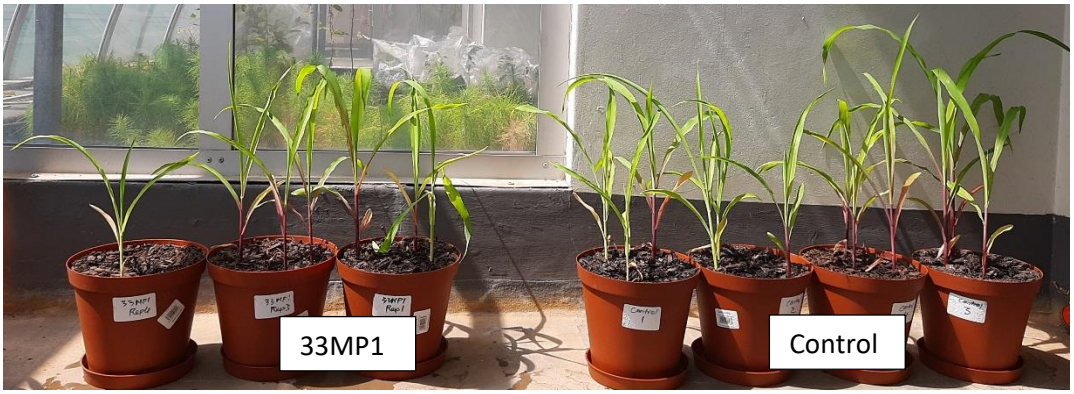


Figure 18: Effects of PGPR isolates on maize plant growth parameters cultivated under greenhouse conditions, with 80 % soil WHC and temperature of 25/23 °C. significant differences between treatments were conducted using the student's t-Test $p < 0.05$. Different letters distinguish between statistically different treatments whereas same letters suggest no statistical differences between treatments.

Under non-stressed conditions, the soil water holding capacity was maintained at 80 % and the temperature at 25/23 °C. The plant growth trends of maize inoculated with the bacterial isolates of interest differed from those observed under stressed conditions (Figures 18 and 19). For instance, no significantly enhanced root dry biomass, shoot dry weight and plant height were observed in plants inoculated with the isolates. Even so, isolate 21MN1B improved the shoot dry weight and the plant height of maize. Also, only plants inoculated with 21MN1B, 33MP1 and the mixture of all isolates showed significantly increased relative water content (Figure 18D). Maize plants inoculated with 21MN1, 33MP1, and the mixture had an RWC increase of 15.8, 17.9, and 18.6 % compared to the control plants respectively. The RWC of maize inoculated with 11MN1 was lower by approximately 10 % compared to the control. On the other hand, the RWC of plants inoculated with 36MP8 was relatively the same as the control plants. Correspondingly, no defined visual improvements were observed in maize shoot growth under these non-stressed conditions in the inoculated treatments (Figure 19). Even so, maize plants inoculated with 21MN1B performed the best among the treatments.


11MN1
Control


21MN1B
Control


33MP1
Control


36MP8
Control

Figure 19: Visual depiction of bacterized maize seeds grown under ambient conditions with soil moisture maintained at 80 % WHC and the temperature at 25/23 °C after 32 days.

4.5.Discussion

Plants are constantly exposed to abiotic stresses, such as drought and heat-stresses, that cause serious problems affecting plant growth and development. Moreover, these abiotic stresses commonly occur more frequently in combination than as isolated incidents under field conditions. PGPR inoculation of plants exposed to these abiotic stresses has been shown to mitigate the resultant detrimental effects. According to the literature reviewed in the current study, there are no reports of PGPRs exhibiting characteristics of inducing plant tolerance to the dual effects of drought and heat stress. To Address the issue, the current study evaluated the potential of 4 drought and heat tolerant bacterial isolates exhibiting plant growth-promoting abilities at mitigating stress on maize plants under greenhouse conditions. The isolates 11MN1, 14MN3B, 21MN1B, 33MP1, and 36mp8 were previously identified as *B. thuringiensis*, *B. pseudomycoides*, *Acinetobacter sp.*, *L. amnigena*, and *Leclercia* sp. respectively.

Under the dual stress of drought and heat, plants inoculated with the bacterial isolates showed an improvement in either one or more of the measured plant growth parameters compared to the control (Figure 16). However, *B. thuringiensis* 11MN1 and *B. pseudomycoides* 21MN1B could not improve the root and shoot dry biomass, as well as plant height compared to the control uninoculated plants. Both bacterial strains exhibited tolerance to drought and heat stress, with IAA production (12.78 ug/ml and 11.04 ug/ml respectively) and nitrogen-fixing abilities. *Bacillus thuringiensis* (Bt) has been widely used for 50 years as a safe biopesticide for controlling

agricultural and insect pests, however both species including *B. pseudomycoides* have been found useful as a PGPR. Bacterial strains of the same genus have previously been shown to mitigate the effects of either drought or heat stress and, improve nutrient uptake as well as plant growth when applied singly. *B. thuringiensis* HYDGRFB19 exhibiting multiple PGP traits (IAA production 25 g/mg protein, P-solubilization) increased total plant dry biomass by 46.6 - 59.06% under drought stress (Vardharajula *et al.*, 2011). Similarly, *B. thuringiensis* AZP2 inoculated seedlings tolerated severe drought stress and showed greater plant biomass (78%) and 5-fold higher survivorship compared to uninoculated seedlings (El-Daim and Moustafa, 2015). *Bacillus pseudomycoides* with potassium-solubilization enhanced potassium uptake in tea plants by increasing potassium availability and increased plant height by 45.15 % (D'Amato *et al.*, 2019). The results found in these studies are contradictory to our findings, suggesting that the combined drought and heat stress may be different than the single stresses, thus may need different ameliorating mechanisms.

Even so, maize inoculated with *L. amnigena* 33MP1 and *Leclercia* sp. 36MP8 showed significant differences in shoot dry biomass and plant height. *L. amnigena* and *Leclercia* sp. have only been recently discovered as PGPR and has previously been associated with mitigating abiotic stresses (Danish *et al.*, 2020). *L. amnigena* 15/1 with ACC deaminase alleviated the negative effects of salinity, increased shoot, and root length under lab conditions in Petri-dishes by 20 and 18.5%, and in jar trials by 82.3 and 60% (ATEŞ and Kivanc, 2020). Similarly, in a study by El-Akhdar *et al*. (2020), *L. amnigena* MSR-M49 with multiple plant growth-promoting (PGP) traits was also able to alleviate wheat seedlings from salt stress, improving grain yield (g/plant) by 91% under salt stress (6.0 d/Sm) and 103.5 % under no stress conditions. Similarly, drought tolerant *Leclercia adecarboxylata* has been shown to possess the capability of providing resistance to plants against drought stress. One study showed that an ACC deaminase producing *L. adecarboxylata* could alleviate drought stress by improving root elongation (2.2-fold), shoot dry weight (1.5-fold), root dry weight (1.4-fold), NPK uptake, and possibility decreasing ethylene in plants (Danish *et al.*, 2020). Danish *et al*. (2019) also showed that under osmotic stress, seed inoculation with *L. adecarboxylata* increased root and shoot dry weight, as well as shoot length by 36, 60%, and 40.4% respectively. Likewise, wheat inoculated with ACC deaminase containing *L. adecarboxylata* had significantly improved grain (48.37%) and straw yield (102.13%) under severe drought stress (Danish and Zafar-ul-Hye, 2019). These studies emphasize the potential of *L. adecarboxylata* and

L. amnigena at mitigating the effects of abiotic stress. The current study also notes that 33MP1 and 36MP8 significantly enhanced shoot dry biomass weight, but not root dry weight under concurrent drought and heat stress. These results complement the observations made by Khan *et al*. (2019) who reported that treatment with PGPR significantly increased shoot dry weight compared to the control, but the root dry weight was at par with the control plants under drought stress conditions. This shows that treatment with PGPR for the mitigation of abiotic stresses in plants can favor enhancement of certain measured plant growth parameters over others.

Lelliotia adecarboxylata and *L. amnigena* commonly display multiple plant growth-promoting (PGP) traits such as ACC deaminase activity, and the production of phytohormones (IAA) (ATEŞ and Kivanc, 2020; Danish and Zafar-ul-Hye, 2019; Danish *et al.*, 2020; El-Akhdar *et al.*, 2020). These PGP substances can help plants maintain better nutrition under abiotic stress conditions by influencing the relationship between plants and microbes. For instance, Auxins are key regulators in virtually all aspects of plant growth and development. Numerous studies in the past decade have presented resolute evidence for the role played by auxins in abiotic stress responses (Khan *et al.*, 2019; Gamalero and Glick. 2011; Mohite, 2013; Vardharajula *et al.*, 2011). IAA mediates plant tolerance to drought and heat stress by influencing the antioxidant enzymes, osmoprotectant (proline) synthesis, regulating gene expression, and improvement in the accumulation of photosynthetic pigment (Khan *et al.*, 2020). Similarly, ACC-deaminase activity has been linked to root and shoot growth under drought or heat stress. The enzyme ACC-deaminase reduces the accumulation of ethylene by breaking down its precursor, 1-aminocyclopropane-1-carboxylic acid, into α- ketobutyrate and NH3 (Glick *et al*., 1999; Zafar-ul-Hye *et al.*, 2014). As a result, the low accumulation of stress generating ethylene in roots and shoots improves root and shoot biomass. Similar claims of improvement in plant growth attributes have been documented by many scientists where plants were inoculated with ACC-deaminase containing PGPRs under abiotic stress (Chandra *et al.*, 2018; Zahir *et al*., 2009; Zhang *et al.*, 2018). It should also be noted that inoculation with PGPR compensates for the effects of drought or heat stress and improves the growth and developments of plants by increasing the production of soluble sugars, essential amino acids, and proline enhancing the absorption of soil nutrients and water (Vardharajula *et al.*, 2011). This improvement in plant growth is also concurrent with changes in endogenous phytohormones (IAA, abscisic acid, or salicylic acid), antioxidants (LPO, GSH, SOD, or APX) and heat shock proteins and the expression of stress response genes (Khan *et al*., 2020).

All inoculated plants under combined drought and heat stress showed significant increases in leaf relative water content. The leaf relative water content reflects the balance between water supply to the leaf tissue and transpiration rate. Moreover, it is the most appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. A previous study showed that seedlings inoculated with *B. thuringiensis* AZP2, improved RWC and a delayed response to drought stress was observed. Furthermore, 40% of AZP2 inoculated seedlings survived for up to 8 days without irrigation, whereas 0% survived in control plants (El-Daim and Moustafa, 2015). In the current study, *B. thuringiensis* 11MN1 and *B. pseudomycoides* 21MN1B only enhanced the leaf RWC. Even so, it is likely that the survivorship would improve if irrigation were to be halted for a certain period compared to the control. Thus, even though no physical changes were observed in maize plants inoculated with these 11MN1 and 21MN1B, it is likely that changes occurred at the cellular and molecular level. Interestingly, inoculation of maize with the mixture/consortium (11MN1, 21MN1, 33MP1, 36MP8) performed better than the single isolate inoculation. All measured plant growth parameters were significantly increased under concurrent drought and heat-stressed conditions. Similarly, the dual inoculation of *L. amnigena* with a *Bacillus* strain led to greater effects on the measured plant growth parameters than the single application of these strains (El-Akhdar *et al.*, 2020). Dual inoculation improved grain yield (g/plant) by 116 % under salt stress (6.0 d/Sm) and by 124 % under no stress conditions (El-Akhdar *et al.*, 2020). This suggests that a synergy between the two strains resulted in enhanced growth of wheat plants on saline soil. Correspondingly, Chickpea sensitive and tolerant genotypes showed increased root (58.94 and 156.38 % respectively) and shoot dry weight (93.8 and 250.48 % respectively) accumulation under drought stress due to consortium (*Bacillus subtilis, Bacillus thuringiensis*, and *Bacillus megaterium*) treatment, and the increase was higher in the sensitive genotype than the tolerant one (Khan *et al*., 2019). In addition, the consortium increased RWC for the sensitive type by 137.04% and the tolerant type by 43.6 % (Khan *et al*., 2019). In general, consortium treatments helped the plant to maintain growth by accumulating higher biomass under drought conditions. This may be largely contributed by the greater functional diversity resulting from genetic diversity from the different microorganisms that now reside in the rhizosphere. Baig *et al.* (2011) showed that bacterial isolates with dual PGP traits outperformed isolates with single traits in pot experiments, with increased dry shoot weight (37.6%), root weight (3.9-fold), and root elongation (3.8-fold), and grain yield (38.5%). This suggests that the more diverse the PGP traits, the greater

the improvements on plant growth. Inoculation with a consortium of microorganisms increases this PGP diversity, hence the compounded improvements in plant growth compared to single isolate inoculations.

Under no stress conditions, no significant or apparent increases in plant growth were observed. However, there were significant improvements in RWC for maize plants inoculated with 21MN1, 33MP1, and the mixture. Likewise, suggesting PGPR-induced changes that occurred at the cellular and molecular level. Plant inoculation with potential PGPR has been reported to greatly improve yields when evaluated even under ideal climatic situations (de Carvalho *et al*., 2020). Even so, previous studies have repeatedly shown that the greatest benefits of PGPR occur when crops encounter stressful conditions, especially for prolonged periods (Egamberdiyeva and Hoflich 2004; Vardharajula *et al.*, 2011). This phenomenon better explains the results obtained in the current study, where significant changes in maize seedlings under stress conditions were observed but not under non-stressed conditions. In addition, plant susceptibility or tolerance to the environment also significantly influences plant responses to PGPR inoculation. For instance, a study conducted by (Vives-Peris *et al.*, 2018) showed that the root exudates from heat-stressed plants had a positive effect on bacterial growth, and this characteristic was more evident in the heat-sensitive plants. The study concluded that root exudates modulate bacterial growth depending on the growth conditions and the plant genotype. Therefore, successful colonization of the rhizosphere is dictated largely by root exudation, which in turn dictates the type of effect inoculation with PGPR might have on plants. Possibly, the experimental conditions under no stress conditions might not have been adequate for a more beneficial plant-microbe relation similar to that observed under stressed conditions.

4.6.Conclusions

Overall, the results showed that the bacterial isolates *B. thuringiensis* 11MN1, *B. pseudomycoides* 21MN1B, *L. amnigena* 33MP1, and *Leclercia sp.* 36MP8 have great potential to improve the growth of maize under the dual stress induced by drought and heat stresses in soil. Based on the results, isolates *L. amnigena, Leclercia sp.* and the co-inoculation of all the isolates show the best potential of inferring tolerance in maize plants under dual drought and heat stress. Inoculation increases in different agronomical parameters leading to increased plant growth. The current study is possibly the first to associate these bacterial species with the alleviation of dual

drought and heat stress on maize plants. The study introduces an alternate way to maximally increase maize production under these adverse climate conditions for commercial and smallholder farmers. Moreover, these PGPR isolates have the potential to reduce the need for extensive irrigation of maize crops throughout their growth cycle. The project will aid poor black communities who use conventional agricultural methods which do not address the effects brought by climate change and typically result in lower yields. To elucidate PGPR-induced molecular changes in maize, targeted transcriptomic analysis on the stress response genes will be conducted. The results demonstrated in this research provide a promising environmentally-friendly solution for increasing crop yields in semi-arid regions.

4.7.References

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5. Chapter 5: The regulation of selected stress response genes in PGPR inoculated maize plants under concurrent drought and heat stress.

5.1.Abstract

Under field conditions, drought and heat stress are more prevalent in combination than as isolated phenomena. The combined effects of heat and drought stress have unique effects on various plant physiological and molecular response parameters suggesting that plants have different response mechanisms to combined stresses than their individual stress. The use of plant growth-promoting rhizobacteria is thought to be key to the adaptation and survival of plants to abiotic stresses. Therefore, the aim of the study was to observe the expression of selected stress response genes in PGPR inoculated maize plants under concurrent drought and heat stress, as well as non-stressed conditions. The total RNA of maize leaves was isolated, and the quantitative real-time PCR was conducted for the relative quantification of selected stress response genes. Under dual drought and heats stress, the *DHN2* gene was statistically ($P < 0.05$) downregulated in maize plants inoculated with isolate 11MN1 (by 0.33-fold) and the mixture (by 0.88-fold) compared to the control, however, plants inoculated with 21MN1B, 33MP1 and 36MP8, the gene was upregulated, with 21MN1B being the only isolate that statistically upregulated *DHN2* gene (by 0.78-fold). The *HSP70* gene was statistically ($P < 0.05$) downregulated by 0.72, 0.32 and 0.65-fold for plants inoculated with 11MN1, 33MP1 and the mixture compared to the control plants under dual drought and heat stress, respectively. The *CAT2* gene was downregulated in maize plants inoculated with isolate 11MN1 and 21MN1B, whereas those inoculated with 33MP1, 36MP8 and the mixture (MIX) showed upregulation of the gene. Under non stress conditions, maize plants exhibited downregulation of all the stress response genes except for the *CAT2* gene on plants inoculated with the mixture which was on par with the control uninoculated plants. In conclusion, these rhizobacterium appear to confer tolerance to concurrent drought and heat partly by modulating the *CAT2 and DHN2* stress response genes. *Bacillus thuringiensis* 11MN1, *B. pseudomycoides* 21MN1B, *L. amnigena* 33MP1, and *Leclercia* sp. 36MP8 and the mixture of all these isolates promotes growth and protects maize plants from damage to concurrent drought and heat stress.

5.2.Introduction

The combination of stresses instead of individual stresses is the greatest limiting factor for plant growth and crop productivity (Kapoor *et al*., 2020). Under field conditions, drought and heat stress

are more prevalent in combination than as isolated phenomena (Meseka *et al.*, 2018). Research has however not been conducted extensively on how stress combination affects crop productivity, as compared to singly isolated effects, especially in maize. Maize (*Zea mays* L.) is an essential staple grain crop in Latin America, Asia and Sub-Saharan Africa and is primarily used for human consumption and animal feed production (Department of Agriculture, Forestry and Fishery, 2016). More than 73% of the maize growing areas are in developing countries, where maize is a vital staple food crop for millions of people (Shiferaw *et al.*, 2011; Tesfaye *et al*., 2018). Studies that were aimed at characterizing the consequences of both heat and drought stress on plants reported significantly enhanced negative effects on the growth and productivity of crops for the combined efforts of these stresses compared to each stress applied in isolation (Jian and Haung, 2001; Prasad and Staggenborg, 2008). Furthermore, land affected by combined stress is likely to increase given the anticipated climate changes, therefore the occurrence of concurrent heat and drought stress is likely to become an increasingly common scenario in the future (Lobell *et al*., 2011; Nelimor*et al*., 2019).

The combined effects of heat and drought stress have unique effects on various plant physiological, developmental, growth, yield, and genetic parameters suggesting that plants have different response mechanisms to combined stresses than their individual stress (Hussain *et al*., 2019). The transcriptomic analysis of combined heat and drought stressed plants has revealed a combination of shared and unique transcriptomic changes. The shared response under combined drought and heat stress normally includes the induction of heat shock proteins (HSPs), ROS detoxification enzymes, late embryogenesis 7 (LEA7) genes, dehydrin, photosynthesis related genes, transporter proteins, anthocyanin biosynthesis related genes and enzymes involved inglycolysis (Min *et al*., 2015; Pandey *et al*., 2015; Rizhsky*et al*., 2002; Rizhsky*et al*., 2004). Although, these genes are commonly regulated under combined and individual stress response of plants, differences are observed in their expression levels (Johnson *et al*., 2014). Unique genes are also found in the individual and combined stress conditions*i.e.* some are up-regulated exclusively under heat, drought, or combined stress (Pandey *et al*., 2015). Thus, the transcriptional response of plants to a combination of drought and heat stress is unique and cannot simply be extrapolated from the effect of each stress imposed individually (Danish *et al*., 2019). Therefore, plants have novel responses when presented with combined stress.

Plant-microbe interactions are thought to be key to the adaptation and survival of plants to abiotic stresses. A group of microbes known as Plant growth-promoting rhizobacteria (PGPR) have been an area of great concern in past few decades and have been shown to induce tolerance to plants under drought and heat stress (Ali *et al*., 2011; Curá *et al*., 2017; Hassan *et al*., 2020; Mukhtar *et al*. 2020; Zarei *et al*., 2020). The plant growth promotion and mitigation of drought and heat stress has been documented for several PGPR belonging to the genera such as *Pseudomonas, Azotobacter, Azospirillum, Rhizobium, Pantoea, Bacillus, Enterobacter, Bradyrhizobium, Methylobacterium, Burkholderia, Trichoderma*, and cyanobacteria (Meena *et al*., 2017).

These microbes have been shown to induce "Induced Systemic Tolerance" (ISR), a term used for microbe-mediated induction of abiotic stress tolerance in plants. This suggests that an inoculated plant is physiologically primed which leads to increased tolerance against abiotic stresses (Dimkpa*et al*., 2009). The priming phenomenon has not been fully elucidated at the molecular level however, a strong relationship with the accumulation of inactive signaling proteins that are activated and transduced under stress has been shown (Park *et al*., 2017). This is especially true upon future exposure of the plant to the same stress. This enables PGPR to alter the transcriptional expression of plant genes, thereby ameliorating abiotic stresses. Changes in the levels of plant phytohormones, defense-related proteins and enzymes, antioxidants and exopolysaccharides have been observed for PGPR-mediated drought and heat tolerance (Abd El-Daim*et al*., 2018; El-Esawi*et al*., 2018; Meena *et al*., 2017; Naseem and Bano, 2014).

For instance, *Bacillus aryabhattai* SRB02 ameliorated the effects of heat stress, and the plant reportedly had greater accumulation of GmIAA16 and GmIAA9 transcripts, genes that encode the Aux/IAA transcriptional repressor proteins involved in auxin signaling (Park *et al*., 2017). Similarly, *Azospirillum brasilense* NO40 and *Bacillus amyloliquefaciens*5113 could ameliorate drought stress in wheat plants through upregulation of S-adenosylmethionine synthase 1 (SAMS1), ascorbate peroxidase 1 (APX1), and HSP17.8 transcripts in the leaves (Kasim *et al*., 2013). *Bacillus licheniformis* K11 inoculated pepper plants showed an upregulation greater than 1.5-fold in *Capsicum annuum* dehydrin (Cadhn), sHSP, and *Capsicum annuum* pathogenesis-related protein 10 (CaPR-10) compared to control plants under drought stress (Lim and Kim, 2013). In *Bacillus amyloliquefaciens* inoculated rice, the upregulation of dehydrin (DHN), glutathione S-transferase (GST), late embryogenesis abundant (LEA), no apical meristem (NAM),

Rab-like GTPase activators, myotubularin (GRAM) was observed under heat stress and osmotic stress (Tiwari *et al*., 2017). Such strategies make plants more tolerant toward abiotic stresses.

Nevertheless, the combined effects of drought and heat stress on plants, especially maize, and how PGPR induce abiotic tolerance to these stresses is extensively lacking. Laboratory and greenhouse tests have been focused on the single occurrence of these stresses, which does not necessarily correlate to field conditions where plants are more regularly exposed to a combination of these stresses. As such, PGPR fail to induce any positive response in the field primarily because a combinatory stress of drought induces a unique molecular stress response to plants compared to these stresses applied individually. Therefore, studies aimed at mitigating abiotic stresses by using PGPR should be reassessed, including the selection, screening, and application of stress-tolerant microorganisms to multiple abiotic stress. Moreover, the advances in techniques such as metatranscriptomics, metabolomics and metaproteomics for monitoring plant responses at the molecular and gene regulation level, make it possible to more precisely determine how PGPR alter the expression of stress response genes to induce drought and heat tolerance in maize plants (Eastburn *et al*., 2011). Therefore, the aim of the study is to observe the expression of selected stress response genes in PGPR inoculated maize plants under concurrent drought and heat stress, as well as non-stressed conditions.

5.3.Material and methods

5.3.1. Plant Material and RNA Preparation

Maize leaves previously stored at -80°C were ground to fine powder in liquid nitrogen. Total RNA was extracted from individual treatments and replicates using the Trizol Reagent (Thermo Fisher Scientific). Briefly, 1 mL of Trizol Reagent was added to 100 mg of maize leaf tissue samples and homogenized. The sample mixtures were incubated for 5 minutes to permit complete dissociation of the nucleoproteins complex. Subsequently, 0.2 mL of chloroform was added and incubated for 3 minutes. The samples were centrifuged at 12,000 × g at 4°C for 15 minutes. The mixtures separated into a lower red phenol-chloroform, and interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to new tubes. To isolate the RNA, it was precipitated adding 0.5 mL of isopropanol to the aqueous phase. Afterward, the sample mixtures were incubated for 10 minutes, then centrifuged at 12,000 × g at 4°C for 10 minutes. The supernatant was discarded leaving a white-like RNA pellet. The RNA pellet was resuspended in 1

mL 75% ethanol and briefly vortexed, then centrifuged at 7500 × g at 4°C for 5 minutes. The supernatant was discarded, and the RNA pellet was air dried for 10 minutes. The RNA pellet was resuspended 30 µL of RNase-free water. Afterward, the dissolved RNA was incubated in a heat block set at 60°C for 15 minutes. RNA was quantified using the NanoDrop (Thermo Fisher Scientific, USA) and the quality and integrity checked by running in a 1 % agarose gel containing 0.5 % sodium chloride (Aranda *et al*., 2012). The RNA was stored at -80°C until use.

5.3.2. RT-qPCR

For RT-qPCR, cDNA synthesis was conducted from 50ng of total RNA using the LunaScript® RT SuperMix Kit (New England Biolabs, UK) as instructed by the manufacturer. Quantitative real-time PCR was conducted in 96-well plates using Luna Universal qPCR Master Mix in the CFX96 Touch Deep Well Real-Time PCR System (Bio-Rad, USA). Briefly, 1 µL of sample was added to 19µL of Luna Universal qPCR Master Mix and primers (concentration of 0.25µM). The cycling conditions were as follows: an initial denaturation at 95°C for 1min followed by 39 cycles of denaturation at 95°C for 15s and primer annealing at 65°C for 1 min. Each treatment had 3 biological replicates and two technical replicates. Afterward, the melting curves ranging from 60 °C to 95 °C were evaluated in each reaction to check the specificity of the amplicons. The samples were normalized against Tubulin beta (*β-TUB*) and Elongation factor 1 alpha (*EF1a*) as the reference genes and the control uninoculated samples were used as calibrators (Lin*et al*., 2014). The selected stress response genes of interest included, Dehydrin 2 (*DHN2*), Heat Shock Protein 70 (*HSP70*) and Catalase 2 (*CAT2*) (Capelle*et al*., 2010). The comparative CT ($\Delta\Delta^{ct}$) method was used to measure relative expression (Livak and Schmittgen, 2001). To check whether RT-qPCR results were statistically different when comparing inoculated samples to uninoculated control samples, the two tailed Student's *t*-test (unequal variances) was used ($P < 0.05$). Some primers were obtained from previous studies and some were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) and are listed in Table 1. Primer specificity was checked using NCBI's Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 4: Candidate stress response genes and the reference genes, their primer sequences and product sizes.

Gene Name	Primer Name	Primer Sequence	Product size	Reference
Dehydrin 2	dhn2-624F	5'-ACGAAGACTCAGACCCCACCA-3'	104	(Capelle*et al*., 2010)
	dhn2-727R	5'-GCGTCTTCCGGCTTCTTGT-3'		
Heat Shock Protein 70	Hps70F	5'-AGCTAAGACTGGGTGGCTGA- 3'		Designed
	Hsp70R	5'-GTCGTCTTCTCCCTGTGCTC-3'		
Catalase 2	Cat2_F	5'-TCTCTGTCTGCTTTCGCTCA-3'	151	Designed
	Cat2_R	5'-GGACACAGCCAGCCATTATT-3'		
Tubulin beta (β-Tub)	β-TUB_F	5'- CTACCTCACGGCATCTGCTATGT-3'	139	Lin *et al*., 2014
	β-TUB_R	5'- GTCACACACACTCGACTTCACG-3'		
Elongation factor 1 alpha (EF1a)	EF1a_F	5'- TGGGCCTACTGGTCTTACTACTGA-3'	135	Lin *et al*., 2014
	EF1a_R	5'- ACATACCCACGCTTCAGATCCT-3'		

5.4.Results

The previous chapter investigated the effects of potential drought and heat-tolerant PGPR isolates on the early growth of maize seedlings, under the dual effects of drought and heat stress, as well as no-stress. These potential isolates include, 11MN1 (*B. thuringiensis*), 21MN1B (*B. pseudomycoides*), 33MP1 (*L. amnigena*), and 36MP8 (*Leclercia sp.*) and exhibited the potential to alleviate the adverse effects caused by concurrent drought and heat stress in maize seedlings under *in vitro* conditions. Therefore, this warranted further investigation. The current chapter

focuses on the expression of selected stress response genes on the greenhouse grown maize under the conditions stipulated above.

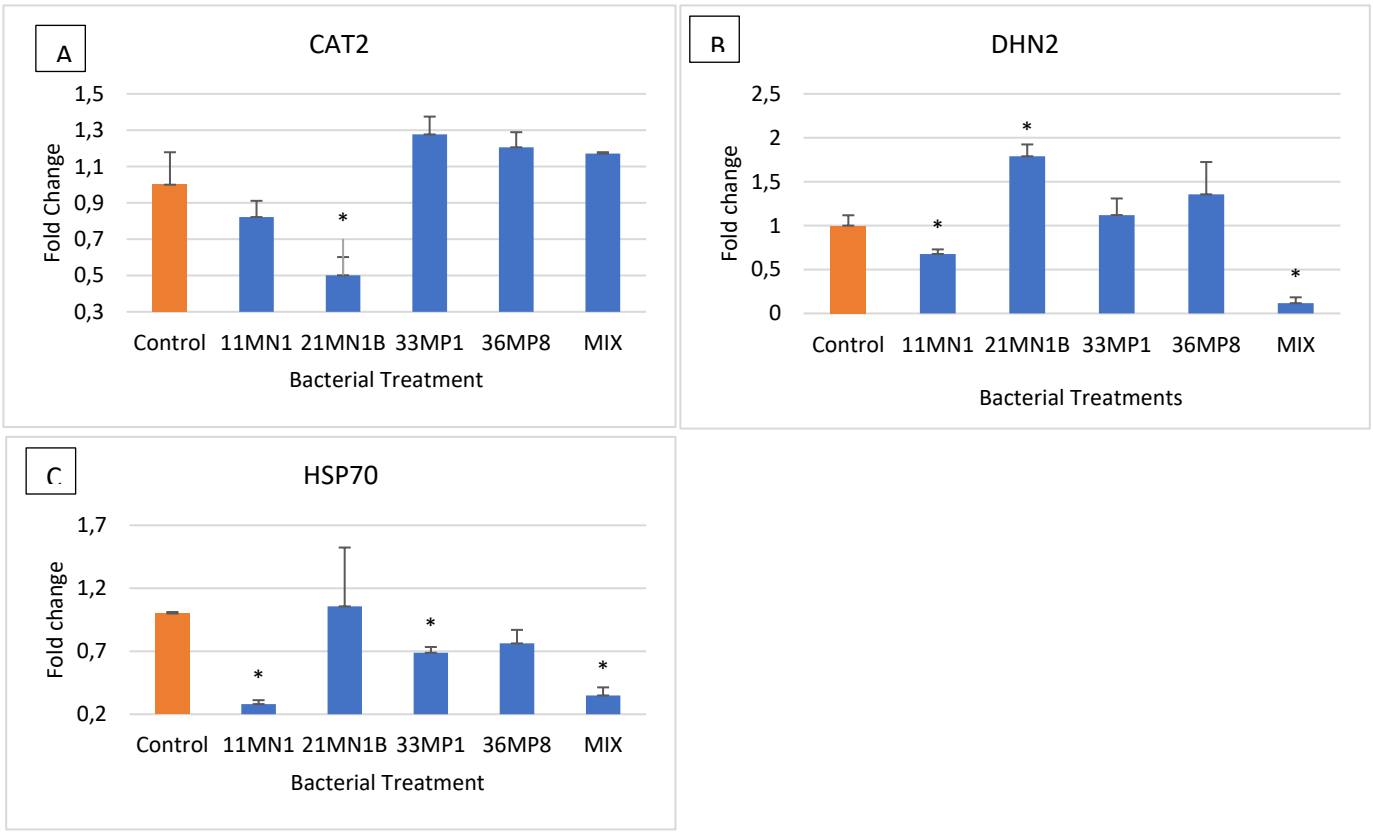

Figure 20: The expression of stress response genes on PGPR inoculated maize plants grown under greenhouse conditions under concurrent drought and heat stress conditions (soil WHC 40% and temperature of 32/28 °C).The genes of interest include Dehydrin 2 (*DHN2*), Heat Shock Protein 70 (*HSP70*) andCatalase 2 (*CAT2*).Tubulin beta (*β-TUB*) and Elongation factor 1 alpha (*EF1a*) were used as the reference genes. Error bars represent the SEM of fold change determined using $2^{-\Delta\Delta CT \pm SEM}$ (n = 3). Significant differences between control sample and inoculated samples were conducted using the student's t-Test, $p < 0.05$.The Asterix (*) distinguishes treatments that are significantly different from controls.

0.33-fold) and the mixture (downregulated by 0.88-fold), however, plants inoculated with 21MN1B, 33MP1 and 36MP8, the gene was upregulated, with 21MN1B being the only isolate that statistically upregulated *DHN2* gene (upregulation by 0.78-fold) (Figure 1B). The downregulation of DHN2 gene was more pronounced in maize plants inoculated with the mixture of all isolates. The *HSP70* gene was downregulated in all maize treatments under the stressed

conditions, except those inoculated with 21MN1B (Figure 1C). Moreover, the *HSP70* gene was statistically downregulated by 0.72, 0.32 and 0.65-fold for plants inoculated with 11MN1, 33MP1 and the mixture, respectively.

Under non-stressed conditions, the soil water holding capacity was maintained at 80% and the temperature at 25/23°C. The expression trends of selected stressresponse genes of PGPR-inoculated maize differed from those observed under stressed conditions (Figure 2). Maize plants exhibited downregulation of all the stress response genes except for the *CAT*2 gene on plants inoculated with the mixture. No statistical differences were observed for the *CAT2* gene in all treatments, compared to the control. The *DHN2* gene was statistically different on maize plants inoculated with 36MP8 and the mixture, with a downregulation of 0.68 and 0.59-fold respectively. The *HSP70* gene was downregulated and statistically different for maize plants inoculated with 21MN1B (0.43-fold), 33MP1 (0.48-fold), 36MP8 (0.68-fold) and the mixture (0.42-fold). Only

11MN1-inoculated maize plants showed no statistical differences in the *HSP70* compared to the control.

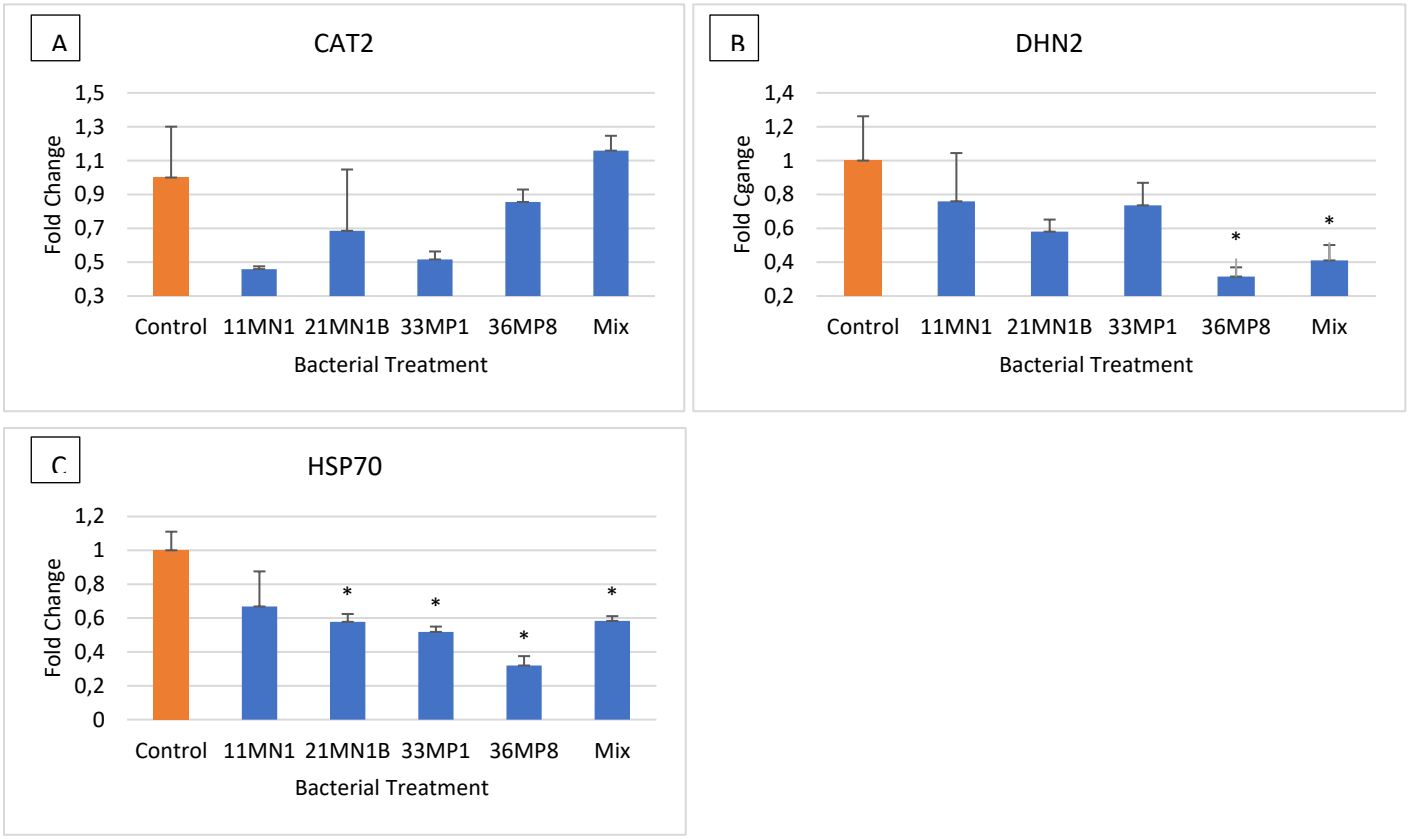

Figure 21: The expression of stress response genes on PGPR inoculated maize plants grown under greenhouse conditions under ambient no stress conditions (soil WHC 80% and temperature of 25/23 °C).The genes of interest include Dehydrin 2 (*DHN2*), Heat Shock Protein 70 (*HSP70*) and Catalase 2 (*CAT2*).Tubulin beta (*β-TUB*) and Elongation factor 1 alpha (*EF1a*) were used as the reference genes. Error bars represent the SEM of fold change determined using $2^{-\Delta\Delta CT \pm SEM}$ (n = 3). Significant differences between control/calibrator sample and inoculated samples were conducted using the student's t-Test, $p < 0.05$.The Asterix (*) distinguishes treatments that are significantly different from controls.

5.5.Discussion

Drought and heat stress affect physiological, biochemical and molecular processes in plants, thus altering their growth and development. These alterations are enhanced under the concurrent occurrence of these stresses. Our previous study demonstrated potential PGPR isolates that could play a role in reducing the detrimental effects of the combination of drought and heat stress. The isolates 11MN1, 21MN1B, 33MP1 and 36mp8 were previously identified as *B. thuringiensis*, *B. pseudomycoides, L. amnigena*, and *Leclercia* sp. respectively. Understanding the effects of PGPR on the molecular response of plants under these combined stresses can contribute greatly in improving the tolerance of economically important crops such as maize. The current study is an effort to identify key stress response genes involved in PGPR (*B. thuringiensis*, *B. pseudomycoides, L. amnigena*, and *Leclercia* sp.) mediated maize tolerance to the effects of dual drought and heat stress, as well as non-stressed conditions. The targeted stress response genes were dehydrin 2 (*DHN2*), heat Shock Protein 70 (*HSP70*) and catalase 2 (*CAT2*).

In the present experiment, PGPR inoculation on maize plants under concurrent drought and heat stress influenced the expression of stress response genes (Figure 1). The *CAT2* gene was downregulated in maize plants inoculated with isolate 11MN1 and 21MN1B, but those inoculated with 33MP1, 36MP8 and the mixture (MIX) showed upregulation of the gene (Figure 1A). In the previous chapter isolate 33MP1, 36MP8 and the mixture (11MN1, 21MN1, 33MP1, 36MP8) significantly improved shoot dry weight and the leaf relative water content (RWC) of maize seedlings, however, maize plants inoculated with 11MN1 and 21MN1B only exhibited improvement in RWC. This suggests a possible link between the increased shoot dry biomass and the upregulation of the *CAT2* gene. Catalase genes encode catalase, a key antioxidant enzyme involved in the catalytic scavenging of hydrogen peroxide (H_2O_2) into water and oxygen (Leung, 2018). H_2O_2 plays a role as a signaling molecule and a regulator of stress response genes during abiotic stress (Mittler, 2017). However, the overproduction of H_2O_2 leads to a disturbance in the cellular redox balance and is highly toxic to cells (Vurukonda *et al*., 2016). Catalase activity is concomitantly increased in plants as a manifestation of the adaptive response of plants to drought and heat stress (Laxa *et al*., 2019; Sofo et *al*., 2015). Plant growth-promoting rhizobacteria have been reported to induce tolerance to plants through enhanced modulation of the catalase enzyme. For instance, *Pseudomonas* application on maize under severe water stress increased the CAT activity by 50% compared to well-watered plants, alleviating the adverse effects of water stress on

physiological characteristics of maize (relative water content (RWC) increase by 8%) (Rezazadeh *et al*., 2019). Similarly, basil plants (*Ocimum basilicum L*.) treated with *Pseudomonas* sp. under drought stress, significantly increased the catalase (*CAT*) enzyme activity (Heidari and Golpayegani, 2011), emphasizing on the importance of this gene in antioxidant activity. Plant growth promoting rhizobacteria are known to mediate abiotic stress tolerance in plants through modulation of ROS-scavenging enzyme expression (Bharti *et al*., 2016; Gururani *et al*., 2013).

The *DHN2* gene was statistically downregulated in maize plants inoculated with isolate 11MN1 (downregulated by 0.33-fold) and the mixture (downregulated by 0.88-fold), however, plants inoculated with 21MN1B, 33MP1 and 36MP8, the gene was upregulated, with 21MN1B being the only isolate that statistically upregulated *DHN2* gene (upregulation by 0.78-fold) (Figure 1B). Dehydrin (*DHN*) genes belong to group 2 LEA (late embryogenesis abundant) proteins and are upregulated in maize in response to abiotic stress such as drought (Camaille *et al*., 2021). Dehydrin proteins have a molecular weight ranging from 5.3 to 66.3 kDa and may bind to the partly dehydrated surfaces of proteins, protecting them from protein denaturation (Shih *et al*., 2008; Xing *et al*., 2011). They may also exhibit ROS scavenging properties. In the present study, *DHN2* expression varied with each PGPR-inoculated maize. *B. thuringiensis* 11MN1 and MIX inoculated maize recorded reduced expression of *DHN2*. However, 21MN1B, 33MP1 and 36MP8 inoculated stressed plants recorded increased *DHN2* expression. The mixture of all isolates which recorded the greatest in root and shoot dry biomass in the previous chapter, may have reduced the dehydration effect by some other mechanism, hence there was no need for upregulation of *DHN2* gene. This is contradictory to the results observed by Sarma and Saika (2014) where mung bean (*Vigna radiata* L) inoculated with *Pseudomonas aeruginosa* GGRJ21 showed an upregulation of three drought stress-responsive genes including dehydrin in inoculated plants in comparison with the uninoculated control plants tested under drought conditions (Sarma and Saikia, 2014).

This phenomenon is also observed with the *HSP70* transcript. The *HSP70* gene was downregulated in all maize treatments under the dual stress conditions, except those inoculated with 21MN1B. The downregulation of the gene is also to varying degrees. HSPs uphold proteins in their functional conformation under stress conditions. The downregulation of *HSP70* transcript in most bacterial treatments, suggests that the environment might have been permissive for protein folding during stress challenge, possibly due to the accumulation of certain compounds with osmolytic properties.

A similar phenomenon was observed in *Bacillus amyloliquefaciens* UCMB5113 and *Azospirillum brasilense* NO40 inoculated wheat plants. Heat increased transcript levels of several stress related genes (*APX1*, *SAMS1*, *HSP17.8*) in the leaves, while expression was lower in inoculated plants (Kasim *et al*., 2013).

Under non-stressed conditions, all the genes evaluated were downregulated in all treatments compared to the control except maize plants inoculated with the mixture, the CAT2 gene was on par with control. The downregulation of these genes was expected as they play major roles in stress response. Overall, the results obtained on the stress response of genes under concurrent drought and heat stress, supports the suggested strain-specific molecular responses of plants to rhizobacterial inoculation (Drogue *et al*., 2012). It has also been suggested that PGPR-mediated plant growth is a multigenic process which may be specific to the participating rhizobacteria and plant species (Bharti *et al*., 2016). The differential expression of *CAT1*, *DHN2* and *HSP70* observed in maize plants under concurrent drought and heat stress, suggests this specificity between PGPR and plant species. This also suggests that PGPR-induced plant growth under dual drought and heat stress is a complex phenomenon and involves a cumulative effect of changes in various genes in the global plant metabolic pathways.

5.6.Conclusion

The combination of stresses instead of individual stresses is the greatest limiting factor for plant growth and crop productivity. Plant growth-promoting rhizobacteria induce abiotic stress tolerance in plants by initiating "Induced Systemic Tolerance". In conclusion, *B. thuringiensis* 11MN1, *B. pseudomycoides* 21MN1B, *L. amnigena* 33MP1, and *Leclercia* sp. 36MP8 and the mixture of all these isolates promotes growth and protects maize plants from damage to concurrent drought and heat stress. These rhizobacterium appear to confer tolerance to concurrent drought and heat partly by modulating the *CAT2 and DHN2* stress response genes. The proteins encoded by these genes are involved in the synthesis of osmoprotectants and antioxidant defence mechanisms to reduce reactive oxygen species. Furthermore, the results suggest that PGPR-induced plant growth under dual drought and heat stress is a complex phenomenon and involves a cumulative effect of changes in various genes. However, further investigations are needed to elucidate the exact molecular changes in maize plants induced by these PGPR under concurrent drought and heat stress. Overall, *L. amnigena* 33MP1, and *Leclercia* sp. 36MP8 and the mixture of all these isolates are promising

candidates as components for the formulation of biofertilizers aimed at mitigating the effects of combined drought and heat stress in maize plants.

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6. Chapter 6: Overview and conclusion

6.1. Summary

Temperature extremes, water deficit conditions, and the combination of these abiotic stresses contribute significantly to the decline of maize productivity in Sub-Saharan Africa. The stress combination instead of the individual presence of these stresses is the greatest limiting factor for maize growth and productivity. Moreover, the predicted increase in their prevalence in future climates will only worsen agricultural losses. Plant growth-promoting rhizobacteria (PGPR) have been shown to enhance plant tolerance to environmental stresses and help limit their adverse impacts on plant growth. The amassed knowledge over the past decades has helped to understand key features regarding the mode of action of plant-PGPR interactions inferring abiotic stress tolerance in plants.

Currently, the focus on laboratory and greenhouse tests has been on the single occurrence of these stresses, which does not necessarily correlate to field conditions where plants are more regularly exposed to a combination of these stresses. As such, PGPR fail to induce any positive response in the field primarily because a combinatory stress of drought and heat is distinctly unique from the response of plants to these stresses applied individually. As such, studies aimed at mitigating abiotic stresses by using PGPR should be reassessed. During early test phases, the effect of combinatory stresses, primarily drought and heat stress on plant metabolism and molecular stress response under such climatic conditions should be considered. As such, the current study aims to screen and identify bacterial isolates with plant growth-promoting attributes and tolerance to drought and heat stress. Furthermore, evaluate the ability of selected drought and heat tolerant PGPR isolates at mitigating the effects of dual drought and heat stress on the early growth of maize, as well as unravel innate maize drought and heat stress response genes which actively participate in PGPR-induced tolerance.

6.2. Objective 1: To screen for bacterial isolates with exceptional plant growth-promoting traits and tolerance to drought and heat stress.

Assessing the plant growth-promoting capabilities of potential PGPR and their tolerance to drought and heat stress

The current study received 104 bacterial isolates that were obtained and screen for plant growth promoting (PGP) attributes in a previous investigation under the dissertation titled "The impact of fertilizer application, tillage systems and crop rotation on soil health and rhizosphere microbial community structure under maize and soybean plantation". The PGPR isolates that demonstrated superior PGP traits were selected, screened for tolerance to drought and heat stress, and identified.

From the 104 isolates obtained, 61 isolates demonstrated superior plant growth-promoting capabilities (Indole acetic acid production, nitrogen fixation, phosphorus solubilization). From the 61, a total of 12 isolates exhibited potential tolerance to both drought and heat stress. Abiotic stresses not only affect plants but also affect microbes. To outlive and to prevail despite the intense competition among microorganisms in the rhizosphere, PGPR must actuate survival induced mechanisms for abiotic resistance. Thus, selecting drought and heat resistant PGPR is crucial when designing new inoculants for use in drought-stricken climates or zones with high temperatures. Inoculation of plants with tolerant PGPR has been shown to have the greatest chance of improving plant response to abiotic stress, therefore the isolated PGPR show great promise.

The isolates also exhibited IAA production, however, of the 12 isolates, nine demonstrated nitrogen-fixing capabilities and, three showed phosphorus solubilizing potential. Plant growth-promoting bacteria associate with plants in the rhizosphere, endophytically within plant roots or on the surface. This association can directly or indirectly facilitate plant growth by under abiotic stress conditions. The ability of the bacterial isolates to produce IAA, fix nitrogen and solubilize phosphorus can potentially facilitate plant growth and the uptake of the nitrogen and phosphorus.

The drought and heat tolerant PGPR were identified as relatives of *Bacillus thuringiensis* (7 isolates), *Bacillus pseudomycoides*, *Lelliottia amnigena*, *Acinetobacter* sp., *Leclercia* sp (2 isolates). Among the seven *B. thuringiensis* and 2 *Leclercia* isolates, 11MN1 and 36MP8 respectively were selected for further evaluation based on their greater adaptability to drought and heat stress. Only one isolate from the species with more than one isolate was selected. Thus, five isolates, namely 11MN1, 14MN3B, 21MN1B, and 36MP8 were used for further investigations. These abiotic tolerant PGPR could thus outperform others and thrive under drought or heat-stressed environment in sufficient numbers to deliver beneficial effects on plants.

6.3.1. Objective 2: To evaluate drought and heat tolerant PGPR isolates at inducing tolerance in maize plants exposed to concurrent drought and heat stress *in vitro*.

Drought and heat tolerant bacteria and their plant growth promoting attributes on maize seedling *in vitro*

The five PGPR isolates, *Bacillus thuringiensis* (11MN1), *Acinetobacter* sp. (14MN3B), *Bacillus pseudomycoides* (21MN1B), *Lelliottia amnigena* (33MP1), and *Leclercia* sp. (36MP8), were evaluated for their potential to alleviate adverse effects induced by the combination drought and heat stress on the early growth of maize seedlings (14 days) *in vitro*. Emphasis on evaluating the early effect of PGPR on plants under various abiotic stresses is crucial. *In vitro* culture techniques have the advantage of more defined and controlled conditions. They also have the added advantage of low cost, ease of handling, being less laborious, and permits the removal of less effective PGPR at the earliest time. In the current study, under the combined drought and heat stress, all evaluated isolates improved either one or more of the measured plant growth parameters in maize seedlings, except for 14MN3B that exhibited pathogenic effects. Thus, showing that the application of drought and heat-tolerant PGPR in agriculture present an opportunity to overcome limits in productivity under drought and heat-struck lands.

6.3.2. Objective 3: To evaluate whether the selected drought and heat tolerant PGPR isolates can be used in combination.

Bacterial isolates that demonstrated the potential to induce tolerance to maize plants subjected to dual drought and heat stress *in vitro* were evaluated for compatibility using the dual culturing technique. Co-inoculation of plants with a bacterial mixture/consortium has been shown to provide greater benefits to plant growth than inoculation with a single bacterial strain. In the current study, the isolates from the *Bacillus* group seem to show slight antagonism against *L. amnigena* and *Leclercia* sp. belonging to the *Enterobacteriaceae* group. However, there was no antagonism between the *Bacillus* spp. and likewise between the *Enterobacteriaceae* group. Compatibility of bacterial strains becomes crucial for formulating bioinoculants that promote plant growth. Even so, some studies have shown that co-cultures of antagonist microbes can provide still greater plant growth than single inoculations. Therefore, with the antagonism displayed by the

Bacillus spp. against *Enterobacteriaceae* group, it is possible their co-inoculation may result in enhanced plant growth under the dual effects of drought and heat stress.

6.4. Objective 4: To assess the phenotypic responses (physiological/physical development) of PGPR-inoculated maize under dual drought and heat stress under greenhouse conditions.

Drought and heat tolerant plant growth-promoting rhizobacteria: Effects on the early growth of maize subjected to dual drought and heat stress

To better understand the effects of these potential PGPR under soil conditions, a greenhouse experiment was conducted simulating dual drought and heat stress, as well as no stress conditions. Sterilized maize seeds were soaked in solutions of the bacterial isolates and planted in plastic pots containing a potting mix. Dual drought and heat stress was induced by maintaining the field water holding capacity (WHC) at 40 % and the temperature at 32/28 °C. Under no stress conditions, the seedlings were grown under greenhouse conditions at 23/25 °C with the WHC maintained at 80 %. After 32 days, the plant growth parameters were determined. A major advantage of greenhouse screening is that environment mimics field conditions, but the environment can be controlled to minimize the effects of confounding biotic and abiotic factors typically experienced under field conditions.

The results show that under simulated concurrent drought and heat stress, isolates *L. amnigena* 33MP1, *Leclercia sp.* 36MP8 and the co-inoculation of all the isolates showed the greatest potential for inducing tolerance in maize plants under dual drought and heat stress. This suggests that these potential PGPR can perform as intended under concurrent abiotic stresses (African climate), outcompete soil endemic microbes, successfully colonize plant roots and enhance growth. The study has putatively identified promising drought and heat tolerant PGPR that can then undergo more extensive field evaluations in subsequent studies.

6.5. Objective 5: To elucidate the expression of selected stress response genes in PGPR-inoculated maize under combined drought and heat stress.

The regulation of selected stress response genes in PGPR inoculated maize plants under concurrent drought and heat stress.

To elucidate expression of selected stress response genes in PGPR-inoculated maize plants under concurrent drought and heat stress, as well as non-stressed conditions, the total RNA of maize leaves was isolated, and the quantitative real-time PCR was conducted for the relative quantification of selected stress response genes (*DHN2*, *CAT2* and *HSP70*). These genes encode proteins that have crucial roles in protecting plant proteins from desiccation and denaturation, as well as oxidative damage. In the current study, these genes were differentially expressed in PGPR-inoculated maize plants under concurrent drought and heat stress. Some of the isolates appear to confer tolerance to concurrent drought and heat by modulating the *CAT2 and DHN2* stress response genes. These results suggest that PGPR-induced plant growth under dual drought and heat stress is specific, thus a complex phenomenon and may involve a cumulative effect of changes in various genes. Under non stress conditions, maize plants exhibited downregulation of all the stress response genes except for the CAT gene on plants inoculated with the mixture which was on par with the control uninoculated plants. This phenomenon was expected since these genes are stress response specific. Overall, the results obtained on the stress response of genes under concurrent drought and heat stress, supports the suggested strain-specific molecular responses of plants to rhizobacterial inoculation.

6.6. Limitations of the study

The limitations mentioned herein are not within the scope of the present study and are in no way expression of scientific incompetencies or admission of failure to undertake any part of the original scope of the study.

The mode of action, especially the PGPR-produced metabolites that play a role in inducing tolerance in maize plants under dual drought and heat stress, is not fully elucidated thus not understood. However, the study has succesfully demonstrated the ability of the PGPR isolates to adapt to drought and heat stress conditions. Moreover, the PGPR concentration for seed inoculation used in the current study was based on literature. The optimal application rate was not investigated. Therefore, even though a response was observed in inoculated maize, it might not have been optimal to induce the most optimal plant response. In addition, only a few maize stress response genes were investigated on PGPR-inoculated maize. It would have been ideal to get a holistic view of the molecular response of maize under these conditions.

6.7. Future studies

The current study demonstrates the potential of selected PGPR isolates for use as potential components in biofertilizers for alleviating and enhancing the growth of maize under adverse climatic conditions. The mode of action, especially the PGPR-produced metabolites that play a role in inducing tolerance in maize plants under dual drought and heat stress should be elucidated. The ideal PGPR concentration for seed inoculation should be determined to get the most optimal plant response. To enhance our understanding of the efficacy of the PGPR isolates, pot experiments should be conducted under drought and heat stress applied as single stresses. Moreover, the stress should be induced using a higher temperature, potentially 33-35 °C, introducing severe heat stress, opposed to the mild form applied in the current study. Metatranscriptomics should be conducted to get a holistic molecular maize response to PGPR inoculation under these climate conditions.

6.8. Conclusion

This study has screened and identified PGPR isolates that can potentially be used as components of biofertilizers formulated for adapting to climate change effects. Moreover, these isolated PGPR could promote the growth of maize plants under dual drought and heat stress in a pot trial experiment. These PGPR induce tolerance to concurrent drought and heat stress tolerance in plants by initiating "Induced Systemic Tolerance and by modulating the *CAT2 and DHN2* stress response genes. The use of such microorganisms is considered an environmentally friendly and sustainable approach. Thus, the study introduces an alternate way to maximally increase maize production in South Africa under these adverse climate conditions for commercial and smallholder farmers. Moreover, these PGPR isolates have the potential to reduce the need for extensive irrigation of maize crops throughout their growth cycle. Considering the global climate change challenge and the consequential lower crop yields being experienced by farmers, the outcome of the presesent study has potential to enhance production capacity of poor communities who use conventional agricultural methods. which do not address the effects brought by climate change and typically result in lower yields. More importantly, unlike some of the products in the market regarded as snake oils and supported by pseudo-science, this would be a potential biofertilizer supported by scientifically sound investigations.

Appendix

Supplementary Table 1: Screened bacterial isolates for drought (40 % PEG 6000) and heat stress (42 ℃) cultured on TSB broth. Bacterial isolates that exhibited an OD above 0.40 for both drought and heat stress were considered drought and heat tolerant. Bacterial isolates coloured green exhibited tolerance to both drought and heat stress

Sample No:	SAMPLE ID	Heat stress		Drought stress	
		Average Optical density (OD)	Standard Deviation	Average Optical density (OD)	Standard Deviation
1	21MN1A	1.09	0.04	0.08	0.18
2	32MN1B	1.10	0.01	0.50	0.09
3	15MN6B	0.71	0.04	0.16	0.05
4	15MN5	1.34	0.10	0.10	0.13
5	32MN2B	1.23	0.10	0.03	0.04
6	14MN3B	1.05	0.17	1.15	0.90
7	22MN2A	0.87	0.11	0.02	0.05
8	14MN3A	1.00	0.10	0.38	0.22
9	14MN5A	1.12	0.00	0.49	0.10
10	14MN5B	0.92	0.08	0.01	0.03
11	22MN2B	1.02	0.03	0.45	0.04
12	11MN3	0.93	0.01	0.42	0.06

13	21MN1B	0.94	0.14	0.45	0.14
14	14MN5A	0.27	__	0.45	0.10
15	11MN1	1.07	0.04	0.53	0.06
16	15MN6B	0.70	0.04	0.15	0.05
17	32MN1A	1.07	0.04	0.37	0.10
18	21MN3	1.00	0.01	0.35	0.10
19	11MN2	1.14	0.04	0.50	0.08
20	32MN3	0.95	0.02	0.37	0.07
21	32MN4	1.10	0.04	0.10	0.06
22	14MN1	1.09	0.01	0.28	0.17
23	22MN2	0.90	0.04	0.33	0.08
24	31MN1B	0.66	0.04	0.50	0.19
25	21MN3S	1.16	0.06	0.34	0.07
26	35MN3	0.89	0.03	0.37	0.14
27	31MN1S	1.30	0.02	0.12	0.02
28	23MN5	0.91	0.03	0.37	0.08
29	14MN2	1.12	0.03	0.24	0.15
30	35MN1	0.85	0.04	0.10	0.09
31	26MP3	1.47	0.08	0.03	0.09
32	20MP2S	1.24	0.03	0.00	__
33	27MP1	0.11	0.03	0.03	0.08
34	33MP1	0.87	0.04	0.54	0.21
35	15MP4	1.00	0.06	-0.06	0.03

36	14MP3	1.06	0.02	0.29	0.06
37	26MP3	1.07	0.01	0.08	0.06
38	30MP4	1.15	0.09	0.14	0.10
39	16MP1	0.87	0.01	0.04	0.16
40	15MP2	0.18	0.04	0.01	0.04
41	36MP8	0.64	0.01	0.96	0.30
42	14MP4	1.22	0.15	0.07	0.04
43	20MP2B	1.14	0.19	0.21	0.14
44	18MP2	1.20	0.05	0.10	0.09
45	12MP2	1.04	0.05	-0.02	0.06
46	23MP1	1.13	0.05	0.16	0.08
47	36MP1	0.74	0.05	0.40	0.20
48	26MP4Y	0.87	0.08	0.22	0.11
49	28MP1W	0.37	0.04	0.14	0.03
50	30MP5	1.00	0.03	0.37	0.05
51	31MP1	0.59	0.04	0.06	0.10
52	30MP3Y	0.93	0.03	0.18	0.03
53	23MP3	0.29	0.07	0.03	0.03
54	34MP2	0.86	0.05	0.48	0.11
55	36MP4	0.89	0.03	0.33	0.12
56	26MP2	0.91	0.03	0.14	0.09
57	21MP2Y	0.01	__	0.14	0.05
58	19MP4Y	0.77	0.41	-0.02	__

59	21MP1	1.08	0.01	0.21	0.04
60	21MP2	-0.08	__	-0.03	0.01
61	19MP4 W	0.27	0.02	-0.02	0.00

Sequence data for species identification

>32MN1B

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