EFFECTS OF ACC DEAMINASE-PRODUCING PLANT GROWTH-PROMOTING BACTERIA FROM *MYROTHAMNUS FLABELLIFOLIUS* (WELW.) ON WHEAT GROWTH UNDER DROUGHT STRESS

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ABSTRACT

The rise in global population compounded by global climatic patterns continue to drastically aggravate arid regions with susceptibility to various abiotic stress conditions. This situation impacts agricultural sustainability, further compromising food security. However, plants growing in hostile environments have become well adapted to their habitats, employing strategies to mitigate the impacts of drought stress by mutualistic association and interaction with 1-aminocyclopropane-1-carboxylate (ACC) deaminase plant growth-promoting bacteria (PGPB). This study was therefore, designed to isolate, characterize and identify bio-prospective ACC deaminase PGPB associated with *Myrothamnus flabellifolius* (Welw.), a Namibian desert-adapted plant. A total of 52 isolates of bacteria were isolated from *M. flabellifolius* of which 33 underwent molecular identification using 16S rRNA, *acdS*, and *nif*H gene sequence alignment, and 29 were further characterized for plant growth promoting (PGP) traits. Ten different genera namely *Bacillus* (12), *Pseudomonas* (6), *Cupriavidus* (5), *Enterobacteriacea* (2), *Kosakonia* (3), *Agrobacterium* (1), *Klebsiella* (1), *Staphylococcus* (1), *Enterobacter* (1) and *Cellulosimicrobium* (1) were identified, with *Bacillus* as the most abundant genera (36.4%). Isolates were selected for their ability to produce ACC deaminase and other PGP traits, and drought tolerance evaluated using four different polyethylene glycol (PEG) 6000 concentrations. From the 29 isolates that were characterized and deemed positive for ACC deaminase, the highest ACC deaminase activity value was exhibited by *Bacillus licheniformis* (R13) 0.0013 ± 0.01 mM of α-ketobutyrate. The highest drought tolerance at 30% PEG 6000 was exhibited by *Staphylococcus hominis* (LB3) and *Bacillus licheniformis* (R13), 0.52 ± 0.37 and 0.35 ± 0.18 respectively. Three ACC deaminase-producing strains, coupled with PGP traits and drought stress tolerance, *Kosakonia* sp., *Cupriavidus metallidurans*, and *Pseudomonas* sp. were selected and evaluated for their response to improve wheat (*Triticum aestivum* L.) germination and early growth under drought stress. Proline levels and enzymatic activity were evaluated on seedlings. Seed treatment with *Kosakonia* sp. strain displayed significant growth enhancement ($P<0.005$) in various growth parameters evaluated under drought stress conditions in contrast to *Pseudomonas* sp. *C. metallidurans* and the control. Proline levels significantly differed across categories of bacterial isolate treatments ($P<0.01$), with *Kosakonia* sp. treated seeds exhibiting the highest level of proline per fresh weight (29.5 µmoles/g) and *Pseudomonas* sp. exhibiting the lowest (6.5 µmoles/g). There was no significant difference in levels of proline produced during various PEG-induced drought conditions. Enzymatic activity (α-amylase) was significantly influenced by bacterial treatments with the control significantly reducing the enzymatic activity in contrast to other treatments. This study showed that reducing water potential decreases germination percentage (GP) and various growth parameters. Additionally, bacterial isolates such as *Kosakonia* sp. have the potential to promote growth and serve as potential microbes in the development of effective bioinoculants for sustainable agricultural crops in arid regions. The study is the first report on prospective microbes from *M. flabellifolius* inhabiting Namibia.

Keywords: *Myrothamnus flabellifolius*, plant growth promoting bacteria, drought, desert

LIST OF PUBLICATION(S)/CONFERENCE(S) PROCEEDINGS

1. Frans, EN; Kwembeya, EG and Uzabakiriho, JD. Drought-tolerant bacteria associated with *Myrothamnus flabellifolius* promote plant growth in arid environments. Oral presentation at the National Students' Research Symposium (21-22 August 2024), Held in Windhoek, Namibia.

2. Frans, EN; Sibanda, T; Kwembeya, EG and Uzabakiriho, JD. Desert adapted *Myrothamnus flabellifolius* welw. rhizosphere and endophytic bacteria exhibit plant drought-stress tolerance promoting traits. Submitted for publication in the South African Journal of Botany in October 2024.

3. Frans, EN; Kwembeya, EG and Uzabakiriho, JD. Drought-tolerant bacteria associated with *Myrothamnus flabellifolius* promote plant growth in arid environments. Oral presentation at the Agricultural and Natural Resources Conference (12-13 November 2024), Held in Ogongo, Namibia

4. Frans, EN; Kwembeya, EG and Uzabakiriho, JD. Effects of ACC deaminase-plant growth promoting bacteria (PGPB) on seed germination and early seedling growth of wheat under simulated drought conditions. Oral presentation at the Agricultural and Natural Resources Conference (12-13 November 2024), Held in Ogongo, Namibia. In preparation for publication.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND/OR ACRONYM

µm: Micromoles

ABA: Abscisic acid

ACC: 1-aminocyclopropane-1-carboxylate

ATP: Adenosine Triphosphate

CAS: Chrome azurol S

CRD: Complete randomized design

DEC: Decentralised Ethics Committee

DF: Dworkin and Foster

DNF: 2,4-dinitrophenylhydrazine

DNS: 3,5-dinitrosalicylic acid

GA: Gibberellic acid

GP: Germination percentage

HCN: Hydrogen cyanide

IAA: Indole-3-acetic acid

ISR: Induced systemic resistance

K: Potassium

LB: Luria bertani

MGT: Mean germination time

mM: Millimolar

N: Nitrogen

NA: Nutrient agar

NBRI: National Botanical Research Institute

NCBI: National Centre of Biotechnology Information

NH3: Ammonia

(NH4)2SO4): Ammonium sulphate

OD: Optical density

P: Phosphorous

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

PGP: Plant growth promoting

PGPB: Plant growth-promoting bacteria

PSI: Phosphate solubilizing index

ROS: Reactive oxygen species

RWC: Relative water content

SAM: S-adenosyl-methionine

SM: Synthetic malate

SVI: Seedling vigour index

TBE: Tris-Borate-EDTA

TSA: Trypticase soya agar

TSB: Tryptic soy broth

VM: VM-ethanol

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Finally, to the Almighty, thank you Lord for your Grace, and for the constant reminder that we are not in charge and that all things happen according to Your will, for showing me that in due time, you will make it happen and that you are in total control of everything.

DEDICATION

I would like to dedicate this study to my three children, Delta, Alpha & Mega, and my dearest loving husband, Tomas Frans, who held my hand throughout the entirety of my studies. Your support and prayers have sustained me this far.

This has been one of the hardest and most emotional academic periods of my life and I would not have made it to the finishing line without your support. Thank you for your patience, motivation, and allowing me to focus on school while you take care of our children. You believed in me even when I doubted myself. Thank you for helping me realize my full potential, I owe it all to you.

To my children, the completion of this Thesis is proof that it is not about how long it takes you to finish, but that you finish what you started, no matter how long it takes you.

DECLARATION

I, Esther Ndatala Frans, hereby declare that this study is my own work and is a true reflection of my research, and that this work, or any part thereof has not been submitted for a degree at any other institution.

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Esther Ndatala Frans April 2025

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Name of student Signature Date

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Drought represents one of the major environmental constraints that adversely affect crop productivity worldwide (Gil, Garrido and Hernández-Mora, 2010; Ngumbi and Kloepper, 2016; Fahad *et al.*, 2017; Bashir *et al.*, 2021; Seleiman *et al.*, 2021). The recurrence of drought periods, especially in arid and semi-arid countries is exacerbated by the ongoing cumulative changing climatic conditions (Osmolovskaya *et al.*, 2018; Byregowda *et al.*, 2022), with about two-thirds of the African region being affected (Eke *et al.*, 2019). By the year 2050, forecasts predict an increase in the impact of drought on more than half of the world's arable lands (Kasim *et al.*, 2013; Vurukonda *et al.*, 2016). Consequently, drought has been a catalyst of global famines in the past and continues to be a critical threat to global food security (Barnawal, Singh and Singh, 2019; Seleiman *et al.*, 2021). This multidimensional stress severely impacts morphological, physiological, and biochemical attributes of crops (Vurukonda *et al.*, 2016; Sánchez-bautista *et al.*, 2018; Sharma *et al.*, 2022).

In plants, water deficit induces osmotic imbalance, resulting in turgor loss. Furthermore, the inhibition of photosynthetic capacity profoundly decreases the availability and assimilation of nutrient elements, leading to hormonal and nutritional alterations in the plant (Vurukonda *et al.*, 2016; Miranda *et al.*, 2023). Additionally, water deficit affects seed germination (Pouri *et al.*, 2019; Pavli *et al.*, 2020; Bogati and Walczak, 2022), a prerequisite and transition stage in seedling establishment, which when hindered, results in late radicle emergence and poor seedling development (Haj Sghaier *et al.*, 2023). Nevertheless, plants possess various adaptive traits to overcome water related constraints (Barnawal, Singh and Singh, 2019; Chukwuneme *et al.*, 2020; Govindasamy *et al.*, 2020). Plants also improve their drought tolerance and avoidance mechanisms to thrive in arid environments through microbe-induced changes in the plants (Barnawal, Singh and Singh, 2019; Byregowda *et al.*, 2022).

Endophytic and rhizospheric microbes colonise plant tissues without causing any harm, and are known to exert a wide range of beneficial effects that could confer plant tolerance to biotic or abiotic stress (Compant, Van Der Heijden and Sessitsch, 2010; Barnawal, Singh and Singh, 2019; Govindasamy *et al.*, 2020; Miranda *et al.*, 2023). They do this through phytohormonal modifications, production of exopolysaccharides, and accumulation of osmolytes such as proline, which acts as a defence mechanism against reactive oxygen species (ROS) and antioxidant enzymes (Zhang *et al.*, 2008; Naylor and Coleman-Derr, 2018).

PGPB have shown to enhance stress tolerance, at least in part, by enhancing the root length, allowing better access to water (Ashry *et al.*, 2022). The application of PGPB as bio-fertilizers has previously been considered as an alternative novel biotechnological approach to improve crop yields in substitution of chemical fertilisers (Riseh *et al.*, 2021; Aloo *et al.*, 2022). To this end, the isolation and exploration of novel desert plant-adapted endophytes could improve crop yields by conveying habitat-adapted symbiotic properties (Byregowda *et al.*, 2022).

During drought stress, there is a high production of ethylene in plants, which is a key regulator of drought tolerance (Murali, Singh, Gowtham, *et al.*, 2021). Therefore, reduction of ethylene concentrations via the hydrolyses of plant ACC by ACC deaminase into α-ketobutyrate and ammonia (NH_3) (Naing, Maung and Kim, 2021), by use of PGPB with ACC deaminase activity is one of the most effective and alternative strategies to induce tolerance in plants against various biotic and abiotic stressed conditions, and increase plant yield (Pandey and Gupta, 2020; Kour *et al.*, 2022). Moreover, the use of PGPB to induce abiotic stress tolerance has previously been used in crops such as wheat (Kasim *et al.*, 2013). This is due to frequent exposure of wheat plants to drought, which adversely affect both yield and seed quality globally (Akbari, Akbari and Golakiya, 2016). Recent reports further indicate a decline in global yield production of wheat by 21% due to drought (Camaille *et al.*, 2021), consequently affecting global food security. Therefore, there is a need for agricultural productivity to be increased (Chandra, Srivastava and Sharma, 2018; Camaille *et al.*, 2021), with 110% by the year 2050 to meet the increasing human population (Chandra, Srivastava and Sharma, 2018).

In Namibia, wheat is one of the highest consumed staple crop, despite accounting for the lowest in terms of production (NAB, 2021). During the 2019/2020 season, NAB (2020) reported a reduction in local wheat production, attributed to a decrease in hectarage, with 4399 tons of wheat harvested on 1011 hectares, resulting in total local production of 4466 tons. According to (NAB, 2021), local wheat production during the 2018/2019 season was 7508 tons, indicating a 40.5% decrease. Therefore, with 89% of Namibia's domestic wheat requirements imported, the country does not meet domestic wheat demands (NAB, 2020, 2021).

Namibia is one of the most arid countries in sub-Saharan Africa, with about 60% of the natives in rural areas dependent on agriculture for subsistence (Wilhelm, 2012). This aridity makes the country susceptible to water shortages for the most part of the year (Koroma, 2016). The aforementioned conditions therefore, make arid environments somewhat inhabitable for living organisms to survive (Eida *et al.*, 2018) due to hostile conditions such as limited nutrients that consequently affect plant growth and survival (Zhang and White, 2021). Consequently, there is a need to identify environmentally friendly approaches to mitigate agricultural drought vulnerability (Mishra and Singh, 2010; Gontia-Mishra *et al.*, 2016). The utilization of PGPB is an environmentally friendly approach to improve crops, providing stress tolerance to plants (Ojuederie and Olanrewaju, 2019). Dry ecosystem plants could be strongly influenced by their interaction with specific microbial endophytes (Wu *et al.*, 2021; Zhang and White, 2021; Miranda *et al.*, 2023) that play a significant role in counteracting the negative effects of stress and maintaining and enhancing growth under biotic and abiotic conditions (Timmusk *et al.*, 2014). Therefore, a comprehensive understanding of plants and microbes that are well adapted to extreme environments is particularly relevant in the face of climate change (Alsharif, Saad and Hirt, 2020).

There are a group of plants inhabiting the most hostile and harsh environments known as ‘resurrection’ plants, with nearly complete desiccation tolerance level (Marks *et al.*, 2022).

Myrothamnus flabellifolius Welw., a Southern African desert-adapted plant, is known as a ‘resurrection’ plant due to its ability to tolerate desiccation (Zhang and Bartels, 2018; Erhabor *et al.,* 2020), as well as its ethnobotanical properties (Moore *et al.*, 2007).

The present study, therefore, focused on the isolation and characterization of ACC deaminase containing bacterial endophytes from *M. flabellifolius*, evaluation of plant growth promoting (PGP) traits associated with the plant, determining the PGP effects of these isolates on early seedling growth, determining physiological status of treated seeds for proline and α-amylase and their potential as bio-inoculants under normal and stressed conditions.

1.2 Statement of the research problem

Wheat production in sub-Saharan Africa has declined due to drought, accentuated by global climate change, and during the 2014-2016 drought period, over one million tons of wheat was imported to meet the demand (Ruwanza, Thondhlana and Falayi, 2022). Unfortunately, due to daily increase in the population coupled with high food demands, agricultural productivity and food security has been compromised (Akbari, Akbari and Golakiya, 2016). Consequently, causing a surge for wheat demand in the sub-Saharan region (Tadesse, Bishaw and Assefa, 2019).

1.3 Objectives of the study

a) To isolate, identify and screen for ACC deaminase activity and drought tolerance of *Myrothamnus flabellifolius* rhizosphere and endophytic bacteria from Namakorabis Farm in Namibia.

b) To screen for plant growth promoting traits (production of indole-3-acetic acid (IAA), hydrogen cyanide (HCN), siderophore, ammonia, nitrogen fixation, phosphate solubilisation) from *M. flabellifolius* ACC deaminase producing bacteria.

c) To determine the effects of ACC deaminase producing PGPB isolated from *M. flabellifolius* on seed germination, early seedling growth (shoot and radicle length, number of shoots and radicles, seedling vigour index, and seedling weight), the levels of α-amylase and proline of wheat under polyethylene glycol (PEG) 6000 simulated drought conditions.

1.4 Significance of the study

Improving the resilience of staple crops growing under Namibia's arid climate to endure drought is of uttermost importance for the country’s food security (Alsharif, Saad and Hirt, 2020). Breeding and genetic approaches for plant drought tolerance have often overlooked the potential of the PGP microbiome (Zia *et al.*, 2021). It is hypothesized that the Namibian desert plant, *M. flabellifolius*, harbours ACC deaminase PGPB that could induce beneficial changes in the morphology, physiology and metabolism of domesticated plants such as wheat, thereby conferring stress tolerance. However, the interaction between *M. flabellifolius* ACC deaminase producing PGPB and wheat under drought conditions have not been explored. This study, therefore, has the potential to improve the understanding of desert microbe-mediated plant resistance to drought improvement in crops in arid and semi-arid areas such as Namibia. It offers a prospect of a better alternative to improving stress tolerance during germination and early growth, as well as in attaining sustainable agriculture by designing potential biological fertilizers. Thus, the present study investigates the potential of these bacterial strains to induce drought tolerance in wheat during seed germination and early seedling growth under PEG 6000-simulated drought conditions.

1.5 Limitations

The methods used in the study are culture dependent and micro-organisms used in the study were used in axenic culture, contrary to natural environments, where they could function in synergy. Culture-dependent method could therefore, impact PGPB isolated and the production of metabolites by the isolated soil microbes.

1.6 Delimitations of the study

Growth promotion of wheat seedlings under various drought conditions was conducted in an *in-vitro* setting, which could not mimic exact field conditions in nature. Also, *in-vitro* findings are comparable to those in the field.

1.7 Ethical considerations

Ethical clearance certificate to do the study was issued by the School of Science, Decentralised Ethics Committee (DEC), University of Namibia, (Reference number: SOS-0137).

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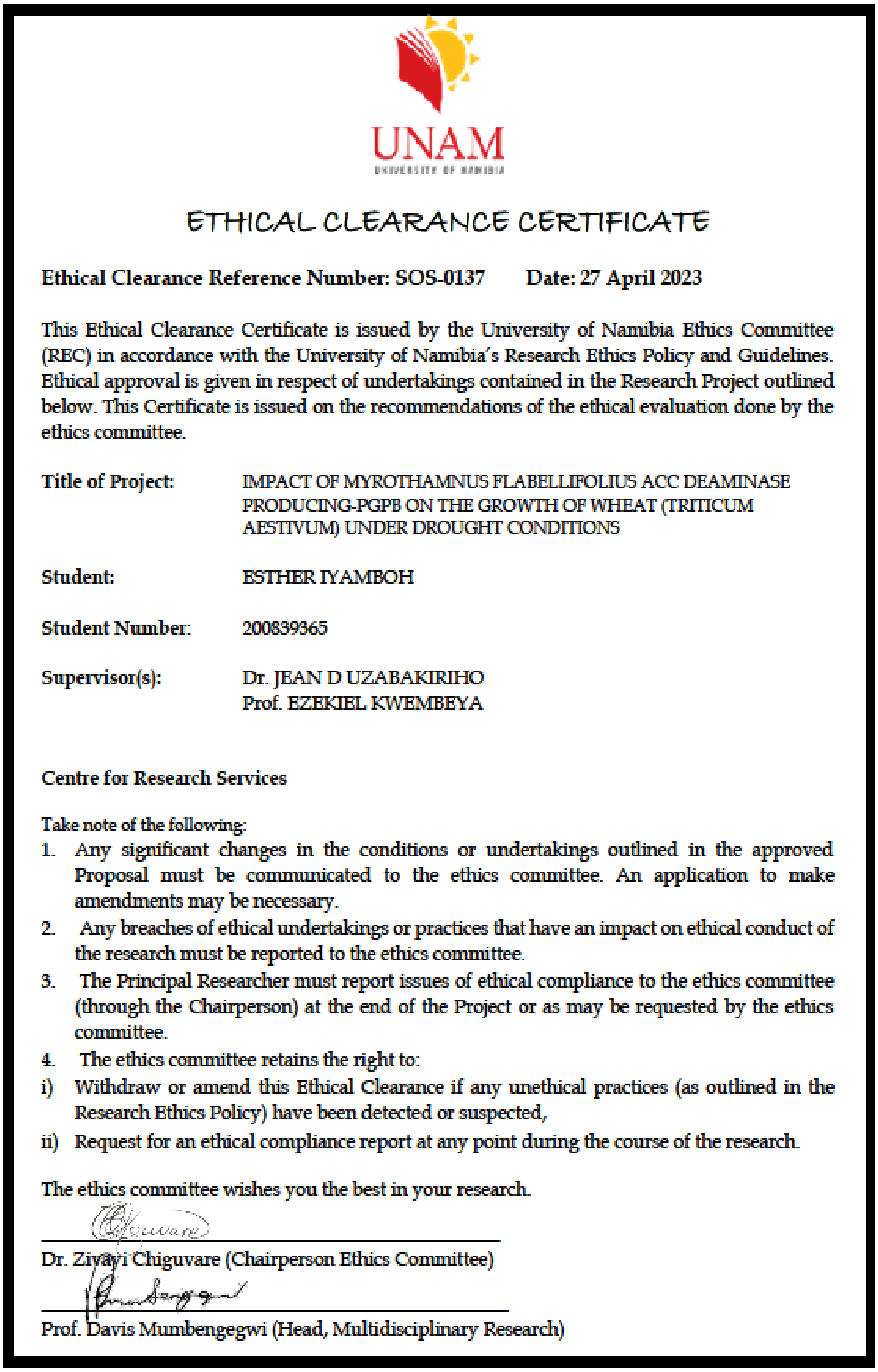
APPENDIX

Ethical clearance certificate



UNAM
UNIVERSITY OF NAMIBIA

ETHICAL CLEARANCE CERTIFICATE

Ethical Clearance Reference Number: SOS-0137 **Date: 27 April 2023**

This Ethical Clearance Certificate is issued by the University of Namibia Ethics Committee (REC) in accordance with the University of Namibia's Research Ethics Policy and Guidelines. Ethical approval is given in respect of undertakings contained in the Research Project outlined below. This Certificate is issued on the recommendations of the ethical evaluation done by the ethics committee.

Title of Project:	IMPACT OF MYROTHAMNUS FLABELLIFOLIUS ACC DEAMINASE PRODUCING-PGPB ON THE GROWTH OF WHEAT (TRITICUM AESTIVUM) UNDER DROUGHT CONDITIONS
Student:	ESTHER IYAMBOH
Student Number:	200839365
Supervisor(s):	Dr. JEAN D UZABAKIRIHO Prof. EZEKIEL KWEMBEYA

Centre for Research Services

Take note of the following:

1. Any significant changes in the conditions or undertakings outlined in the approved Proposal must be communicated to the ethics committee. An application to make amendments may be necessary.
2. Any breaches of ethical undertakings or practices that have an impact on ethical conduct of the research must be reported to the ethics committee.
3. The Principal Researcher must report issues of ethical compliance to the ethics committee (through the Chairperson) at the end of the Project or as may be requested by the ethics committee.
4. The ethics committee retains the right to:

i) Withdraw or amend this Ethical Clearance if any unethical practices (as outlined in the Research Ethics Policy) have been detected or suspected,

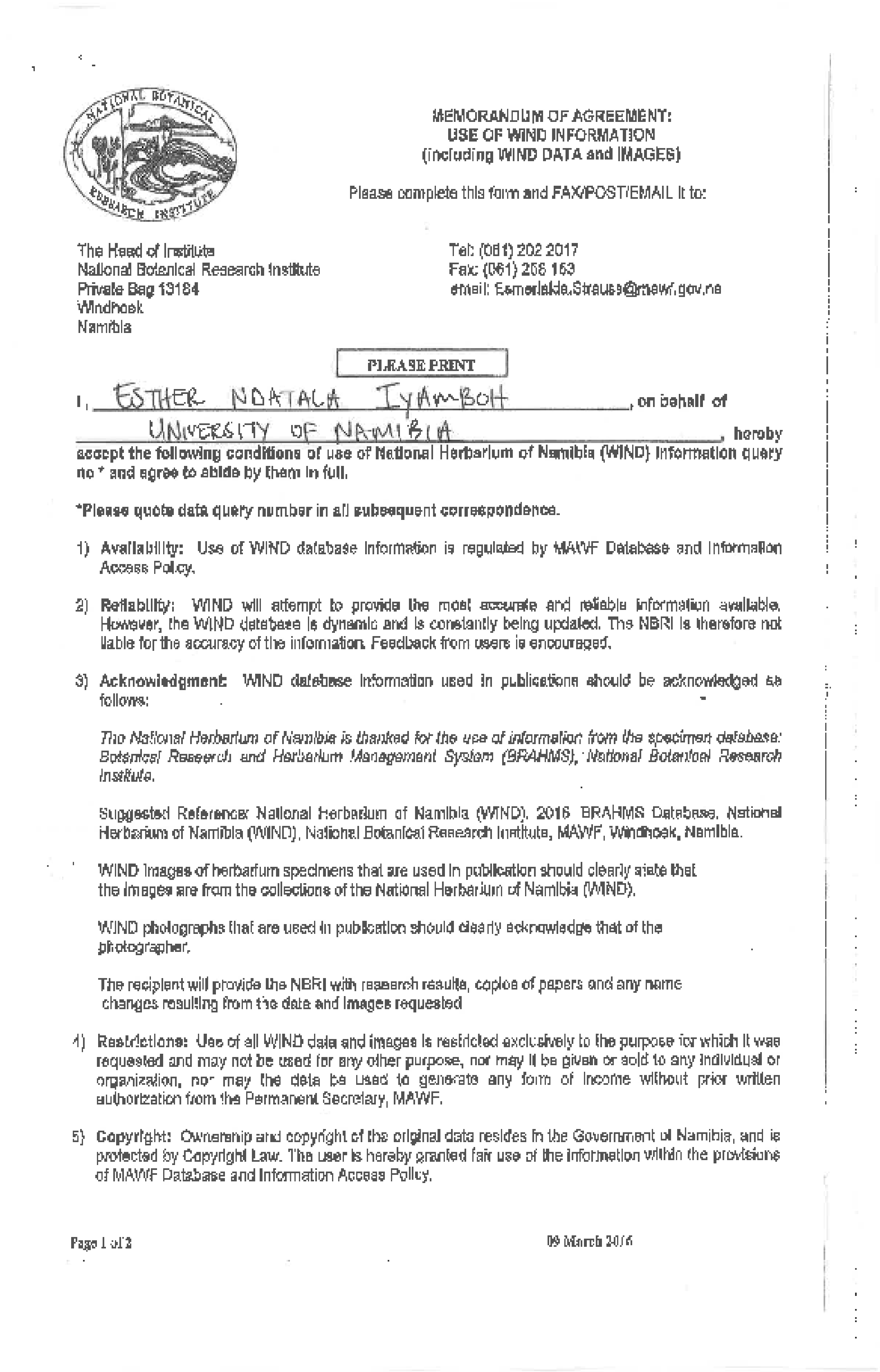
ii) Request for an ethical compliance report at any point during the course of the research.

The ethics committee wishes you the best in your research.

Dr. Zivayi Chiguvare (Chairperson Ethics Committee)

Prof. Davis Mumbengegwi (Head, Multidisciplinary Research)

Permission Letter



MEMORANDUM OF AGREEMENT:
USE OF WIND INFORMATION
(including WIND DATA and IMAGES)

Please complete this form and FAX/POST/EMAIL it to:

The Head of Institute
National Botanical Research Institute
Private Bag 13184
Windhoek
Namibia

Tel: (061) 202 2017
Fax: (061) 258 153
email: Esmeralda.Strauss@mawf.gov.na

PLEASE PRINT

I, ESTHER NDATALA IYAMBOH, **on behalf of** UNIVERSITY OF NAMIBIA, **hereby accept the following conditions of** use of **National** Herbarium of **Namibia (WIND)** information query no * **and agree to abide by them in full.**

***Please quote data query number in all subsequent correspondence.**

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2) **Reliability:** WIND will attempt to provide the most accurate and reliable information available. However, the WIND database is dynamic and is constantly being updated. The NBRI is therefore not liable for the accuracy of the information. Feedback from users is encouraged.

3) **Acknowledgment:** WIND database information used in publications should be acknowledged as follows:

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   WIND images of herbarium specimens that are used in publication should clearly state that the images are from the collections of the National Herbarium of Namibia (WIND).

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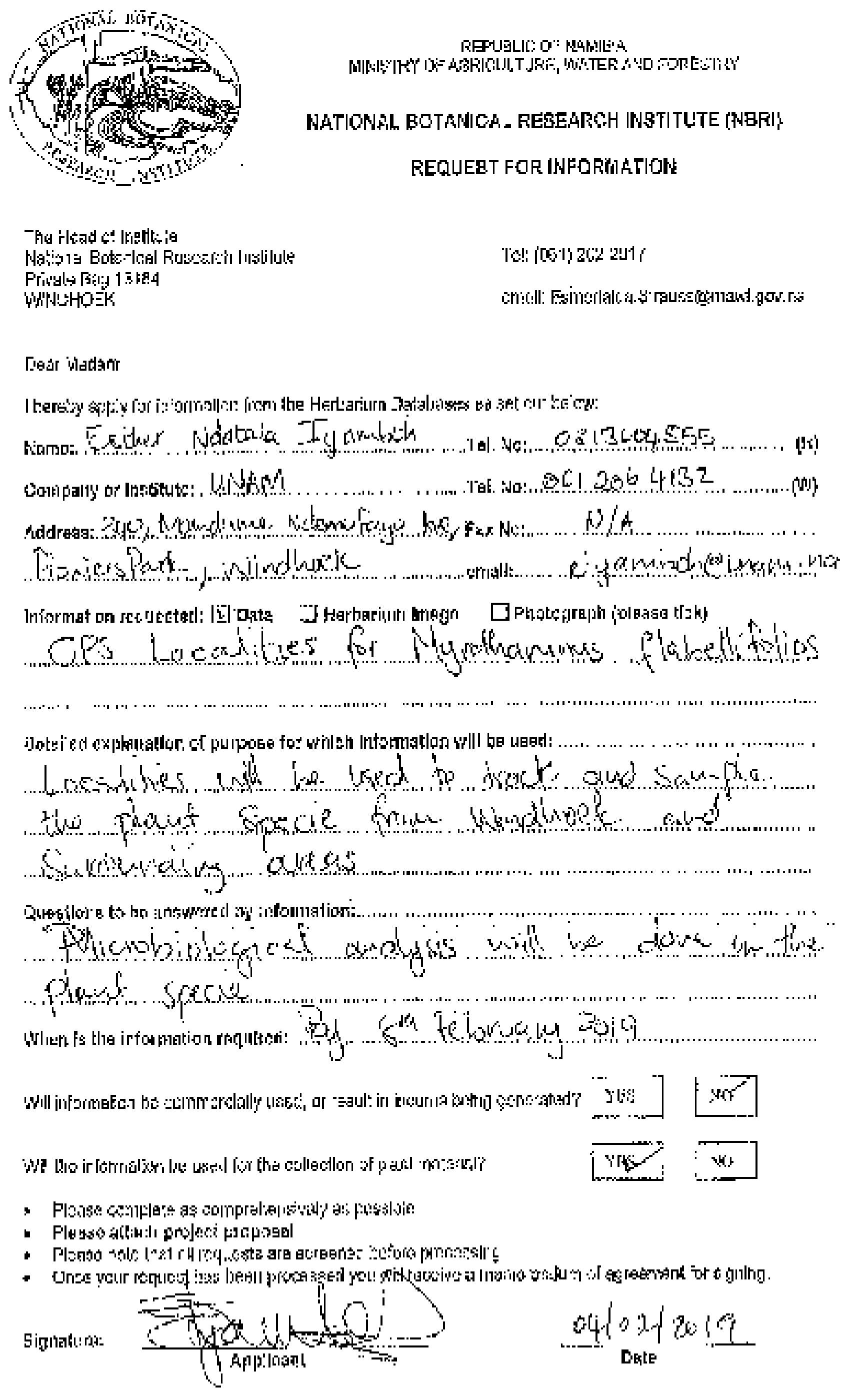
Page 1 of 2 09 March 2016



REPUBLIC OF NAMIBIA
MINISTRY OF AGRICULTURE, WATER AND FORESTRY

NATIONAL BOTANICAL RESEARCH INSTITUTE (NBRI)

REQUEST FOR INFORMATION

The Head of Institute
National Botanical Research Institute
Private Bag 13184
WINDHOEK

Tel: (061) 202 2017

email: Esmerialda.Strauss@mawf.gov.na

Dear Madam

I hereby apply for information from the Herbarium Databases as set out below:

Name: Esther Ndatala Iyambeh **Tel. No:** 0813604555 (H)

Company or Institute: UNAM **Tel. No:** 061 206 4132 (W)

Address: 290, Mandume Ndemufayo Ave, **Fax No:** N/A

Pioneers Park, Windhoek **email:** eiyambeh@unam.na

Information requested: ☒ Data ☐ Herbarium image ☐ Photograph (please tick)

GPS Localities for Myrothamnus flabellifolius

Detailed explanation of purpose for which information will be used:

Localities will be used to track and sample the plant specie from Windhoek and surrounding areas

Questions to be answered by information:

Microbiological analysis will be done on the plant specie

When is the information required: By 6th February 2019

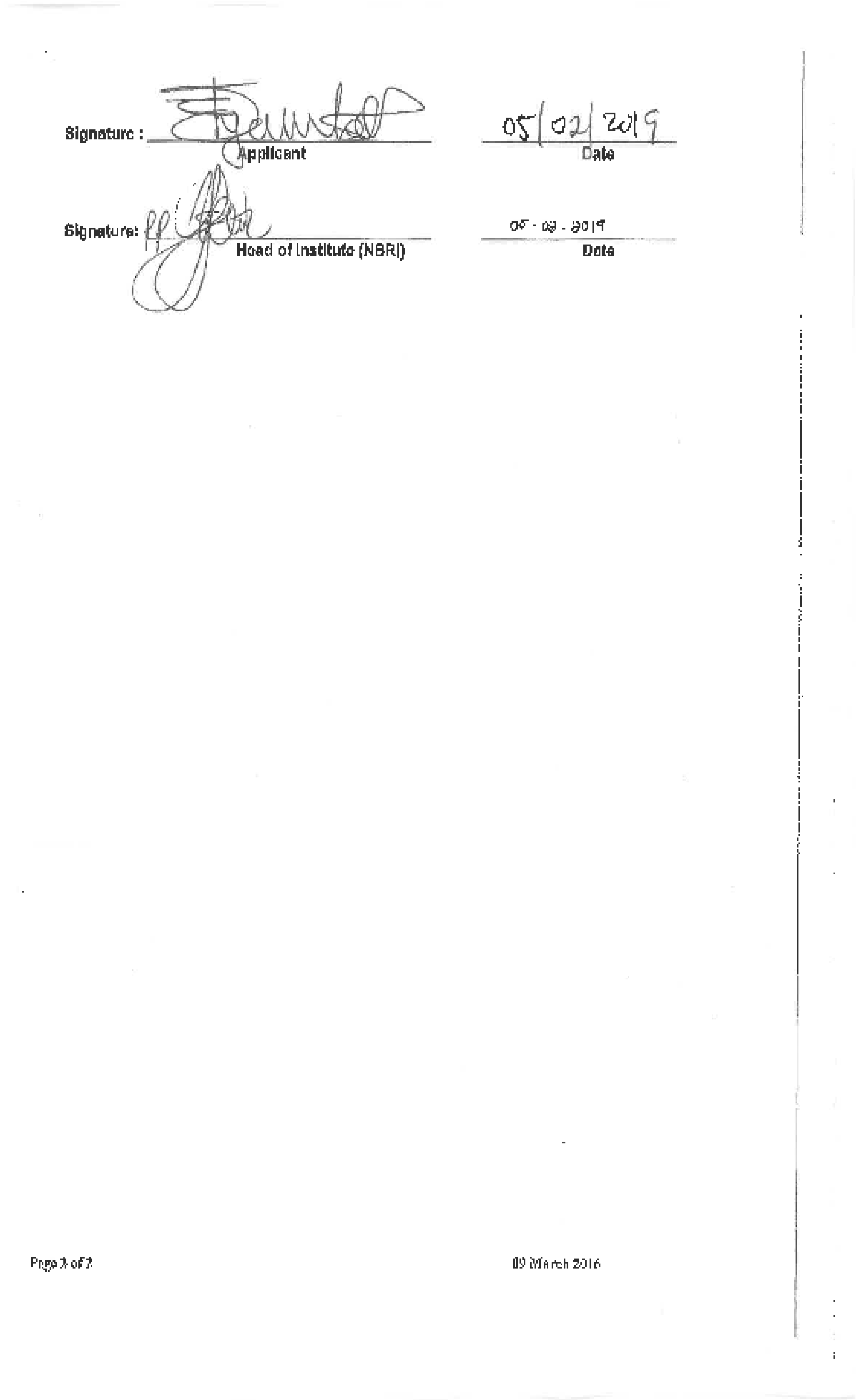
Will information be commercially used, or result in income being generated? YES [✓ NO]

Will the information be used for the collection of plant material? [✓ YES] NO

- Please complete as comprehensively as possible
- Please attach project proposal
- Please note that all requests are screened before processing
- Once your request has been processed you will receive a memorandum of agreement for signing.

Signature: Applicant

04/02/2019
Date



Signature : ____________________ 05/02/2019
Applicant Date

Signature: pp ____________________ 05 - 02 - 2019
Head of Institute (NBRI) Date

Page 2 of 2 09 March 2016

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Global agriculture and food security are presumed to face multiple challenges over the next decades, due to an increase in global population and the effects of climate change (Camaille *et al.*, 2021). Climate change has caused water shortages coupled with heat stress in sub-Saharan Africa, and is limiting agricultural crop production (Tadesse, Bishaw and Assefa, 2019). These effects are acutely manifesting in arid countries such as Namibia, which are susceptible to climate variations (Bombi *et al.*, 2021). It is, therefore, imperative to develop strategies to impart drought tolerance in crop production systems. The use of plant associated microbes, specifically those found inhabiting drought-prone environments as they may have the potential to confer drought tolerance abilities to plants (Eke *et al.*, 2019).

Myrothamnus flabellifolius also known as the "resurrection plant" (opstandingsplant in Afrikaans) has evolved mechanisms to cope with extreme environmental conditions with the ability to tolerate desiccation periods in arid areas for 9-12 months (Tebele, Marks and Farrant, 2023). This plant is known to host a diverse microbiome that could assist the plant in adaptation and survival during drought stress conditions (Tebele, Marks and Farrant, 2023). PGPB naturally inhabit the soil, the rhizosphere, phyllosphere, or internal plant tissues and have the potential to produce different plant growth hormones and other growth-enhancing molecules, which could enhance plant growth under abiotic stresses (Saikia *et al.*, 2018).

The use of PGPB is an eco-friendly strategy with positive effects on plant, with the potential to reduce the use of chemicals and fertilizers, which potentially have negative effects on the environment (Bahlolzada and Hussaini, 2021). In addition, PGPB could reduce stress inducible ethylene levels in plants by producing 1-

aminocyclopropane-1-carboxylate (ACC) deaminase (Singh & Jha, 2015). ACC deaminase is an enzyme that hydrolyses ACC, the direct precursor to the plant hormone (ethylene) produced by plants during stress (Jaya *et al.*, 2019). Therefore, the use of effective PGPB as a tool to decrease the effects of drought stress on crop production in arid areas could result in sustainable agricultural practices (Bahlolzada and Hussaini, 2021). Due to microbial and genetic diversity in desiccation tolerance plants being understudied, (Marks *et al.*, 2021), microbial composition, characterization of taxa and distribution across various plant parts of *M. flabellifolius* could help to screen for microbes involved in enhancing the drought tolerance of the drought adapted plants (Tebele, Marks and Farrant, 2023).

Lastly, due to the negative impacts of climate change, it is imperative now more than ever that agricultural practices are advanced by identifying microbes associated with drought tolerant plants such as *M. flabellifolius* in order to improve crop production (Marks *et al.*, 2021).

2.2 Drought stress and its impact on crop production

Drought is defined as a period of "below-average precipitation" that leads to an inadequate soil and rhizospheric moisture (Mahpara, Hussain and Farooq, 2015; Ilyas *et al.*, 2020). Drought is a climatic phenomenon commonly experienced in arid and semi-arid regions (Mahpara, Hussain and Farooq, 2015). It is one of the most hazardous environmental stress that hampers crop growth and development resulting in a substantial decline in biomass production (Seleiman *et al.*, 2021). The predicted increase from 3 °C to 5 °C in the next 50-100 years coupled with uneven rainfall (Gull, Lone and Wani, 2019) are set to continuously distress crop production in many parts of the world (Serba and Yadav, 2016). Furthermore, over the past half century, grain production has suffered a 10% decline due to the detrimental effects of drought

(Zhang *et al.*, 2018). Data spanning between 1980 and 2015 indicate a worrisome 21% reduction in wheat yields and a staggering 40% reduction in corn production globally due to water scarcity (Fahad *et al.*, 2017; Camaille *et al.*, 2021). Disturbingly, projections indicate that by 2050 more than half of the arable land will experience productivity loss (Kasim *et al.*, 2013; Ilyas *et al.*, 2020; Zhang *et al.*, 2020).

Drought recognised as a multifaceted stress exerts critical impacts on the metabolism of crop plants, triggering remarkable limitations of crop production (Bashir *et al.*, 2021). This impact is manifested through physical damages, physiological functions and biochemical pathway disruptions (**Figure 2.1**) (Fahad *et al.*, 2017). Specifically, drought stress disrupts the plant's water status thereby, adversely affecting plant growth and development (Osmolovskaya *et al.*, 2018). These disruptions echo through crucial physiological processes including photosynthesis, respiration, stomatal closure and reduced mineral nutrients uptake (Osmolovskaya *et al.*, 2018). Additionally, moisture impact on metabolic activities such as respiration, chlorophyll content, sugar metabolism, and nutrient translocation have been investigated (Fadiji *et al.*, 2022). Changes in physiological factors such as relative water content (RWC), are indicative of drought severity (Soltys-Kalina *et al.*, 2016). Therefore, occurrence of physiological alteration such as reduction of turgor pressure in plants lead to reduced chlorophyll levels, reduced photosynthesis and ultimately reduced crop yield (Ojuederie and Olanrewaju, 2019).

Crop yield reduction have been observed in crops such as maize, characterised by leaf rolling (Ojuederie and Olanrewaju, 2019; Seleiman *et al.*, 2021), a size reducing mechanism aimed at minimizing water loss. Furthermore, several factors trigger plant responses to drought, including type of species, plant growth stage, cellular metabolism, crop yields and environmental factors (Fahad *et al.*, 2017; Gull, Lone and Wani, 2019). Crops are critically affected during reproductive and flowering growth stages in contrast to the vegetative stage, consequently affecting the yield as observed in crops such as wheat (Ojuederie and Olanrewaju, 2019).

The impact of drought is further manifested in plant root physiology, leaf structure, nutrient uptake, and seedling germination (Verma *et al.*, 2021). Drought affect plant growth parameters including inhibition of cell expansion, early maturation, reduced germination rates, diminished fresh and dry biomass, impaired water & nutrient uptake, decreased photosynthesis, proline accumulation, abscisic acid (ABA), inorganic ions, and phytohormones within the plant (Bogati and Walczak, 2022). Additionally, nutrient availability in the soil is adversely impacted by drought stress, leading to disruption in impaired transport and nutrient uptake from the roots to the shoot (Kour *et al.*, 2022). Thus, drought impacts overall the plant productivity and, ultimately lead to plant death (Osmolovskaya *et al.*, 2018).

Insufficient moisture remain the primary source of impaired seed germination and seedling establishment (Vurukonda *et al.*, 2016; Fahad *et al.*, 2017; Ojuederie and Olanrewaju, 2019), adversely impacting various growth parameters such as root and shoot dry weight, hypocotyl length, and vegetative growth as reported in field crops (Fahad *et al.*, 2017). These adverse effects impact on common growth parameters including fresh weight and water content (Kasim *et al.*, 2013). Equally significant, the reduction in moisture inflicts re-adjustment of crop physiological and biochemical performance, such as stomatal closure, which restricts photorespiration, resulting in permanent wilting, stunted growth, reduction in quality and yield performance (Eke *et al.*, 2019; Ojuederie and Olanrewaju, 2019) in addition to yellowing of leaves and leaf scorching (Seleiman *et al.*, 2021).

Drought has therefore, been reported as one of the major limitations of crop production affecting agricultural productivity globally (Singh *et al.*, 2015; Camaille *et al.*, 2021). In order to meet the global food demand, it is imperative to double the world food production (Singh *et al.*, 2015). Hence, providing sufficient food for an ever-increasing population necessitate diverse strategies and approaches, of which the increased use of PGPB, an inexpensive, enviro-friendly and sustainable approach to enhance sustainable crop production under environmental stress is worth exploring (Ojuederie and Olanrewaju, 2019).

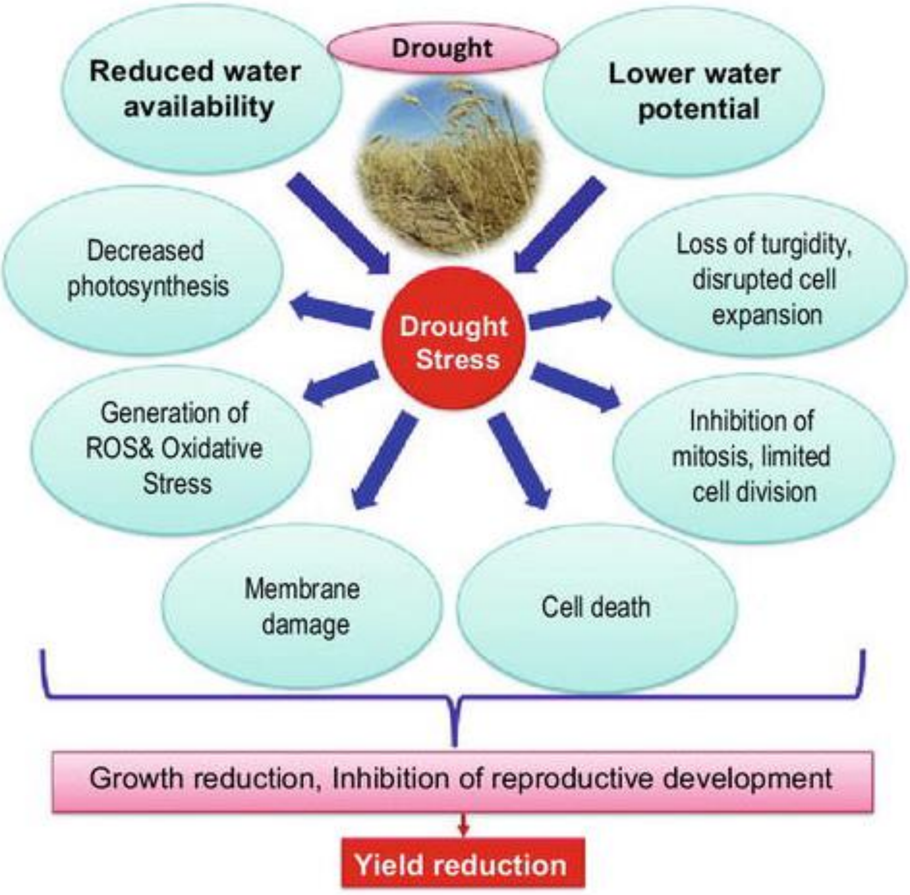


Figure 2.1: A summary of the effect of drought on physiological processes, growth and yield. Adapted from Ali and Akmal (2021).

2.3 Plant microbe interaction as a potential tool for drought mitigation

Arid plants surviving in drought-prone areas are presumed to have microbes conferring beneficial effects, as seen by the association of drought tolerant bacteria with plants adapted to water deficit environments (Eke *et al.*, 2019). Microbes inhabiting the soil rhizosphere of arid environments have the ability to mitigate drought stress through adapting their structural and functional compositions (Bogati and Walczak, 2022).

PGPB are a group of free-living bacteria found in the plant root rhizospheric region, and in endophytic associations (Orozco-Mosqueda, Glick and Santoyo, 2020). They can either directly or indirectly facilitate plant growth in stress conditions (Ali, Sandhya and Rao, 2014; Niu *et al.*, 2018). They aggressively colonize the rhizosphere and have beneficial effects on plants (Jogaiah *et al.*, 2010; Majeed *et al.*, 2015). Abiotic stress factors represent the main contributing factors to the decline in agricultural production globally (Bahlolzada and Hussaini, 2021). They encompass, heat, salinity, flooding, low temperatures, nutrient deficiency and particularly drought (Bahlolzada and Hussaini, 2021; Camaille *et al.*, 2021), whereas biotic stresses are instigated by biological agents such as bacteria, viruses, fungi, and insects (Camaille *et al.*, 2021).

It is documented that soil microbes could induce drought stress tolerance in plants through the production of polysaccharides that enhance soil structure and water holding capacity (Bogati and Walczak, 2022). Furthermore, PGPB-mediated drought tolerance may include root-architecture modification of the plant, the production of ACC deaminase, antioxidants synthesis, and osmolytes accumulation (Fadiji *et al.*, 2022).

Plants produce a range of phytohormones imperative to plant growth, development and abiotic stress tolerance. These phytohormones include ethylene, abscisic acid, cytokinins, gibberellins, and IAA (Vurukonda *et al.*, 2016; Fadiji *et al.*, 2022; Kour *et al.*, 2022). The interaction of plant-microbiome associations in drought conditions is facilitated by the expression of several genes known as *acdS* that encode the enzyme ACC deaminase (Checcucci *et al.*, 2017), a vital enzyme possessed by certain PGPB, to regulate production of ethylene to help plants withstand various stresses (Ali, Sandhya and Rao, 2014). The interaction between plants and microbes has evolved over the years, attributing to the significant role of endophytic bacteria in improving plant survival and adaptation (Wu *et al.*, 2021). Hence, plant microbiome continues to play a significant role in maintaining and enhancing growth and fitness of the hosts under various biotic and abiotic stress conditions (Verma *et al.*, 2021).

2.4 Plant growth promoting bacteria

2.4.1 Functions and mechanisms of plant growth promotion by PGPB

PGPB constitute a diverse group of bacteria dwelling in the plant rhizosphere, epiphytic surfaces and endophytes plant niches microorganisms (Souza, Ambrosini and Passaglia, 2015). Endophytes are microorganisms (bacteria and fungi) that reside in plant tissues without causing any visible harm to the host (El-Deeb, Fayez and Gherbawy, 2013; War Nongkhlaw and Joshi, 2014).

Certain microorganisms such as the PGPB are endowed with the ability to promote plant growth and development (Majeed *et al.*, 2015; Olanrewaju, Glick and Babalola, 2017; Saikia *et al.*, 2018). PGPB achieve this remarkable ability through direct and indirect mechanisms (**Figures 2.2 and 2.3**) (Wang and Ouyang, 2014; Olanrewaju, Glick and Babalola, 2017). Direct mechanisms include the production of auxins (IAA), cytokinin, gibberellin, siderophores, nitrogen fixation and phosphorous solubilisation (Olanrewaju, Glick and Babalola, 2017). These mechanisms encompass plant growth promotion by facilitating nutrient uptake of nitrogen, iron, and phosphorous, coupled with increased phytohormone synthesis (Dutilloy *et al.*, 2022; Fadiji *et al.*, 2022). Indirect plant growth promotion mechanisms include disease suppression through induced systemic resistance (ISR) (Dutilloy *et al.*, 2022) a physiological state of enhanced defence triggered by biological or chemical inducers, and protecting plant tissues indirectly against future attacks by pathogens (Romera *et al.*, 2019). These microbes are also known to produce compounds such as hydrogen cyanide, antibiotics, synthesis of ACC deaminase, and lytic enzymes (Ojuederie and Olanrewaju, 2019).

Additionally, PGPB possessing phosphate solubilising potentials and other PGP traits have enhanced crop growth and yield (Ojuederie and Olanrewaju, 2019). These microbes include those from the genera *Bacillus*, *Rhizobium*, and *Pseudomonas* (Olanrewaju, Glick and Babalola, 2017). Moreover, a recent study by Fadiji *et al*. (2022) showed that multiple plant-associated microorganisms from the genera *Azospirillum*, *Bacillus*, *Enterobacter*, *Methylobacterium*, and *Pseudomonas* enhanced drought tolerance and improved plant growth. In addition, bacteria such as *Bacillus circulans*, *Bacillus megaterium*, *Agrobacterium radiobacter*, *Pseudomonas putida*, and *Enterobacter sakazaki* have been identified to confer drought resistance in plants, with *Pseudomonas* as the most dominant genera found in the rhizosphere and endosphere of most plants, and commonly associated with multiple PGP traits (Naderi *et al.*, 2022).

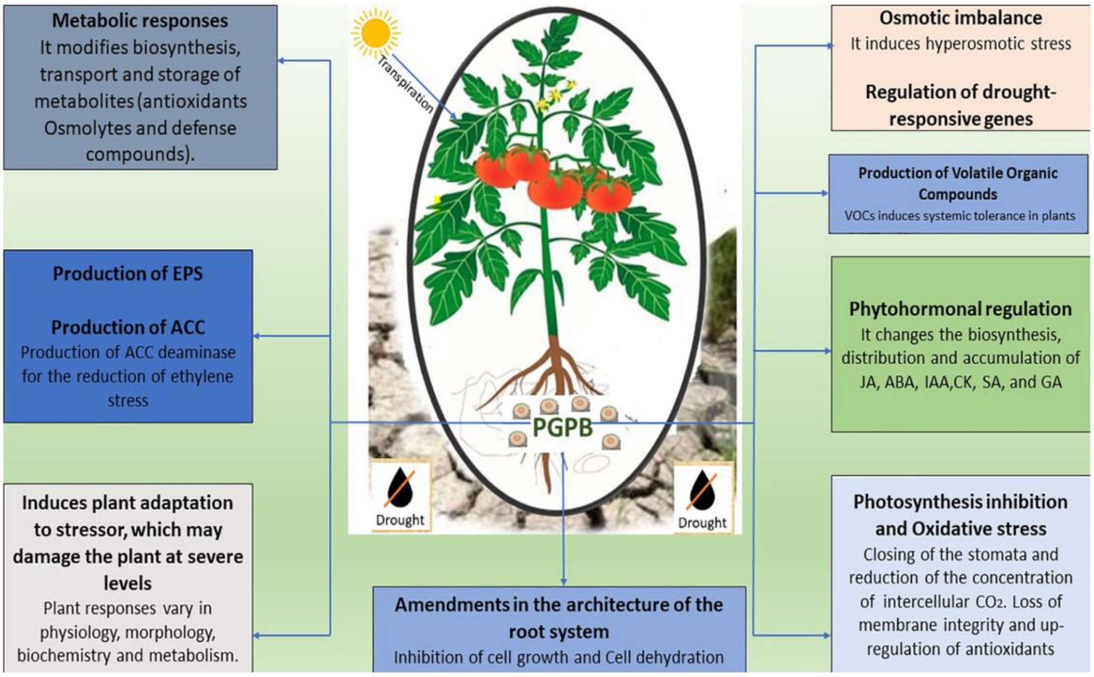


Figure 2.2: Representation of the different potential mechanisms employed by plant growth-promoting bacteria in the amelioration of drought stress in crops. Adapted from Fadiji *et al*. (2022).

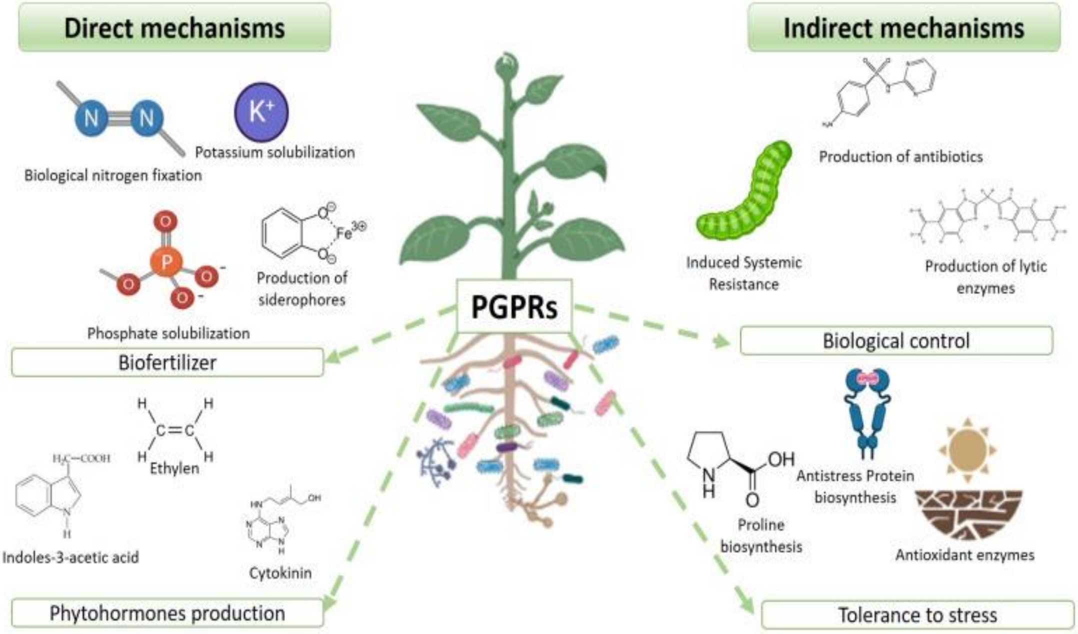


Figure 2.3: Direct and indirect mechanisms employed by beneficial rhizospheric bacteria. Adapted from Gómez-Godínez *et al.* (2023).

2.4.2 Role of PGPB in enhancing abiotic stress tolerance

Owing to their sessile nature, plants are easily confronted with abiotic stresses (Ojuederie and Olanrewaju, 2019), and consequently, plants have to adapt and tolerate severe environmental conditions (Kasim *et al.*, 2013). In order to positively influence plants subjected to abiotic stress, PGPB must induce physiological changes that enhance plant stress tolerance (Nordstedt and Jones, 2020). One effective strategy used by PGPB to enhance stress tolerance in plants is the production of antioxidants, metabolites and osmolytes (Fadiji *et al.*, 2022).

In addition, production of IAA and accumulation of osmolytes such as proline have the potential to improve root growth as a response to drought stress (Abdelaal *et al.*, 2021). Furthermore, accumulation of proline during oxidative stress has previously been linked to drought resistance (Fadiji *et al.*, 2022). Under water stress, proline levels tend to increase during the tillering stage, increasing turgor within cells, thus enabling adaptation to drought stress (Ojuederie and Olanrewaju, 2019). Additionally, the expression of ACC deaminase by PGPB also has the potential to enhance absorption of major nutrients such as Nitrogen (N), Phosphorous (P) and Potassium (K), thereby promoting plant growth under stressful conditions (Abdelaal *et al.*, 2021). Furthermore, drought tolerance may be enhanced by PGPB-mediated production of endogenous phytohormones (Fadiji *et al.*, 2022). Some examples of phytohormone-producing microbes include *Pseudomonas aeruginosa*, *Bacillus endophyticus*, *Bacillus tequilensis* (Riseh *et al.*, 2021), as well as *Rhizobium*, which are drought tolerant and have the ability to alleviate drought stress in crops through various mechanisms (Kour *et al.*, 2022). A key mechanism for PGPB to induce stress tolerance in plants is phytohormone level modification, resulting in enhanced growth (Nordstedt and Jones, 2020). Phytohormones such IAA may mitigate stressful conditions through the formation of lateral roots and root hairs, further increasing water and nutrient uptake (Vurukonda *et al.*, 2016).

In addition, modifications in the elasticity of the root cell membrane by PGPB could improve the stability of the plant cell membrane, further enhancing drought stress tolerance (Vurukonda *et al.*, 2016). As a strategy to enhance abiotic stress tolerance, PGPB therefore, induce ACC deaminase activity directly or indirectly by consuming ACC prior to oxidation by ACC oxidases, resulting in the reduction of ethylene levels (Abdelaal *et al.*, 2021). Also, this stress-induced ethylene in plants may be reduced by PGPB via the cleaving of ACC-by-ACC deaminase (Nordstedt and Jones, 2020).

2.5 IAA activity

PGPB are known to modify phytohormone levels via ACC deaminase and IAA production (Carlos *et al.*, 2016). This has resulted in better crop growth and yield through the production of these phytohormones (Barnawal, Singh and Singh, 2019; Camaille *et al.*, 2021).

Production of IAA stimulates the synthesis of ACC deaminase, an enzyme that hydrolyses ACC, the direct precursor to the plant hormone (ethylene) produced by plants during stress (Asaf *et al.*, 2017). IAA is synthesised and catabolized by soil microorganisms such as *Bacillus japonicum* and *Pseudomonas putida* (Souza, Ambrosini and Passaglia, 2015). IAA is the most active auxin responsible for plant growth and development (Vurukonda *et al.*, 2016).

2.6 ACC deaminase and its role in stress alleviation

2.6.1 ACC deaminase enzyme: function and regulation

Possession of ACC deaminase, a vital pyridoxal phosphate-dependent enzyme ubiquitously found in bacterial and fungal species, is a common trait found among PGPB (Singh and Jha, 2015). This enzyme was first isolated in 1978 from *Pseudomonas* sp. and *Hansenula satrunus* (yeast) and has since been detected in fungi and in a limited number of other bacteria due to its significant role in sustaining plant growth and development under biotic and abiotic stress conditions (Saraf, Jha and Patel, 2010; Ali, Sandhya and Rao, 2014).

According to Saraf, Jha and Patel (2010), this multimeric enzyme has only been found in microorganisms, and is characterized in such way that the substrate ACC must be exuded by plant tissues and subsequently taken up by an ACC deaminase-containing microorganism before it is cleaved.

PGPB expressing ACC deaminase protect plants against the growth inhibition that could result following flooding, high temperature, organic and inorganic toxicants, phyto-pathogens, drought or high salt concentrations (Saraf, Jha and Patel, 2010). Therefore, as a mitigative strategy to circumvent the effects of drought, ACC deaminase regulate ethylene biosynthesis, which inhibit plant growth (Vurukonda *et al.*, 2016). The synergistic interaction between ACC deaminase, the plant and bacterial auxin IAA results in the optimal functioning of the PGPB (Glick, 2014) as illustrated below in **Figure 2.4**.

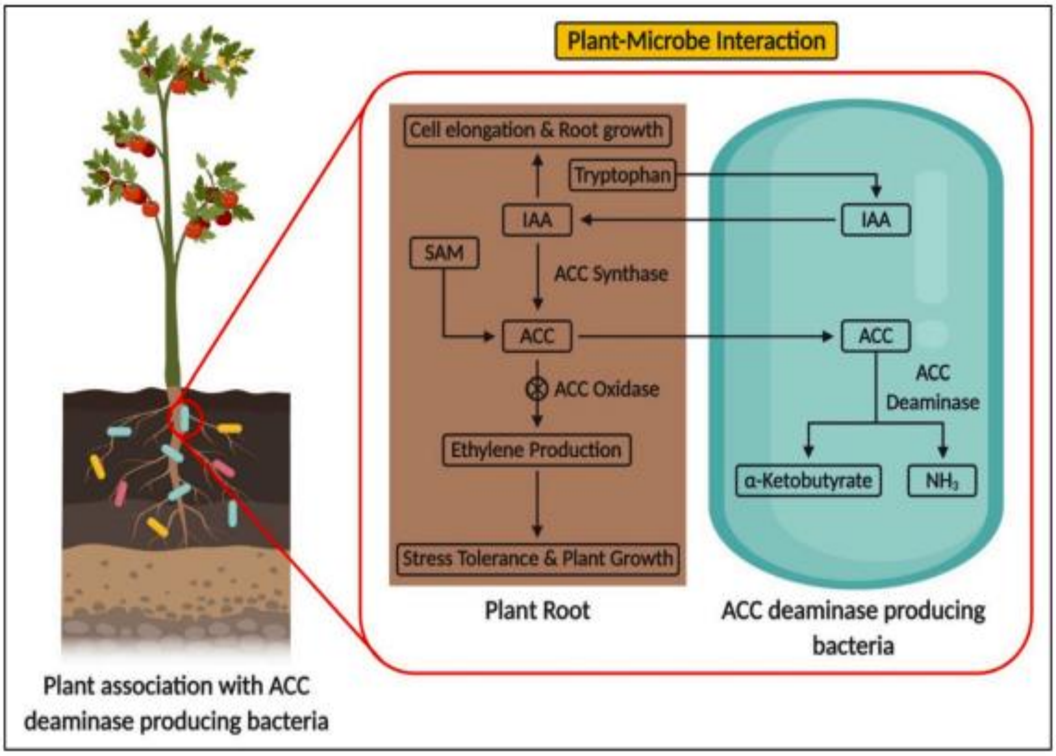


Figure 2.4: Mode of ACC deaminase producing bacteria in mitigating ethylene stress under abiotic stress. Adapted from Murali, Gowtham, Singh, *et al*. (2021).

PGPB possess ACC deaminase, which hydrolyses plant ACC (ethylene precursor) to ammonia and α-ketobutyrate, thereby lowering the level of ethylene under various biotic and abiotic stresses (Ali, Sandhya and Rao, 2014).

2.6.2 Mechanisms of ACC deaminase-mediated stress alleviation

The role of bacterial ACC deaminase under stress conditions includes imparting drought resistance to abiotic stresses (Saraf, Jha and Patel, 2010). As illustrated in **Figure 2.4**, PGPB bind to the surface of the plant in response to tryptophan and other small molecules in the seed or root exudates, further secreting IAA, which is absorbed by the plant (Saraf, Jha and Patel, 2010). Bacterial IAA together with endogenous IAA therefore, stimulate plant cell proliferation and elongation, further activating biosynthesis of ACC from ACC synthase (Saraf, Jha and Patel, 2010;

Glick, 2014; Orozco-Mosqueda, Glick and Santoyo, 2020). However, excess IAA levels could activate transcription of ACC synthase, resulting in high levels of deleterious ethylene in plants (Ojuederie and Olanrewaju, 2019). Additionally, ACC (the immediate precursor of ethylene) is produced via the conversion of S-adenosyl-methionine (SAM) by ACC synthase during plant stress, thereby increasing the levels of ACC concentration (Ojuederie and Olanrewaju, 2019; Orozco-Mosqueda, Glick and Santoyo, 2020).

Plant ACC is then exuded along with various root exudates and assimilated by the PGPB which is further cleaved by bacterial ACC deaminase to form α-ketobutyrate and ammonia (Saraf, Jha and Patel, 2010). Additionally, biosynthesis of ethylene in plants starts with the production of SAM from methionine and Adenosine triphosphate (ATP), which is converted to ACC by ACC synthase, and later converted to ethylene by ACC oxidase (Orozco-Mosqueda, Glick and Santoyo, 2020). Ultimately, ethylene levels are increased (Saraf, Jha and Patel, 2010; Orozco-Mosqueda, Glick and Santoyo, 2020). Consequently, the amount of harmful plant ethylene is reduced, thus increasing plant growth particularly under stress conditions (Orozco-Mosqueda, Glick and Santoyo, 2020; Camaille *et al.*, 2021). When ACC deaminase-containing PGPB are bound to a plant, they act as a sink for ACC ensuring that plant ethylene levels do not increase (**Figure 2.4**) (Saraf, Jha and Patel, 2010), thereby maintaining equilibrium for internal and external plant ethylene (Jaya *et al.*, 2019). The excessive production of ethylene results in unfavourable "stress ethylene", responsible for initiating senescence, chlorosis and leaf abscission, thereby, hindering plant growth and development (Singh *et al.*, 2015).

PGPB endowed with ACC deaminase activity contribute to mitigating ethylene levels and alleviate effects of stress, promote growth, and enhance plant adaptation and survival (Orozco-Mosqueda, Glick and Santoyo, 2020). Subsequently, in times of stress, the levels of ACC are lowered by ACC deaminase-containing PGPB (Ali, Sandhya and Rao, 2014). Arguably, possession of ACC deaminase in bacterial strains is pivotal in facilitating plant growth by relieving plants from the deleterious effects of ethylene (Ali, Sandhya and Rao, 2014; Glick, 2014; Priyanka *et al.*, 2017).

2.6.3 Ethylene and its involvement in plant stress responses

Ethylene is a phytohormone produced by all higher plants and mobilized through plant tissues by passive diffusion (Orozco-Mosqueda, Glick and Santoyo, 2020). This phytohormone is responsible for the regulation of various physiological responses in different developmental stages (such as seed germination, root elongation, and initiation of flowering) in low concentrations (Arshad, Shaharoona and Mahmood, 2008; Munir *et al.*, 2008; Vurukonda *et al.*, 2016; Naing, Maung and Kim, 2021).

Biosynthesis of ethylene is increased by various biotic and abiotic stresses (Vurukonda *et al.*, 2016), hence previously regarded as a ‘stress hormone’ by Munir *et al*. (2008). Ethylene regulates plants response to various biotic and abiotic stress conditions (Ali, Sandhya and Rao, 2014). Therefore, as a response to a variety of stress signals, it is synthesized at an accelerated rate by plants (Arshad, Shaharoona and Mahmood, 2008; Orozco-Mosqueda, Glick and Santoyo, 2020; Naing, Maung and Kim, 2021; Fadiji *et al.*, 2022). This results in the significant increase of endogenous ethylene production, adversely affecting plant growth and development (Ali, Sandhya and Rao, 2014). Additionally, an increase in ethylene levels triggers a series of physiological modifications to the plant such as leaf yellowing, senescence, abscission of leaves, petals and flowers, as well as premature death (Orozco-Mosqueda, Glick and Santoyo, 2020). As observed during drought stress, plant production of ethylene increases until a threshold is reached, subsequently leading to physiological modifications (Camaille *et al.*, 2021).

Despite the aforementioned, ethylene plays a major role in plant growth and development as it regulates drought stress tolerance in various plants (Murali, Gowtham, Singh, *et al.*, 2021). However, during various biotic and abiotic stresses, the levels of the ethylene precursor ACC in plants is decreased by ACC deaminase-containing PGPB (Ali, Sandhya and Rao, 2014; Ojuederie and Olanrewaju, 2019). This reduction of ethylene is thus caused by the degradation of plant ACC by ACC deaminase PGPB, allowing plant tolerance to a variety of stress factors (Murali, Gowtham, Singh, *et al.*, 2021).

2.7 Diversity and potential application of PGPB in agriculture

Achieving high-yielding crops under water shortage has become one of the main challenges for agriculture (Timmusk *et al.*, 2014). It is therefore, important to understand the biological mechanisms employed by plants to adapt to unfavourable environments (Marks *et al.*, 2022). Several studies have focused on plants living in hostile conditions such as the desert, along with their associated microbial communities in order to develop strategies for a sustainable agriculture (Alsharif, Saad and Hirt, 2020). There are beneficial microorganisms inhabiting the roots and the rhizosphere with growth promoting capabilities such as, nitrogen fixation, disease mitigation, enhancing nutrient and water uptake, phosphate solubilization, and production of phytohormones (Tebele, Marks and Farrant, 2023). In addition, studies indicate that PGPB isolated from plants grown in semi-arid agroecosystems possess the ability to alleviate drought stress by producing ACC deaminase (Riseh *et al.*, 2021).

PGPB are known to confer positive influences on plants, which potentially stimulate growth, making them bio-fertilizers due to their ability to invigorate plant growth and enhancing nutrient availability in plants as microbial inoculants (Aloo *et al.*, 2022). Hence, inoculation of these beneficial microbes has the potential to increase germination of seeds, seedling vigour index, and ultimately plant growth as a response to osmotic stress (Fadiji *et al.*, 2022). As a result, use of PGPB for the enhancement of sustainable agricultural production could become a widely accepted practice in many parts of the world (Zahid *et al.*, 2015), especially those faced with harsh environmental constraints affecting agricultural production.

During stressful conditions, PGPB have further manifested their effectiveness by improving growth of various plants such as legumes, cereals, and vegetables (Abdelaal *et al.*, 2021). In addition, these PGPB may increase adaptation to drought stress in crops such as wheat and stimulate plant growth (Vurukonda *et al.*, 2016; Fadiji *et al.*, 2022). Furthermore, successful application of PGPB has shown positive outcomes in various crops such as maize, soybean, barley, and wheat by enhancing drought stress tolerance and promoting growth (Fadiji *et al.*, 2022). However, there is still a large pool of unexplored microbial diversity (Timmusk *et al.*, 2014). This could be attributed to the fact that, drought-tolerant bacteria associated with crop species, which are naturally adapted to drought have not yet been fully explored (Niu *et al.*, 2018), and are yet to be exploited for drought management in agriculture (Eke *et al.*, 2019).

Therefore, any efforts to increase the practical use of PGPB should start with a better understanding of how these bacteria promote plant growth (Glick, 2014), understanding how stress-adapted species could be utilized to improve resilience in crops during harsh environmental conditions (Marks *et al.*, 2022). Furthermore, the understanding of the fundamental mechanisms employed using PGPB could potentially hasten the acceptance of these organisms as suitable and effective adjuncts to agricultural practices (Glick, 2014). Nonetheless, the use of PGPB has steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements (Khan *et al.*, 2016). Therefore, use of PGPB could hold promise for plant growth promotion and alleviation of plant drought stress (Raheem *et al.*, 2018).

PGPB have great potential for biotechnological applications in drought-stressed agricultural systems (Niu *et al.*, 2018) and thus the selection and use of ACC deaminase-producing drought tolerant PGPB, with multiple plant growth promoting activities for the facilitation of plant growth in drought environments is a fertile ground for future research (Ali, Sandhya and Rao, 2014). Some well-known PGPB include organisms belonging to the genera *Pseudomonas*, *Bacillus*, *Azotobacter*, *Klebsiella* (Jogaiah *et al.*, 2010), including *Stenotrophomonas* amongst many others that have been successfully commercialised in attempts to control plant pathogens and used to increase plant growth (Ahemad and Kibret, 2014) as seen in **Table 2.1** below. Therefore, the utilisation of beneficial microorganisms with multiple PGP traits continues to gain importance in managing biotic and abiotic stresses (Vurukonda *et al.*, 2016).

Table 2.1: Plant growth-promoting (PGP) traits of culturable plant growth promoting bacteria (PGPB).

PGPB	Plant growth promoting traits	References
Pseudomonas putida	IAA, siderophores, HCN, ammonia, systemic resistance, exopolysaccharides, phosphate solubilisation	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014; Eid *et al.*, 2021)
***Klebsiella* sp.**	IAA, siderophores, HCN, ammonia, systemic resistance, exopolysaccharides, phosphate solubilisation Phosphate solubilization, Nitrogen fixation, IAA	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014) (Di Benedetto *et al.*, 2017; Eid *et al.*, 2021)
***Rhizobium* sp.**	IAA, siderophores, HCN, ammonia, exopolysaccharides, systemic resistance Nitrogen fixation, Phosphate solubilization	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014; Eid *et al.*, 2021)
***Bacillus* sp.**	IAA, siderophores, HCN, ammonia, systemic resistance	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014; Fadiji *et al.*, 2022)
Pseudomonas fluorescens	ACC deaminase, phosphate solubilization, systemic resistance	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014; Di Benedetto *et al.*, 2017; Fadiji *et al.*, 2022)
***Enterobacter* sp.**	ACC deaminase, IAA, siderophore, phosphate solubilisation, systemic resistance	(Jogaiah *et al.*, 2010; Ahemad and Kibret, 2014; Fadiji *et al.*, 2022)
Pseudomonas fluorescence	ACC deaminase, IAA, Siderophore, Antifungal activity IAA Phosphate solubilization	(Souza, Ambrosini and Passaglia, 2015; Eid *et al.*, 2021) (Di Benedetto *et al.*, 2017) (Çakmakçı *et al.*, 2017)
***Rhizobium* spp.**	Nitrogen fixation, IAA, Siderophores, Phosphate solubilization ACC deaminase	(Souza, Ambrosini and Passaglia, 2015; Eid *et al.*, 2021) (Saraf, Jha and Patel, 2010)
Bacillus subtilis	Nitrogen fixation, biocontrol activity	(Souza, Ambrosini and Passaglia, 2015)
Bacillus amyloliquefaciens	Siderophore, IAA, ACC deaminase Phosphate solubilization, IAA, Antifungal activity	(Souza, Ambrosini and Passaglia, 2015) (Eid *et al.*, 2021)
***Pseudomonas* spp.**	Siderophores, IAA, ACC deaminase, Nitrogen fixation, Phosphate solubilization, antibiotic production, salt tolerance, trace metal tolerance	(Çakmakçı *et al.*, 2017; Bahlolzada and Hussaini, 2021; Eid *et al.*, 2021)
Bacillus paralicheniformis	Nitrogen fixation	(Eid *et al.*, 2021)

Table 2.1
(continued).

Bacillus **sp.**	IAA, ACC deaminase, amylase, phosphate solubilization, nitrogen fixation	(Di Benedetto *et al.*, 2017; Eid *et al.*, 2021)
	HCN, Siderophore, Ammonia	(Çakmakçı *et al.*, 2017)
Staphylococcus	IAA, Nitrogen fixation, ACC deaminase	(Eid *et al.*, 2021)
Bacillus velezensis	Biocontrol activity	(Eid *et al.*, 2021)
Burkholderia phytofirmans	ACC deaminase, Siderophores	(Di Benedetto *et al.*, 2017; Bahlolzada and Hussaini, 2021)
Acinetobacter **spp.**	ACC deaminase	(Di Benedetto *et al.*, 2017)
	IAA, Siderophores, Phosphate solubilization, Antibiotic production	(Eid *et al.*, 2021)
Pseudomonas putida	ACC deaminase	(Saraf, Jha and Patel, 2010)
	IAA	(Di Benedetto *et al.*, 2017)
Agrobacterium **spp.**	ACC deaminase, IAA	(Di Benedetto *et al.*, 2017)
	Nitrogen fixation, Siderophores, Phosphate solubilization, antibiotic production	(Eid *et al.*, 2021)
Serratia	ACC deaminase, Phosphate solubilization	(Çakmakçı *et al.*, 2017; Di Benedetto *et al.*, 2017)
	IAA, Nitrogen fixation	(Eid *et al.*, 2021)
Kosakonia	IAA, Nitrogen fixation	(Eid *et al.*, 2021)
Kosakonia cowardii	Antagonistic activity against fungi	(Eid *et al.*, 2021)
Azotobacter	Phosphate solubilization, Nitrogen fixation, Antifungal activity	(Çakmakçı *et al.*, 2017; Di Benedetto *et al.*, 2017)
Stenotrophomonas rhizophila	IAA, Siderophores, Phosphate solubilization, Antibiotic production, ACC deaminase	(Eid *et al.*, 2021)

2.8 Wheat as an economically important agricultural crop

Wheat belongs to the genus *Triticum*, which has approximately 300 species adapted to a diverse range of habitats (Dutilloy *et al.*, 2022). Two major species of wheat stand out, namely the common wheat (*Triticum aestivum*) mainly grown in northern and eastern Europe, and durum wheat (*Triticum durum*), which thrives in warmer regions (Dutilloy *et al.*, 2022). *Triticum aestivum* L. is the most widely grown and consumed cereal worldwide (Mahpara, Hussain and Farooq, 2015). It is crucial globally as a staple food crop encompassing primary nutriment of calories and proteins in both human food animal (Dutilloy *et al.*, 2022). In addition, 85% of the global population depends on wheat for basic calories and 82% for protein intake (Caverzan, Casassola and Brammer, 2016). However, wheat production has declined in various parts of the world due to increasing water stress due to persistent rainfall reduction and drought (Mishra and Singh, 2010). According to FOASTAT data, between the year 2000 and 2010, there was a decline in global production share of wheat in regions such as Europe, Americas and Oceania, with 0.6%, 3.4%, and 19%, respectively (FAO, 2023). Consequently, wheat production demands are thus expected to increase in tandem with the rise in global population (Ilyas *et al.*, 2020).

2.8.1 Wheat cultivation in Southern Africa

Due to the increase in population coupled with increased change in food habits and urbanization, there has been a surge for wheat demand in the sub-Saharan region (Tadesse, Bishaw and Assefa, 2019). Africa produces more than 25 million tons of wheat accounting for 3.3% of the total wheat production globally (Tadesse, Bishaw and Assefa, 2019; NAB, 2021). In sub-Saharan Africa, wheat production stands at 7.5 million tons, accounting for 40% of the total continental wheat production (Tadesse, Bishaw and Assefa, 2019). The most important wheat producing countries in Southern Africa include South Africa, Zimbabwe, and Zambia, in descending order of production (NAB, 2021). South Africa is the biggest producer of wheat in Southern Africa, with an estimated projection of 2.148 million tons of wheat for the 2019/2020 season (NAB, 2021). However, wheat production has experienced a decline in Southern Africa because of drought, especially in South Africa. This situation has resulted in the importation of over one million tons of wheat during the 2014-2016 drought period (Ruwanza, Thondhlana and Falayi, 2022). According to NAB (2020), in Namibia, local wheat production is grown under 1011 hectares of land. Local production is at 11% while 89% of the domestic wheat requirements is imported (NAB, 2021).

2.8.2 Wheat production challenges and constraints

Wheat is the most cultivated staple food crop (Faisal, Mujtaba and Mahboob, 2019), with global production estimated at 765 million tons in 2020 (Dutilloy *et al.*, 2022). However, daily increase in global population has inferred major challenges that has compromised agricultural productivity and food security at large (Akbari, Akbari and Golakiya, 2016). Wheat production in sub-Saharan Africa has been constrained by various biotic and abiotic stress factors accentuated by the onset of climate change including climate-induced high temperatures, erratic rainfall, flooding, pests and diseases, limited availability and high price of inputs such as fertilizers. Wheat production in developing countries is estimated to decrease by as much as 20-30% (Tadesse, Bishaw and Assefa, 2019). It is predicted that in order to meet market demands in the next 10 years, 60% increase in wheat production is required (Kasim *et al.*, 2013; Tadesse, Bishaw and Assefa, 2019).

As one of the most essential cereal in the world, wheat is grown in a wide range of environmental conditions, salinity and heat (Kizilgeci *et al.*, 2017), with nearly 50% of the wheat area cultivated subjected to periodic droughts (Fahad *et al.*, 2017). Cereals such as wheat are highly sensitive to drought alongside various biotic and abiotic stresses, particularly during their developmental stages (Zia *et al.*, 2021; Dutilloy *et al.*, 2022). Fungal diseases such as *Fusarium* and Net blotch inflict 30-70% and 50% yield losses respectively as they target various plant organs (Dutilloy *et al.*, 2022). Net blotch, the *Ascomycetes* fungus, causes significant economic losses with damage to leaves, stems, and kernels (Dutilloy *et al.*, 2022).

Recurrent drought conditions associated with climate change have become one of the principal constraints to global wheat production (Zia *et al.*, 2021), resulting in 57% yield reduction (Fahad *et al.*, 2017). Wheat is often susceptible to drought, adversely affecting both yield and seed quality, consequently destabilizing wheat production (Akbari, Akbari and Golakiya, 2016). Recurrent droughts have resulted in significant decline in wheat production, loss of revenue and food price speculations (Ruwanza, Thondhlana and Falayi, 2022).

Countries in Southern Africa including Namibia do not meet wheat domestic demands leading to relying on imports (NAB, 2021). Namibia's agricultural sector grapples with various abiotic threats such as drought and floods (Koroma, 2016). In addition, climate variability inevitably causes fluctuations in the performance of agricultural production (Shifiona, Dongyang and Zhiquan, 2016). Consequently, this is likely to perpetuate the country's dependence on imports in order to meet its wheat domestic consumption needs (Shifiona, Dongyang and Zhiquan, 2016). Hence the need to identify microorganisms capable to mitigate drought in wheat growing in arid and semi-arid environments.

2.8.3 Drought stress and its impact on wheat germination and early growth

As one of the major food staples globally, the yield of wheat has been adversely affected by recurrent droughts (Guo *et al.*, 2017; Ojuederie and Olanrewaju, 2019). This has thus resulted in the decline of wheat productivity due to the negative effects of drought stress (Sharma *et al.*, 2022).

Drought-induced stress has the ability to constrain growth and development of wheat through reduced seed germination, shoot length, root length, and the tillering stage (Duan *et al.,* 2017; Kizilgeci *et al.,* 2017; Sharma *et al.*, 2022; Evamoni *et al.*, 2023). Seedling emergence in wheat is one of the most sensitive and critical developmental stages impaired by water stress (Kizilgeci *et al.,* 2017; Turkoglu *et al.,* 2022; Evamoni *et al.*, 2023). Furthermore, the vegetative stage of wheat is significantly affected by inadequate water, ultimately affecting yield and the yield attributes (Ali & Akmal, 2021).

Moreover, drought stress inhibits mitosis, negatively affecting elongation and cell division in wheat, and consequently affecting cellular differentiation and ultimately plant growth (Abdelaal *et al.*, 2021; Camaille *et al.*, 2021). Water shortages in wheat continue to affect plant growth, ultimately triggering a delay in germination (Camaille *et al.*, 2021). This is attributed by the fact that wheat is known to experience drought stress at various growth stages, particularly during germination (Kizilgeci *et al.*, 2017), with seed germination as one of the most critical phase in a plant’s life cycle (Kizilgeci *et al.*, 2017; Ghosh, Shahed and Robin, 2020).

A study by Duan *et al.* (2017) demonstrated that germination rate decreases with increased drought frequency. In support of this, Kizilgeci *et al.* (2017) reiterated that germination percentage decreases with high drought stress. This was demonstrated in wheat cultivars, where germination rate significantly decrease with increased drought simulation using PEG 6000 (Duan *et al.*, 2017). Furthermore, during seed germination, amylase enzymes play a significant role by hydrolysing endosperm starch into a soluble sugar, which potentially provide energy for the growing seed (Abido and Zsombik, 2020). However, during water stress, amylase enzyme is reduced, further inhibiting germination and early seedling growth.

2.8.4 Physiological responses of wheat to drought stress in seed germination and early seedling

Plants have employed strategies such as drought tolerance to protect themselves against water deficits (Osmolovskaya *et al.*, 2018). It is observed that crops such as wheat are severely affected during the reproductive or flowering stages of growth in contrast to the vegetative stage, which invariably affects the yield (Ojuederie and Olanrewaju, 2019). Photosynthetic rate and stomatal conductance are reduced due to low carbon dioxide under severe and moderate water stress, and accrual of compatible solutes such as proline, which act as osmo-protectants, shielding cells from dehydration and aiding the detoxification of stressed cells from levels of reactive oxygen species (ROS) (Ojuederie and Olanrewaju, 2019). Lastly, some wheat cultivars respond by high water use efficiency during drought stress (Fahad *et al.*, 2017).

2.8.5 Wheat and the impact of global climate crop production

Wheat is typically grown and cultivated under rain-fed conditions and is often prone to water deficits (Caverzan, Casassola and Brammer, 2016; Salem *et al.*, 2018). As such, the primary constraint limiting wheat production in semi-arid regions is moisture availability (Mahpara, Hussain and Farooq, 2015). Global climatic changes coupled with increased water shortage have a strong influence on wheat production (Mahpara, Hussain and Farooq, 2015), with recent studies indicating about 40% of yield reductions in wheat due to the impact of global drought (Fahad *et al.*, 2017). Additionally, continuous exposure to various biotic and abiotic stress factors may affect wheat and other crops by reducing plant growth and productivity (Ilyas *et al.*, 2020). Hence, climate variability is the common limiting factor that affects wheat production in Namibia (NAB, 2021), due to the large arid and semi-arid climatic region that constitutes the country (Koroma, 2016).

2.8.6 Mechanisms of adaptation to drought stress by plants

In order to survive hostile conditions, plants acclimatize themselves to hostile conditions upon experiencing abnormalities in their normal physiological function (Vurukonda *et al.*, 2016; Ojuederie and Olanrewaju, 2019; Bashir *et al.*, 2021). Currently, efforts aimed at increasing the plants' ability to tolerate drought stress and enhancing the production of high yielding drought resistant crops involve the use of water-saving irrigation, conventional plant breeding, and genetic engineering of drought-tolerant transgenic plants (Ngumbi and Kloepper, 2016; Niu *et al.*, 2018). However, these methods are labour intensive and difficult to apply in practice (Ngumbi and Kloepper, 2016; Niu *et al.*, 2018).

Drought is the most limiting factor affecting crop production in arid and semi-arid regions globally (Kizilgeci *et al.*, 2017), and is considered to affect the metabolism of plants at different developmental stages (Evamoni *et al.*, 2023). Due to the adverse impact of drought on crops, plants have therefore employed specific mechanisms to circumvent water stress, which include modification of various physiological, biochemical and molecular processes occurring at cellular level (Bashir *et al.*, 2021). Therefore, causing the plant to enter physiological phases such as adaptation, avoidance and tolerance for survival (Shaffique *et al.*, 2022).

Plants may under-go a period of inactivity prior to drought commencement (Vurukonda *et al.*, 2016; Ojuederie and Olanrewaju, 2019), and complete its life cycle before the start of the dry season (Ojuederie and Olanrewaju, 2019). This is in addition to growth arrest, which involves restricting shoot development to reduce metabolic demands of the plant, and drought escape, which involves early flowering (Bashir *et al.*, 2021). In addition, plants could under-go drought avoidance, which involves the capacity to withstand any kind of water status e.g., having an expensive root system to enhance water uptake (Ojuederie and Olanrewaju, 2019; Bashir *et al.*, 2021). Furthermore, plants may produce compatible solutes during reduced water potential in order to sustain its metabolic activities (Ojuederie and Olanrewaju, 2019). In order to escape, tolerate or adapt to drought stress, some desert plants keep seeds in a dormant state and only germinate when water is available (Alsharif, Saad and Hirt, 2020).

Metabolic changes and gene expression may occur within the plant as a response to drought, further regulating the mechanisms of adaptation (Ojuederie and Olanrewaju, 2019; Bashir *et al.*, 2021; Seleiman *et al.*, 2021). In addition, metagenomic analysis of rhizospheric microorganisms indicate that changes at the genome level enables microbes to cope with drought stress by enhancement of functional genes encoding for ACC deaminase (Bogati and Walczak, 2022), a cytoplasmic enzyme that is encoded by the *acdS* gene (Jaya *et al.*, 2019), which is known to reduce harmful effects of ethylene synthesis when expressed (Singh *et al.*, 2015). Additionally, there may be genes responsible for adaptation and tolerance, which may encode for enzymes involved in the biosynthesis of osmolytes, antioxidants, and stress-induced proteins (Ojuederie and Olanrewaju, 2019).

Furthermore, in order to adapt to drought stress, plants produce and accumulate solutes such as proline for osmotic adjustment as manifested in cereal crops such as wheat, maize, and barley (Ojuederie and Olanrewaju, 2019; Alsharif, Saad and Hirt, 2020; Bashir *et al.*, 2021). This strategy helps to limit loss of water by modifying osmotic potential to maintain osmolarity and avoid dehydration (Camaille *et al.*, 2021). Proline, a prominent osmolyte that act as an osmo-protectant is produced during drought stress and is known to scavenge free radicles, ROS, thus reducing the damage of oxidative stress induced by drought, and stabilizing sub-cellular structures (Ábrahám, Hourton-cabassa and Erdei, 2010; Ilyas *et al.*, 2020). Moreover, proline has a protective role in the adaptation of plants, making it the most compatible osmolyte in plants under stress (Jain *et al.*, 2013). This is due to its ability to hydrate and bind to proteins, thus stabilizing biological macromolecules and maintaining their cell membrane structures (Bogati and Walczak, 2022).

PGPB are known to effectively mitigate drought stress in wheat plants (Shaffique *et al.*, 2022). However, mechanisms of wheat seedling in response to drought stress is complex (Duan *et al.*, 2017), with varied responses by morphological and physiological features in the wheat assumed to be attributable to differences in genotypes and varieties (Sharma *et al.*, 2022). Therefore, in order to have vital and efficient wheat genotypes, it is imperative to select drought resistant genotypes during early developmental stages (Evamoni *et al.*, 2023).

The above-mentioned strategies are all essential for the sustenance of plants under various stresses. However, the degree of drought stress experienced by the plant determines the drought mitigation strategy (Vurukonda *et al.*, 2016; Ojuederie and Olanrewaju, 2019). Consequently, more studies are required to understand various adaptive mechanisms evolved in these organisms (Bogati and Walczak, 2022).

2.8.7 Previous studies on PGPB and drought tolerance in wheat plants

Previous investigations into ACC deaminase-producing *Klebsiella* strains, isolated from desert environments, have primarily focused on their interaction with wheat seedlings. These studies have revealed a noteworthy augmentation in plant biomass when subjected to salt stress conditions, thereby supporting the notion of desert-adapted PGPB to bolster plant salt stress tolerance (Alsharif, Saad and Hirt, 2020). This adaptability extends the potential applicability of these PGPB to potentially mitigate various other abiotic stressors.

Moreover, parallel research involving PGPB strains isolated from xerophytic plants, such as *Acacia* species, has revealed their potential to enhance wheat yields under drought stress conditions (Riseh *et al.*, 2021). Notably, studies focused on the inoculation of wheat plants under drought stress with PGPB strains, particularly those belonging to the *Pantoea* genus, have demonstrated their efficacy in promoting wheat growth while concurrently reducing proline levels—a key indicator of drought stress tolerance (Alsharif, Saad and Hirt, 2020). Moreover, *Pantoea's* ability to enhance growth of wheat seedlings under drought conditions align with previous studies (Verma *et al.*, 2021).

Furthermore, PGP bacterial strains belonging to the *Bacillus*, *Enterobacter*, and *Pseudomonas* genera that have been isolated from xerophytic plants have revealed the ability to improve yield of wheat under drought stress conditions (Riseh *et al.*, 2021). It was also reported that *Bacillus* spp. improved wheat photosynthetic capacity due to their siderophore producing abilities, mitigating iron deficiency in plants and lessen oxidative stress by protecting IAA from oxidative damage (Mahpara, Hussain and Farooq, 2015). Under drought stress, treatment of wheat plants with *Bacillus* spp. has been reported to yield a 78% higher biomass plants and an increase in shoot length in contrast to non-treated, confirming the potential of

PGPB to enhance plant performance under drought stress conditions (Trivedi *et al.*, 2017).

Siderophore producing *Azospirillum* strains are resistant to drought stress and used as inoculants for wheat to alleviate drought stress and improve plant growth and yield (Bogati and Walczak, 2022). Application of *Azospirillum* strains on wheat yields in both greenhouse and field conditions has exhibited beneficial effects upon inoculation, yielding 30% grain increase (Hayat *et al.*, 2010). Furthermore, inoculation of wheat plants with PGPB such as *Pseudomonas* has previously exhibited to increase biomass and root length, with proline levels increasing by twofold, as well as increased levels of auxin (Fadiji *et al.*, 2022).

2.9 The use of Polyethylene glycol (PEG 6000) drought simulation to screen tolerance in wheat

The effects of drought stress on plants varies across different tissues and depends on the duration, intensity and plants development stage (Faisal, Mujtaba and Mahboob, 2019; Persic *et al.*, 2022; Licaj *et al.*, 2023). Germination and seedling establishment are the most vulnerable stages of plant growth and development (Persic *et al.*, 2022).

When developing drought-resistant cultivars during water limitations, it is necessary to test the performance of all varieties at different stages and intensities of drought (Persic *et al.*, 2022). However, selecting wheat genotypes with drought tolerance capabilities has become critical especially because of the challenges in maintaining consistent and controlled drought field conditions (Faisal, Mujtaba and Mahboob, 2019). Understanding the molecular basis of how wheat varieties respond to water stress is fundamental to improve tolerance, helping with the selection and characterization of drought-tolerant wheat varieties to help identify peculiar traits that could be used as markers in breeding programs (Licaj *et al.*, 2023).

In vitro screening techniques have thus proved to be effective in selecting drought tolerant varieties by use of PEG 6000 (Faisal, Mujtaba and Mahboob, 2019). PEG 6000 is a non-ionic water polymer that mimics an osmotica by reducing water potential, with the ability to penetrate into plant tissues creating water stress via the outward flow of water from plant tissues to the concentrated PEG (Duan *et al.*, 2017; Faisal, Mujtaba and Mahboob, 2019; Ghosh, Shahed and Robin, 2020; Evamoni *et al.*, 2023). PEG 6000 induces physiological drought, and is commonly used to induce drought under controlled laboratory conditions (Faisal, Mujtaba and Mahboob, 2019; Ghosh, Shahed and Robin, 2020) as well as to screen out drought resistant varieties at the early stage of development (Ghosh, Shahed and Robin, 2020).

The use of PEG 6000 has been used to simulate environments of plants such as wheat, rice, and maize in an affordable and efficient way to help screen and select drought tolerant genotypes (Duan *et al.*, 2017; Sharma *et al.*, 2022; Evamoni *et al.*, 2023). Lastly, PEG-induced drought has previously been used to develop and improve screening methods for identifying and evaluating functional relationships useful for adaptation of winter wheat drought-tolerant cultivars (Persic *et al.*, 2022).

2.10 The desiccation-tolerant *Myrothamnus flabellifolius* (Welw.)

2.10.1 Botanical description, habitat and distribution

Myrothamnus flabellifolius (Welw.) (Gunnerales, Myrothamnaceae) is a dioecious woody shrub of height between 0.5 m and 1.5 m., with a distinctive aromatic fan-like wedge-shaped leaves (**Figures 2.5 and 2.6**) (Viljoen *et al.*, 2002; Moore *et al.*, 2007, 2011). The plant’s most remarkable feature is the ability of the leaves to fold parallel to the leaf face upon dehydration due to the unique arrangement of alternating ridges and furrows (Viljoen *et al.*, 2002; Moore *et al.*, 2007). The plant is widely distributed in Southern Africa, thriving in regions with low summer rainfall, nutrient-poor rocky inselbergs and sandy soils (Viljoen *et al.*, 2002; Huang *et al.*, 2020). In addition, it may be described as a geophyte, with its extensive root system, which extends into the rock crevices upon which it grows (Moore *et al.*, 2007).

The occurrence of *M. flabellifolius* in Namibia demonstrates its clear preference for regions characterized by unpredictable rainfall patterns, with some places receiving annual rainfall as low as ±238 mm (Bentley, Moore and Farrant, 2019). Moreover, the plant's distribution is fragmented, particularly along the western coastlines of Namibia (Moore *et al.*, 2006). The plant has three subspecies, namely *M. flabellifolia sensu stricto*, *M. flabellifolia elongate* and *M. flabellifolia robusta*, with the former two subspecies found inhabiting the Kalahari Desert (Moore *et al.*, 2007).

Figure 2.5: *Myrothamnus flabellifolius* inhabiting rock crevices. Adapted from (Bentley and Farrant (2020).

Figure 2.6: Aerial parts of *M. flabellifolius* **(a**), **(b)** and **(c)**. dehydrated **(d)** and **(e)** hydrated parts of the plant. Adapted from Erhabor *et al.* (2020).

2.10.2 Mechanisms underlying drought tolerance in *M. flabellifolius* (Welw.)

Drought tolerance is a complex trait controlled by multiple genes, production of ROS and osmolytes, metabolic adaptations, gene transcription and hormonal signalling (Licaj *et al.*, 2023). *Myrothamnus flabellifolius* is known to be the most primitive angiosperm with extreme drought tolerance, found in Central and Southern Africa (Moore *et al.*, 2006; Huang *et al.*, 2020). This remarkable resurrection plant can tolerate desiccation for up to 12 months due to its woody stem (Tebele, Marks and Farrant, 2023). Recent studies on resurrection plants have demonstrated that plant desiccation tolerance may be attributed to the accumulation of sugars such as sucrose, large scale production of proteins, and adjustment of cell structures such as the cell wall (Farrant, 2000; Zhang and Bartels, 2018). Upon dehydration, a series of physiological activities, such as photosynthesis and respiration are rearranged, of which resurrection plants reduce their RWC, resulting in leaf cells losing water and the accumulation of protective metabolites at a significantly higher speed (Zhang and Bartels, 2018).

There are various mechanisms that protect resurrection plants, particularly *Myrothamnus flabellifolius* from desiccation. Some of the drought tolerance mechanisms include closing of leaves in a fan-like manner during desiccation (Tebele, Marks and Farrant, 2023), leaf folding to shade leaves from light during dehydration, production of anthocyanins, which prevent light-chlorophyll interaction (both preventing photosynthesis from taking place), cell wall folding to prevent development of tension within cells, which could result in plasma membrane rupture, and lastly, production of antioxidants to scavenge ROS free radicles produced by unregulated metabolic activities (Farrant, 2000). The presence of anthocyanins in the plant is believed to decrease the osmotic potential of the leaves and stomatal conductance, thereby minimising water loss via transpiration during hot conditions (Bentley, Moore and Farrant, 2019).

The plant has an extensive root system that enables it to extends around and into rocks (Tebele, Marks and Farrant, 2023), allowing roots to intercept and absorb water (Moore *et al.*, 2007). Additionally, during water stress, respiration is less affected and its continuation to low water content is necessary to provide energy for the acquisition of subcellular protection (Farrant, 2000). During the dry state, chlorophyll is retained and protective pigments such as phenols (3,4,5 tri-O-galloylquinic acid) are synthesized to protect the membranes of the plant from desiccation and prevent photo-oxidative damage (Jaspal *et al.*, 2018). Apart from the physiological and morphological changes, root microbes minimize drought induced damage to the plant host via mobilization of ROS scavenging systems and producing anti-oxidative and protective metabolites (Bentley, Moore and Farrant, 2019; Tebele, Marks and Farrant, 2023) resulting in drought tolerance improvement.

2.10.3 Rationale for plant selection: mechanisms for tolerance

Myrothamnus flabellifolius is a medicinal plant that has been widely used in Africa, specifically due to production of secondary metabolites that could be synthesised from endophytic bacteria with growth promoting properties (Erhabor *et al.*, 2020). In addition, PGPB such as *Bacillus* have previously been associated with *Myrothamnus* leaf surfaces of the plant, colonizing the epidermal surfaces of the flowers (Moore *et al.*, 2011). Hence its desiccation tolerance is presumed to be attributed to bacteria that have adapted to the harsh environmental conditions the plant is subjected to. This makes the plant microbiome a reservoir for potential microbes to be utilised and engineered in agricultural practices for developing drought tolerant crops (Orozco-Mosqueda, Glick and Santoyo, 2020). Most research on *M. flabellifolius* was centred around leaf phytochemical properties, medicinal value, as well as the physiological and biochemical mechanisms of desiccation tolerance (Tebele, Marks and Farrant, 2023).

According to a study by Wu *et al.* (2021), there has been relatively less research on medicinal plants and their microbial communities, particularly endophytes and thus more future research is expected to focus on these microbes. Also, these microbes are unexplored and could have the potential to mitigate abiotic stress in agricultural crops (Eke *et al.*, 2019). Lastly, the mechanisms associated with drought tolerance of *M. flabellifolius* are largely unknown and could potentially be used in agriculture to develop and improve drought tolerant crops (Tebele, Marks and Farrant, 2023).

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CHAPTER 3: DESERT ADAPTED *MYROTHAMNUS FLABELLIFOLIUS* Welw. RHIZOSPHERE AND ENDOPHYTIC BACTERIA EXHIBIT DROUGHT-TOLERANCE PLANT GROWTH-PROMOTING TRAITS

ABSTRACT

Myrothamnus flabellifolius Welw. is a desiccation-tolerant medicinal plant found in Southern Africa with the ability to revive and resurrect upon exposure to water. Various strategies have been employed to increase drought tolerance in plants. One strategy involves the use of bacteria with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, exerting beneficial effects onto plants under various abiotic stress conditions by reducing stress ethylene levels, and resulting in better plant growth. Therefore, the main aim of the study was to isolate, identify, characterize potential rhizospheric and endophytic bacteria with ACC deaminase activity from *Myrothamnus flabellifolius* and screen them based on plant growth promoting (PGP) traits such as production of indole-3-acetic acid (IAA), siderophore, hydrogen cyanide (HCN), nitrogen fixation, ammonia, and phosphate solubilisation. A total of 33 isolates were identified by sequencing the 16S rRNA gene region. BLAST analysis showed that the isolates belonged to 10 different genera, with *Bacillus* being the most abundant with 36.4%. All characterized isolates tested positive for ACC deaminase activity, with the highest level of α-ketobutyrate produced via deamination of ACC observed in *Bacillus licheniformis* (R13). The highest ACC deaminase activity values were exhibited by *Bacillus licheniformis* (R13), *Enterobacteriaceae* (LA3) and *Staphylococcus hominis* (LB3) with 0.0013 ± 0.01 mM, 0.0010 ± 0.00 mM, and 0.0010 ± 0.00 mM respectively. These findings suggest that the presence of ACC deaminase activity coupled with multifarious growth promoting traits in various bacterial strains possess the potential to serve as an effective alternative to promote plant growth in extreme environmental conditions. Additionally, this study illustrated that strains isolated from extreme environments have the ability to grow and withstand drought conditions.

Keywords: ACC deaminase, endophytic, drought tolerance, *Myrothamnus flabellifolius*

3.1 INTRODUCTION

Desert plants such as *Myrothamnus flabellifolius* Welw. are commonly termed resurrection plants (Moore *et al.*, 2006; Nantapo and Marume, 2022). They can display desiccation tolerance and resume normal physiological activities such as photosynthesis upon rehydration without suffering permanent injury (Moore *et al.*, 2006; Nantapo and Marume, 2022). *Myrothamnus flabellifolius* is found in Southern Africa, namely; South Africa, Botswana, Namibia, and Zimbabwe (Erhabor *et al.*, 2020) as well as in arid and semi-arid habitats (Farrant and Kruger, 2001; Moore *et al.*, 2011). In Namibia, these woody shrubs inhabit rocky inselbergs (Moore *et al.*, 2007) due to the presence of an extensive root system that penetrates the rock crevices and well adapted to capture water draining into the hollows of the rock (Erhabor *et al.*, 2020). Additionally, due to their phenolic properties, the leaves and twigs of *Myrothamnus flabellifolius* have been used in traditional and commercial teas and cosmetics (Bentley, Moore and Farrant, 2019).

Desert plants have developed strategies to adapt to these extreme environmental conditions by use of their microbiota (Eida *et al.*, 2018; Alkahtani *et al.*, 2020). However, there is a limited understanding of the relationship between desert plants such as *M. flabellifolius* and their associated microbiota (Moore *et al.*, 2011). A study previously conducted by (Li, Cheng and An, 2017) on a desert plant such as *Lepidium perfoliatum*, has revealed that their rhizospheric and endophytic bacteria could mitigate various stresses including salt, drought, and heat, providing significant benefits to the host plant.

Plants release root exudates into the rhizosphere as a source of carbon, recruiting soil microorganisms, which later form the root and endophytic microbiome (Eida *et al.*, 2018). These microorganisms colonize the rhizosphere (Jogaiah *et al.*, 2010; Zahid *et al.*, 2015), internal tissues (endophytes) and the surface of leaves and stems (epiphytes) (Orozco-Mosqueda, Glick and Santoyo, 2020) throughout the plants life without causing any harm to the host (Asaf *et al.*, 2017). These beneficial bacteria are termed plant growth-promoting bacteria (PGPB) (Ali, Sandhya and Rao, 2014; Niu *et al.*, 2018). Additionally, PGPB have been used to improve plant growth in extreme environments (Niu *et al.*, 2018; Osmolovskaya *et al.*, 2018). The use of beneficial microorganisms with PGP traits serves as an appropriate alternative to alleviate drought stress (Naderi *et al.*, 2022).

In this study it was hypothesized that Namibian desert adapted plants harbor microbiomes that possess PGP traits that would enable plants to acquire nutrients, adapt and survive under desert prone conditions (Eida *et al.*, 2018; Yousef, 2018). PGP traits include ammonia, hydrogen cyanide, siderophores, phytohormones production such as IAA, as well as phosphate solubilisation (Singh and Jha, 2015). Additionally, these PGPB produce ACC deaminase activity, positively affecting the adaptation and tolerance of plants to environmental stress conditions (Bal *et al.*, 2013; Naderi *et al.*, 2022).

ACC deaminase, is an enzyme that is encoded by the *acdS* gene and its activity is broadly distributed within desert plants associated bacteria (Orozco-Mosqueda, Glick and Santoyo, 2020). ACC deaminase activity has been found in several bacterial species such as *Rhizobium*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Leclercia*, and *Pseudomonas* (Orozco-Mosqueda, Glick and Santoyo, 2020), with species from *Pseudomonas* and *Bacillus* known to impose drought tolerance in plants (Riseh *et al.*, 2021).

During drought stress, there is an increase in the biosynthesis of ethylene, which has the potential to inhibit plant growth (Shiva *et al.*, 2016). Bacteria with ACC deaminase activity exert beneficial effects on plants under various abiotic stress conditions by reducing stress ethylene levels and conferring resistance and resulting in better growth (Bal *et al.*, 2013). The application of PGPB with multifarious growth promoting traits has thus been suggested to improve and enhance plant growth and crop yield by suppressing the negative effects of biotic and abiotic stress conditions (Gupta and Pandey, 2019). These beneficial microbes have been considered a promising tool (Costa-Gutierrez *et al.*, 2022).

Literature seems to suggest that plant growth and yield promoting and drought mitigating traits of endophytic and rhizospheric bacteria associated with *Myrothamnus flabellifolius* have not been explored.

3.2 MATERIALS AND METHODS

3.2.1 Sampling and site description

A total of eight samples of *Myrothamnus flabellifolius* Welw. (**Figure 3.1**) consisting of rhizosphere soil, stems, roots and leaves were collected from Namakorabis Farm (Nauchas area: 23°41’24’’ S, 16°16’22.7’’ E) in the Namib Naukluft National Park

(Khomas region), Namibia, in April 2019. This region is characterized by hot and extremely dry climate with little rainfall.

The plant samples were carefully uprooted with soil from the rock crevices and stored in sterile polyethylene bags. They were then transported in a cooling chamber maintained at 4 °C to the Microbiology laboratory of the Department of Biochemistry, Microbiology and Biotechnology at the University of Namibia for further analysis. The formal plant identification was carried out at the herbarium of National Botanical Research Institute (NBRI) in Windhoek, where plant herbarium specimens were deposited under specimen number ENF 01, Report No: 2023//444.

Figure 3.1: *Myrothamnus flabellifolius* (Welw.) growing in rock crevices. Picture taken by author (E.N Frans).

3.2.2 Isolation of PGPB from rhizospheric soil of *Myrothamnus flabellifolius* Welw.

Adhering soil was removed by gently shaking the plant (Niu *et al.*, 2018). The rhizospheric soil were strongly shaken in 50 ml falcon tubes containing 20 ml sterile phosphate buffered saline (PBS) (Sutra, Risede and Gardan, 2000). Roots were then placed in a new falcon tube with sterile PBS for endophytic isolation.

Leaves, stems and root samples of each plant were gently rinsed separately in sterile distilled water to remove adhering epiphytes and soil particles followed by immersing in 1% sodium hypochlorite solution for 5 minutes and 70% (v/v) ethanol for 30 seconds (Anjum and Chandra, 2015). Finally, all sample were washed 3 times with sterile distilled water and the last 200 µl washing water aliquots were spread onto trypticase soya agar (TSA) and nutrient agar (NA) and incubated at 28 °C for 72 hours to confirm sterility.

The leaves, roots and stem samples were aseptically cut into small pieces and macerated with 5 ml PBS, using a sterile mortar and pestle (Niu *et al.*, 2018) and allowed to stand for 30 minutes. About 3 ml of crushed solution was transferred to an enriched Synthetic malate (SM) medium, a semi sold medium in test tubes and incubated at 30 °C for 14 days. This was followed by series of dilutions ranging between 10^{-1} and 10^{-6}, and aliquoting 0.1 ml of the suspension onto VM-ethanol (VM) agar, and incubating at 30 °C for 7 days according to (Reinhold-hurek, Haiyambo and Chimwamurombe, 2015).

3.2.3 Molecular characterization and identification of isolated strains

3.2.3.1 DNA extraction

Preserved pure cultures (stored below 5 °C in tryptic soy broth (TSB)) were revived in fresh TSB and incubated at 150 rpm on a shaker at 30 °C for 24 hours. Bacterial cells were centrifuged at maximum speed for 5 minutes. Total genomic DNA of each bacterial isolate was extracted using the Zymo Research ZR Quick-DNA TM Fungal Bacterial MiniPrep Extraction kit (Zymo research, USA) as per the manufacturer's instructions.

Gel electrophoresis was performed using 1% agarose gel and 100 ml Tris-Borate-EDTA (TBE) stained with 2 µl SYBR Green loading dye for visualisation under ultraviolet light. This was carried out to compare the 100 bp DNA ladder (ThermoFisher Scientific, USA) with the fragment size to be amplified.

3.2.3.2 Amplification of 16S rRNA, *acdS*, and *nif*H genes

The polymerase chain reaction (PCR) was carried out in a 50 µl volume of PCR reaction mixture consisting of a 25 µl master mix, 1 µl of each primer, 5 µl DNA template and 18 µl nuclease-free water.

A partial 16S rDNA fragment was PCR amplified using the bacterial universal primers 27F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R 5' -GGT TAC CTT GTT ACG ACT T-3' (Niu *et al.*, 2018).

The amplification of 16S rRNA gene was carried out with the following PCR conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min in a thermocycler (Niu *et al.*, 2018). This was accompanied by the amplification of the a*cdS* gene by PCR using the primers AccF- ATG AAT CTG AAT CGT TTT GAA C and AccR- TCA GCC GTT GCG GAA CAG according to the manufacturer. The PCR conditions for *acdS* gene were the same as 16S rRNA, with modifications at annealing temperature of 50 °C for 45 seconds.

Amplification of the *nif*H gene was carried out using the nested PCR method with the following primers; primary primers FGPH19- TAC GGC AAR GGT GGN ATH G and PolR- ATS GCC ATC ATY NTC RCC GGA and Secondary primers PolF- TGC GAY CCS AAR GCB GAC TC-3' and AQER- GAC GAT GTA GAT YTC

CTG. Amplification was carried out during a 2-step procedure with the following conditions: step 1, initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 5 min. For step 2, all conditions were the same as in the first step except the annealing temperature set to 56 °C instead of 55 °C (Haiyambo, Reinhold-Hurek and Chimwamurombe, 2015). Gel electrophoresis was performed to confirm amplification of the correct DNA fragment.

3.2.3.3 Sequencing and isolate identification

Amplified PCR products were sequenced by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) and consensus sequences generated for each isolate by alignment using the CHROMAS 2.6.6 software (Niu *et al.*, 2018). Sequences were then compared with the nucleotide database in the National Centre of Biotechnology Information (NCBI) GenBank using BLAST to determine and identify the closest match on maximal percentage identity. This was followed by constructing a phylogenetic tree by the neighbour-joining method using MEGA X software (Pandey and Gupta, 2020). The accession numbers of the reference sequences used in the phylogenetic alignment are listed in **Appendix Table 3.1**.

3.2.4 *In vitro* screening of plant growth promoting traits

ACC deaminase activity was determined by preparing Dworkin and Foster (DF) minimal salt medium containing 3 mM ACC as sole nitrogen source according to the method described by (Kandjimi, Uzabakiriho and Chimwamurombe, 2015).

Bacterial cultures were previously grown in flasks containing 5 ml TSB, incubated at 30 °C for 48 hours under shaking conditions of 100 rpm (Shahzad *et al.*, 2010). Bacterial cells were harvested by centrifugation at 3000 *g* for 5 minutes, washed twice with sterile 0.1 M Tris-HCl, and later re-suspended in 1 ml of 0.1 M Tris-HCl and spot inoculated with 5 µl of bacterial isolate onto DF salt minimal medium with 3 mM ACC (Ali, Sandhya and Rao, 2014b). Plates were incubated at 28 °C for 72 hours and growth monitored thereafter (Ali, Sandhya and Rao, 2014b; Kandjimi, Uzabakiriho and Chimwamurombe, 2015; Gupta and Pandey, 2019). Growth and colony diameter were monitored and isolates growing on agar supplemented with ACC were considered to be ACC deaminase producers (Gupta and Pandey, 2019).

Plates containing only DF minimal salt medium without any nitrogen source were used as negative control while plates with DF minimal salt agar medium supplemented with $(NH_4)_2SO_4$ (ammonium sulphate) instead of ACC as a source of nitrogen acted as positive control (Niu *et al.*, 2018).

3.2.4.1 Estimation of ACC deaminase activity

ACC deaminase activity was determined by measuring the production of α-ketobutyrate and ammonia (Ali, Sandhya and Rao, 2014). The induced bacterial cells grown in TSB were harvested by centrifugation at 3000 *g* for 5 minutes and cells later washed twice with 0.1 M Tris-HCl (pH 7.6) (Penrose and Glick, 2003; Ali, Sandhya and Rao, 2014). Cells were then centrifuged at 16000 *g* for 5 minutes and supernatant removed. Pellets suspended in 600 µl Tris-HCl (pH 8.5) with addition of 30 µl of toluene and mixture vortexed at the highest speed for 30 seconds (Penrose and Glick, 2003).

About 200 µl of cells with toluene were placed in a new centrifuge tube and 20 µl of 0.5 M ACC added to the suspension. This was followed by a brief vortex of the suspension and incubating it at 30 °C for 15 minutes (Penrose and Glick, 2003; Ali, Sandhya and Rao, 2014).

About 0.5 ml of 0.56 M HCl was added and the mixture vortexed and centrifuged for 5 minutes at 16000 *g*. About 0.5 ml supernatant was removed and 400 µl of 0.56 M HCl was added to the tube, followed by addition of 150 µl of 2,4-dinitrophenylhydrazine (DNF) and incubated at 30 °C for 30 minutes (Penrose and Glick, 2003; Ali, Sandhya and Rao, 2014).

One ml of 2 N NaOH added to the tube after incubation and absorbance of the mixture read at 540 nm using a micro plate reader (Penrose and Glick, 2003). Sample measurements carried out in duplicate. The negative control consisted of 50 µl cells without ACC while blank consisted of 50 µl of 0.1 M Tris-HCl (pH 8.5) with 5 µl ACC (Ali, Sandhya and Rao, 2014).

The values of absorbance versus α-ketobutyrate concentration Millimolar (mM) were used to construct a standard curve of α-ketobutyrate ranging between 0.0001 mM and 0.001 mM (Bal *et al.*, 2013). Concentration of α-ketobutyrate in each sample was determined by comparison with a standard curve generated according to the method in (Ali, Sandhya and Rao, 2014). ACC deaminase activity expressed as the amount of α-ketobutyrate produce per mg of protein per hour (Bal *et al.*, 2013). The enzyme activity was expressed as micromoles (µm) of α-ketobutyrate per mg of protein per hour (Niu *et al.*, 2018).

3.2.4.2 Inorganic phosphate solubilisation assay

Bacterial isolates were screened on Pikovskaya medium supplemented with bromophenol blue as an indicator. Isolates were spot inoculated with 5 µl and incubated at 28 °C for 7 days (Joe *et al.*, 2018). Formation of clear zones around the colonies was measured to assess the ability to solubilize inorganic phosphate (Alkahtani *et al.*, 2020). Clear zones were determined by subtracting the total zone diameter from the colony diameter (Joe *et al.*, 2018). Solubilisation index calculated according to the formula in (Kandjimi, Uzabakiriho and Chimwamurombe, 2015; Reinhold-hurek, Haiyambo and Chimwamurombe, 2015; Gupta and Pandey, 2019).

Phosphate solubilizing index (PSI) = (Colony diameter + Halo zone diameter)/ Colony diameter

3.2.4.3 Ammonia production

A qualitative assay for ammonia production was performed. Bacterial isolates were grown in 5 ml peptone water broth and incubated at 37 °C for 48 hours (Singh and Jha, 2015). About 1 ml of Nessler's reagent was added to the test tube containing the culture supernatant. The development of a yellow to brownish colour indicated production of ammonia (Gupta and Pandey, 2019). Un-inoculated broth was used as a negative control.

3.2.4.4 HCN production

Selected bacterial isolates were plated on King's B medium supplemented with 0.4% (w/v) glycine. A Whatman filter paper saturated with picric acid solution (2% Na_2CO_3 in 0.5% picric acid) was placed on the upper lids of the petri dishes and incubated invertedly for 4 days at 28 °C. Development of red-brown to yellow colour of filter paper indicated HCN production (Gupta and Pandey, 2019).

3.2.4.5 Screening PGPR for IAA production

Both the qualitative and quantitative determination of IAA production was done as described by Gordon and Weber (1951) with minor modifications. Bacterial isolates were grown in 50 ml Luria Bertani (LB) medium amended with 5 mM Tryptophan at 28 °C for 48 hours on an orbital shaker at 100 rpm (Ali, Sandhya and Rao, 2014).

Bacterial cells were then harvested by centrifugation at 3000 *g* for 20 minutes (Ali, Sandhya and Rao, 2014). About 1 ml of supernatant was decanted in a separate tube and mixed with 2 ml of Salkowskis reagent (Singh and Jha, 2015). The mixture was then incubated at room temperature for 30 minutes in the dark (Maharana, 2019). The occurrence of a red-pinkish colour denoted IAA production.

The quantification and concentration of IAA in each sample was determined from a standard curve of a known IAA concentration ranging between 0 and 100 µg ml^{-1} (Patel *et al.*, 2015; Gupta and Pandey, 2019) by spectrophotometrically measuring the optical density (OD) at 530 nm (Singh and Jha, 2015; Gupta and Pandey, 2019).

3.2.4.6 Siderophore production

Production of siderophore was tested by the chrome azurol S (CAS) assay. One µl bacterial isolates were spot inoculated on CAS agar plates and incubated at 28 °C for 4 days in the dark (Singh and Jha, 2015; Gupta and Pandey, 2019). The development of orange-yellow halo around the bacterial growth indicated siderophore production (Gupta and Pandey, 2019).

3.3 RESULTS

A total of 52 isolates were isolated (from dry and fresh leaves, stems, roots, and rhizosphere samples), of which 33 were identified and only 29 were characterized. Sixty four percent (64%) were isolated from fresh samples, with most of the endophytic bacterial genera belonging to the genus *Bacillus* (38%), while dry samples accounted for 36.4% of the total number of isolates with *Cupriavidus metallidurans* and *Bacillus* accounting for 33% each (**Table 3.1**). The endophytic occurrence was the highest in roots (58%), followed by leaves (21%), stems (15%), and the rhizosphere with only (6%) isolates. The results suggested that there was a higher diversity of bacteria in roots compared to the other tissues, with *Bacillus* being the major genera.

3.3.1 Taxonomic composition of culturable bacterial endophytes isolated from *Myrothamnus flabellifolius*

The 16S rRNA gene sequences of the desert-adapted PGPB were analysed, and a phylogenetic tree was constructed (**Figure 3.2**). Based on 16S rDNA gene sequence analysis, 10 different genera of which *Bacillus* was the dominant genus accounting for 36% and, *Pseudomonas* (18.2%), *Cupriavidus* (15.2%), *Kosakonia* (9.1%), *Enterobacteriaceae* (6.1%) whereas *Agrobacterium*, *Staphylococcus*, *Enterobacter*, *Klebsiella* and *Cellulosimicrobium* were represented by (3%) each by a single isolate.

The most abundant bacterial genus in both root and stem tissues were *Bacillus*, 47% and 40% respectively. In leaves, the most predominant genera were from *Cupriavidus metallidurans* (43%) whereas in the rhizosphere *Pseudomonas* and *Cupriavidus metallidurans* were equally (50%) represented. Interestingly, *Pseudomonas* was the only isolate found inhabiting roots, leaves, stems and rhizosphere of plants.

The endophytes were considered conspecific at a threshold identity of ≥99%. GenBank accession numbers of bacterial isolates are listed in **Table 3.1** below.

Table 3.1: Molecular identification of plant growth-promoting bacteria (PGPB) isolated from *Myrothamnus flabellifolius* (Welw.) and their closest strain in the NCBI GenBank based on sequence similarity.

Sample Reference	Sample origin	Sample type	Isolate ID	NCBI Accession Number	Percentage Similarity (%)	*16S rRNA*	*acdS*	*nif*H
R2	Roots	Fresh	*Agrobacterium pusense*	OR518545	100	+	+	+
R3	Roots	Fresh	*Kosakonia* sp.	OR518546	100	+	+	+
R4	Roots	Fresh	*Kosakonia cowanii*	OR518548	100	+	+	+
R5	Roots	Fresh	*Kosakonia* sp.	OR518547	100	+	+	+
R6	Roots	Fresh	*Bacillus licheniformis*	OR518550	100	+	+	+
R7	Roots	Fresh	*Bacillus licheniformis*	OR518549	100	+	+	+
R8	Roots	Fresh	*Bacillus* sp.	OR518552	99	+	+	+
R11	Roots	Fresh	*Enterobacteriaceae*	OR518551	100	+	+	+
R13	Roots	Fresh	*Bacillus licheniformis*	OR518556	100	+	+	+
R14	Roots	Fresh	*Pseudomonas neuropathica*	OR518557	100	+	+	+
R15	Roots	Fresh	*Bacillus cereus*	OR519873	94	+	+	+
R16	Roots	Fresh	*Pseudomonas* sp.	OR518558	100	+	+	+
R17	Roots	Fresh	*Bacillus safensis*	PV451626	97	+	+	+
R18	Roots	Fresh	*Enterobacter cloacae*	OR623052	100	+	+	+
R19	Roots	Fresh	*Bacillus safensis*	OR518559	100	+	+	+
R21	Roots	Fresh	*Klebsiella aerogenes*	OR518560	100	+	+	+
LA3	leaves	Fresh	*Enterobacteriaceae*	OR518561	100	+	+	+
LA7	leaves	Fresh	*Cupriavidus metallidurans*	OR518562	100	+	+	+
Leaves A1	leaves	Fresh	*Pseudomonas* sp.	OR518563	100	+	+	+
6 Stem A	stem	Fresh	*Bacillus subtilis*	OR518566	100	+	+	+
S8 stem A	stem	Fresh	*Pseudomonas lactis*	OR518564	100	+	+	-

Table 3.1 (continued).

LB1	leaves	Dry	*Cupriavidus metallidurans*	OR518567	100	+	+	+
LB3	leaves	Dry	*Staphylococcus hominis*	OR518565	100	+	+	+
LB7	leaves	Dry	*Cupriavidus metallidurans*	OR518568	100	+	+	+
LB21	leaves	Dry	*Bacillus licheniformis*	OR518569	100	+	+	+
9 stem B	stem	Dry	*Cupriavidus metallidurans*	OR518570	100	+	+	-
S2 stem B	stem	Dry	*Bacillus pumilus*	OR518544	100	+	+	+
Stem B S10	stem	Dry	*Cellulosimicrobium funkei*	OR518592	100	+	+	+
RzBO	rhizosphere	Dry	*Pseudomonas japonica*	OR518658	100	+	+	+
RzW	rhizosphere	Dry	*Cupriavidus metallidurans*	OR519607	100	+	+	+
R9	Roots	Dry	*Bacillus siamensis*	OR519608	100	+	+	+
R10	Roots	Dry	*Pseudomonas* sp.	OR519631	100	+	+	+
R12	Roots	Dry	*Bacillus amyloliquefaciens*	OR519718	100	+	+	+

3.3.1.1 Phylogenetic analysis

Phylogenetic analysis using the Neighbour joining algorithm resulted in a cladogram with 11 clades (**Figure 3.2**). In clade 1, isolate (Leaves A1) *Pseudomonas* sp. clustered with *Pseudomonas putida* a known strain that shares 100% similarity after BLAST, indicating that it is likely a species of *Pseudomonas*. The second clade consisted of *Kosakonia cowanii*, demonstrating to be phylogenetically related to *Cupriavidus metallidurans*. Clade 3 comprised *Enterobacter cloacae* and *Bacillus cereus*. In clade 5, *Cupriavidus metallidurans* from the rhizosphere and *Bacillus safensis* from the roots came out as sister taxa, forming a separate distinct monophyletic cluster with 3 *Cupriavidus metallidurans* isolates from the leaves in clade 4.

The different clusters show that the microbial communities are different, where the *Cupriavidus* species from the rhizosphere branched out from those isolated from the leaves, further demonstrating to have possibly evolved from a different ancestor. Clade 6 comprised *Bacillus cereus* and *Cupriavidus metallidurans* with no known isolate that share similarities. The isolates in clade 7, 8 and 9 clustered together at a bootstrap support value of 100%, but not with any known strain. This could suggest novel species.

Clade 9 shows *Bacillus safensis* and *Bacillus pumilus* clustered together with a bootstrap support value of 100%. Clade 10 consisted of two *Pseudomonas* sp. isolated from roots, and the node has a 100% bootstrap support value. This could suggest that the samples are identical given their origin.

Clade 11 consisted of 5 different species of *Bacillus* isolated from different tissues clustered together at a bootstrap support value of 100%. This demonstrated their close relationship, despite being isolated from different tissues as seen with *B. licheniformis* from roots (R6 and R13) and *B. licheniformis* from the leaves (LB21).

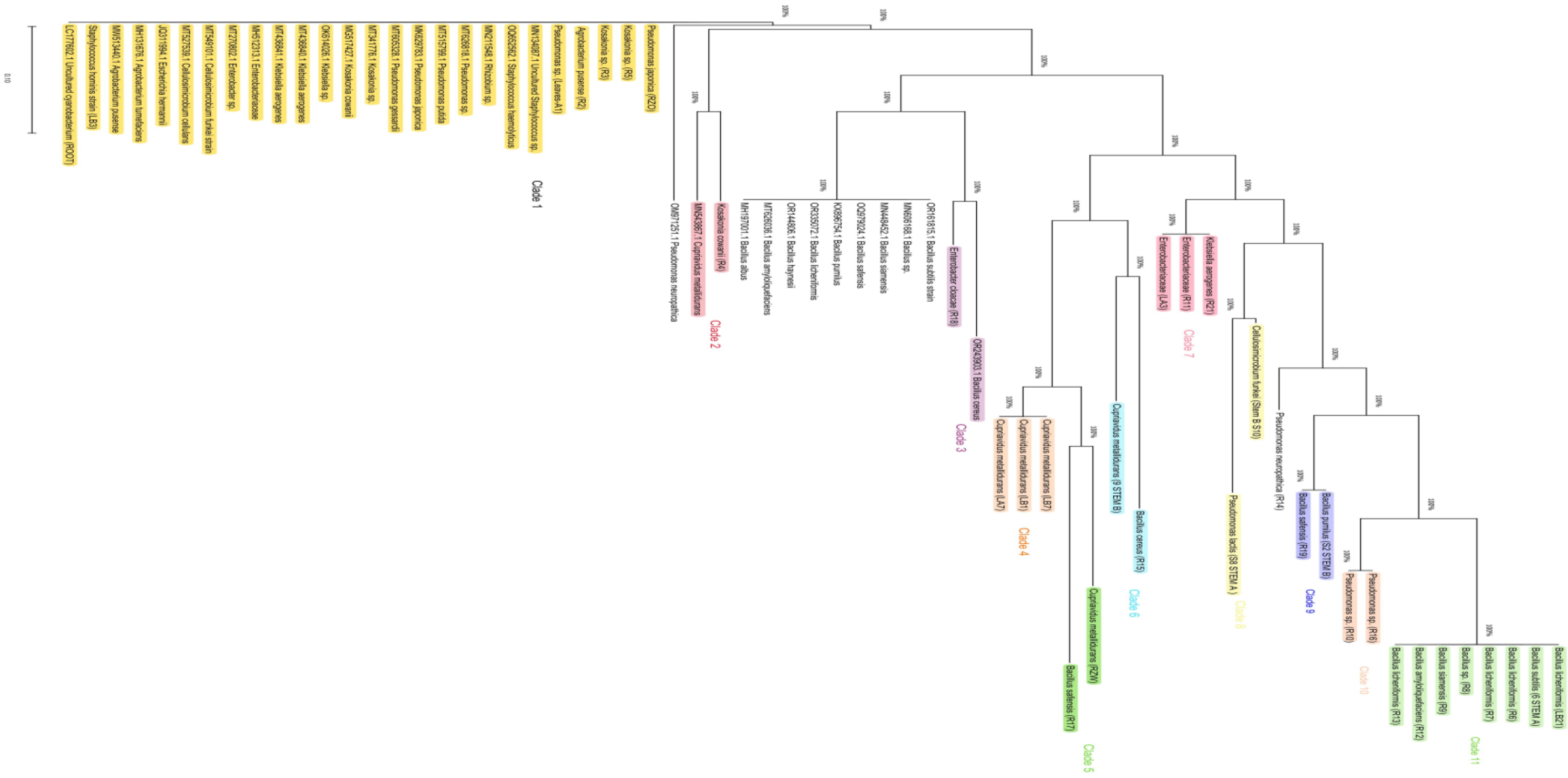
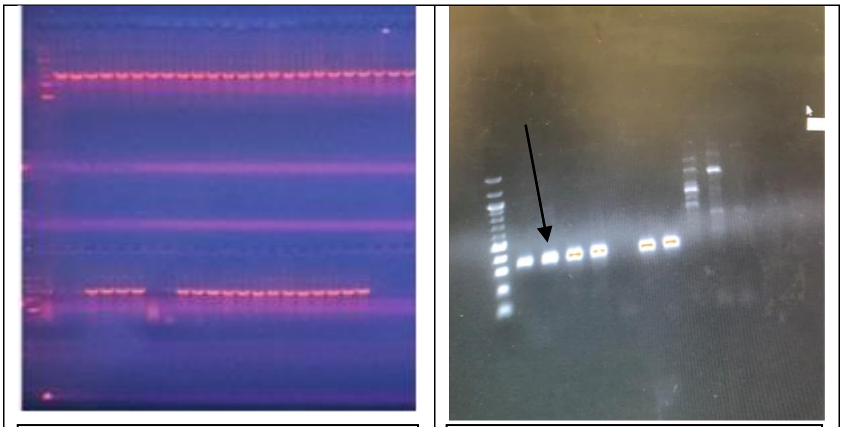


Figure 3.2: Phylogenetic tree based on the partial 16S rRNA nucleotide sequences showing a relationship between various strains from *Myrothamnus flabellifolius* (Welw.) with their reference strains. This phylogenetic tree was inferred using MEGA-X software, with bootstrap performed with 1000 replications. *Cyanobacterium* was used as the outgroup.

3.3.2 Detection of *nif*H and *acdS* genes

The *nif*H gene serves as a biomarker to study the ecology of nitrogen-fixing bacteria (Gaby and Buckley, 2014) and the presence of *nif*H gene served as an indication of nitrogen fixation. Out of the 33 identified isolates, only 2 were negative for nitrogen fixation gene, *Cupriavidus metallidurans* and *Pseudomonas lactis*. Expected amplification of ~900 bp and ~300 bp of *acdS* and *nif*H gene, respectively. Molecular identification results are indicated in **Figure 3.3** below.



(A) *acdS* gene

(B) *nif*H gene

Figure 3.3: Visualisation of amplified gene fragments of various plant growth promoting bacteria on 1% (w/v) agarose by gel electrophoresis **(A)** *acdS* gene **(B)** expression of *nif*H gene at ~300 bp by *Bacillus pumilus* (S2 stem B) as indicated by arrow.

3.3.3 Qualitative assessment of PGP traits

All 33 isolates showed at least one trait as shown in (**Table 3.2**) below when screened for various PGP traits. The assessment revealed that the strains had various plant growth promoting activities. The bacterial isolates were confirmed to be endophytes as no bacterial colonies were observed in the control plates. Based on the results, only (R21) *Klebsiella aerogenes* had the capacity to produce the most PGP traits such as production of NH_3, IAA, siderophore, phosphate solubilisation and ACC deaminase activity.

3.3.3.1 ACC deaminase assay

A total of 33 bacterial strains were isolated from the roots, stems, rhizosphere and leaves of *Myrothamnus flabellifolius* with only 29 of them further characterized. All 29 isolates were deemed positive for ACC deaminase as they all grew on DF minimal salts medium supplemented with 3 mM ACC as the sole nitrogen source. This was also complimented with the expression of the *acdS* gene detected in all isolates.

3.3.3.2 Estimation and quantification of ACC deaminase

The ACC deaminase activity was determined by measuring the amount of α-ketobutyrate generated upon hydrolysis of ACC. The highest activity of α-ketobutyrate was exhibited by bacterial strain *Bacillus licheniformis* (R13) with 0.0013 mM α-ketobutyrate/mg protein/hour. The isolated bacteria in (**Table 3.2**) below showed variation in ACC deaminase activity in the range of 0.0005-0.0013 mM α-ketobutyrate/mg protein/hour.

Table 3.2: Biochemical analysis of rhizosphere and endophytic bacterial isolates obtained from *Myrothamnus flabellifolius.*

Isolates	**Name of bacterial species**	**Phosphate solubilisation**					**NH$_3$**	**HCN**	**Siderophore**		**IAA Production**		**ACC activity (a-keto prod)**	
		growth	Halo zone	Colony diameter (mm)	Halo zone diameter (mm)	PSI Index			Growth	Halo zone	Trypt.	µg/ml	Prs	mM
R2	*Agrobacterium pusense*	+	+	5	10.5	2.1	+	+	-	-	-		+	0.0007 ± 0.00
R3	*Kosakonia* sp.	+	-				+	-	+	-	+	65.98	+	0.0009 ± 0.00
R4	*Kosakonia cowanii*	+	-				+	-	-	-	-		+	0.0008 ± 0.00
R5	*Kosakonia* sp.	+	-				+	-	+	-	+	39.35	+	0.0007 ± 0.00
R6	*Bacillus licheniformis*	+	+	8	12.5	1.6	+	-	+	-	-		+	0.0007 ± 0.00
R7	*Bacillus licheniformis*	+	-				+	+	+	-	-		+	0.0007 ± 0.00
R8	*Bacillus* sp.	+	+	10	19.5	2	+	-	+	-	+	5.02	+	0.0008 ± 0.00
R11	*Enterobacteriaceae*	+	-				+	-	-	-	-		+	0.0005 ± 0.00
R13	*Bacillus licheniformis*	+	+	8	13	1.6	+	-	+	-	-		+	0.0013 ± 0.01

Table 3.2 (continued).

R14	*Pseudomonas neuropathica*	+	-				-	-	+	-	-		+	0.0009 ± 0.00
R15	*Bacillus cereus*	-	-				-	-	-	-	-		+	0.0009 ± 0.00
R16	*Pseudomonas* sp.	-	-				-	-	-	-	-		+	0.0005 ± 0.00
R18	*Enterobacter cloacae*	+	-				+	-	-	-	-		+	0.0007 ± 0.00
R19	*Bacillus safensis*	+	+	5	8.5	1.7	+	-	-	-	-		+	0.0008 ± 0.00
R21	*Klebsiella aerogenes*	+	+	6	11	1.8	+	-	+	+	+	17.47	+	0.0006 ± 0.00
LA3	*Enterobacteriaceae*	-	-				-	-	-	-	-		+	0.0010 ± 0.00
LA7	*Cupriavidus metallidurans*	-	+	8.5	12.5	1.5	-	-	-	-	-		-	-
LB7	*Cupriavidus metallidurans*	+	+	8	32	4	-	-	+	+	+	37.41	+	0.0008 ± 0.00
LB1	*Cupriavidus metallidurans*	+	+	8.5	15	1.8	+	-	+	-	-		+	0.0009 ± 0.00
LB3	*Staphylococcus hominis*	+	+	7	13.5	1.9	+	-	-	-	-		+	0.0010 ± 0.00
LB21	*Bacillus licheniformis*	+	+	6.5	12	1.8	+	-	-	-	-		+	0.0008 ± 0.00
S8 stem A	*Pseudomonas lactis*	+	-				+	-	+	-	+	2.08	-	-

S10/ STEM B S10	*Cellulosimicrobium funkei*	+	-				-	+	-	-	-		-	-
leaves A1	*Pseudomonas* sp.	+	-				+	-	+	+	-		-	-
RzB O	*Pseudomonas japonica*	+	-				-	+	+	+	+	247.29	-	-
RzB W	*Cupriavidus metallidurans*	-	-				-	-	+	-	-		-	-
9 stem B	*Cupriavidus metallidurans*	-	-				-	-	-	-	-		-	-
6 stem A	*Bacillus subtilis*	+	+	9	14	1.6	+	-	+	-	-		-	-
S2 stem B	*Bacillus pumilis*	+	-				+	-	-	-	-		-	-

*Some bacterial isolates were designated similar names by 16S rRNA sequence due to initial selection based on colony morphology

(-) stands for negative test result and (+) stands for positive test result.

IAA: Indole Acetic Acid; HCN: Hydrogen cyanide; NH$_3$: Ammonia; Trypt: Tryptophan; Mm: Millimolar; PSI: Phosphate solubilizing index; Prs: Presence

Error Bars Mean of ±SD (n=3).

Table 3.3: ACC deaminase activities displayed by plant growth-promoting bacteria (PGPB) from *M. flabellifolius*.

Isolates	**Sample origin**	**ACC utilisation of isolates**			
		growth on ACC supplemented medium (Dworkin and Foster minimal salt medium)	**growth on Ammonium sulphate supplemented medium (Dworkin and Foster minimal salt medium)**	**Media with no Nitrogen source**	**Clear zone on ACC media**
R2 *Agrobacterium pusense*	Roots	+	+	-	-
R3 *Kosakonia* sp.	Roots	+	+	+	+
R4 *Kosakonia cowanii*	Roots	+	+	+	+
R5 *Kosakonia* sp.	Roots	+	+	+	+
R6 *Bacillus licheniformis*	Roots	+	+	+	-
R7 *Bacillus licheniformis*	Roots	+	+	-	-
R8 *Bacillus* sp.	Roots	+	+	+	+
R11 *Enterobacteriaceae*	Roots	+	+	+	+
R13 *Bacillus licheniformis*	Roots	+	+	-	-

Table 3.3 (continued).

R14 ***Pseudomonas neuropathica***	Roots	+	+	+	+
R15 ***Bacillus cereus***	Roots	+	+	+	+
R16 ***Pseudomonas*** **sp.**	Roots	+	+	+	+
R18. ***Enterobacter cloacae***	Roots	+	+	+	+
R19 ***Bacillus safensis***	Roots	+	+	-	-
R21 ***Klebsiella aerogenes***	Roots	+	+	+	-
LA3 ***Enterobacteriaceae***	Leaves	+	+	+	+
LA7 ***Cupriavidus metallidurans***	Leaves	+	+	+	+
LB7 I ***Cupriavidus metallidurans***	Leaves	+	+	+	+
LB1 ***Cupriavidus metallidurans***	Leaves	+	+	+	-
LB3 ***Staphylococcus hominis***	Leaves	+	+	-	+
LB21 ***Bacillus licheniformis***	Leaves	+	+	-	+
S8 ***Pseudomonas lactis***	Stem	+	+	+	+

Table 3.3 (continued).

STEM B S10 ***Cellulosimicrobium funkei***	Stem	+	+	+	+
Leaves A1 ***Pseudomonas sp.***	Leaves	+	+	+	+
RzB O ***Pseudomonas japonica***	Rhizosphere	+	+	-	-
RzB W ***Cupriavidus metallidurans***	Rhizosphere	+	+	-	+
9 stem B ***Cupriavidus metallidurans***	Stem	+	+	+	-
6 stem A ***Bacillus subtilis***	Stem	+	+	+	-
S2 stem B ***Bacillus pumilus***	Stem	+	+	-	-

3.3.4 Inorganic phosphate solubilisation

From the 29 isolates characterized, only 12 (41%) formed halo zones around the colonies on Pikovskaya's agar medium supplemented with bromophenol, indicating phosphate solubilisation as seen in (**Figure 3.4**). Relative abundance of solubilisation was observed in roots (50%), followed by leaves (42%), and stems (8%) with no observations in rhizosphere samples. Relative abundance was observed with 50% of solubilizers from *Bacillus*, followed by 25% *Cupriavidus*, and 8.3% in *Agrobacterium*, *Klebsiella*, and *Staphylococcus* respectively. The highest phosphate solubilising activity was observed in isolate LB7 *Cupriavidus metallidurans*, with PSI of 4, followed by *Agrobacterium* R2 with PSI 2.1 and *Bacillus* sp. R8 with PSI 2 as seen in (**Table 3.2**).

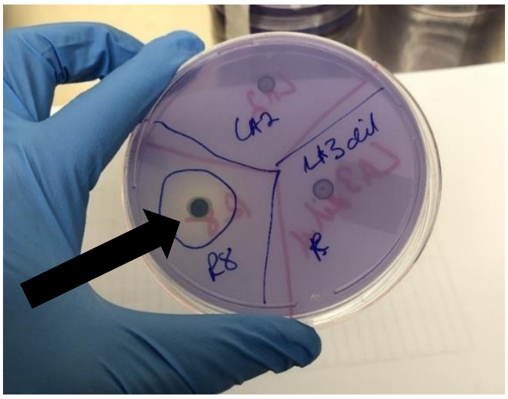


Figure 3.4: Solubilisation of inorganic phosphate by isolate (R8) *Bacillus* sp. With halo zone formation around the colony.

Isolate (R8) *Bacillus* sp. was positive for phosphate solubilisation, as indicated by the appearance of a well-developed clear zone on Pikovskaya's agar medium supplemented with bromophenol blue. The isolate formed a clear zone of 19.5 mm in diameter.

3.3.5 Ammonia production

It was observed that 19 isolates (65.5%) were positive for ammonia production with 63.2% of these ammonia-producing bacteria isolated from the roots, 21% from the leaves, and 15.8% from the stems and none from the rhizosphere. It was further observed that out of these isolates, relative abundance was from isolates belonging to the *Bacillus* genera with 42.1%, followed by *Kosakonia* 15.7%, and *Pseudomonas* 10.5%. The remaining genera, *Agrobacterium*, *Klebsiella*, *Enterobacteriaceae, Enterobacter, Staphylococcus,* and *Cupriavidus* were each 5.3%.

3.3.6 Hydrogen cyanide production

Observations show that only 4 isolates (13.8%) were positive for production of HCN. This included isolates from *Agrobacterium* (roots) *Bacillus* (roots), *Cellulosimicrobium* (stem) and *Pseudomonas* genera (rhizosphere). Isolate RzBO *Pseudomonas japonica* produced the most distinct overcast of orange pigment in contrast to the other isolates. Two of the isolates R2 *Agrobacterium pusense* and R7 *Bacillus licheniformis* both produced Ammonium and HCN as shown in **Table 3.2**.

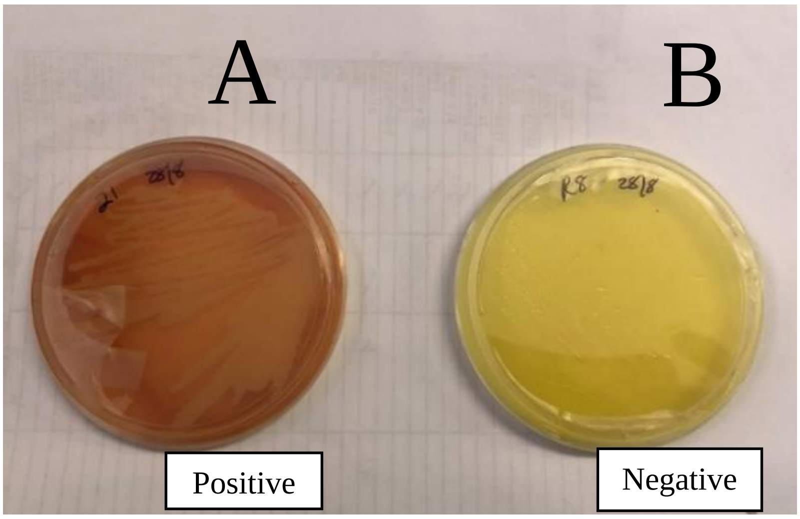


Figure 3.5: Production of Hydrogen cyanide (HCN) observed in isolate (RzBO) *Pseudomonas japonica* **(A)**. Yellow colour indicating negative reaction from (R8) *Bacillus* sp. **(B)**.

3.3.7 Production of IAA

Seven out of 29 isolates (24%) have shown the ability to produce IAA within the range of 2.08-247.29 µg/ml in the presence of the IAA precursor, tryptophan. This constituted 57.1% isolated from the roots, 14.3% isolated from leaves, 14.3% stems and 14.3% rhizosphere. From the 24% of isolates producing IAA in the presence of tryptophan, relative abundance of IAA-producing bacteria was detected from isolates belonging to *Kosakonia* 29% and *Peudomonas* 29%, followed by *Bacillus* 14%, *Klebsiella* 14%, and *Cupriavidus* with 14%. The lowest and highest concentration produced in the presence of tryptophan was exhibited by *Pseudomonas lactis* (S8 stem A) with 2.08 µg/ml and *Pseudomonas japonica* (RzBO) with 247.29 µg/ml as seen in (**Table 3.4**).

Moreover, 8 out of 29 isolates (27.6%) were able to produce IAA in the absence of tryptophan. This constituted 50% isolated from the roots, 25% from the leaves, and

25% from the rhizosphere. The highest concentration of IAA produced was exhibited by *Pseudomonas japonica* (RzBO) isolated from the rhizosphere, with 32.32 µg/ml (Data not shown).

It was further observed that the lowest concentrations of IAA were produced by *Pseudomonas neuropathica* (R14) 2.14 µg/ml; followed by *Kosakonia* sp. (R3) 9.83 µg/ml; *Bacillus cereus* (R15) 11.38 µg/ml; *Bacillus licheniformis* (LB21) 12.42 µg/ml; *Kosakonia cowanii* (R5) 15.11 µg/ml; *Cupriavidus metallidurans* (RzW) 17.63 µg/ml and *Pseudomonas putida* (Leaves A1) 18.99 µg/ml (Data not shown). Fifty percent of these IAA producers were isolated from the roots, leaves (25%) and rhizosphere (25%).

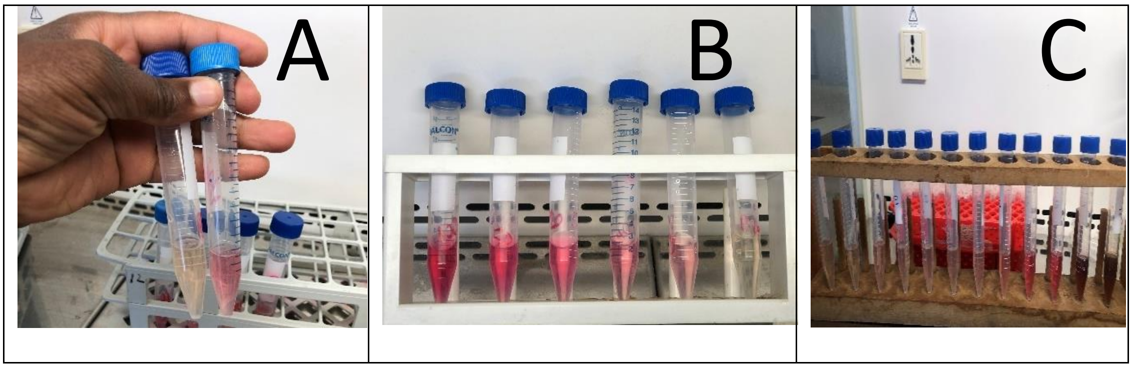


Figure 3.6: Quantitative assay of IAA production. Tube on left is the negative control and tube on right is the positive control **(A)**. IAA standard solutions **(B)**. IAA-producing isolates **(C)**.

Table 3.4: IAA production of plant growth-promoting bacteria with standard deviation; n=3.

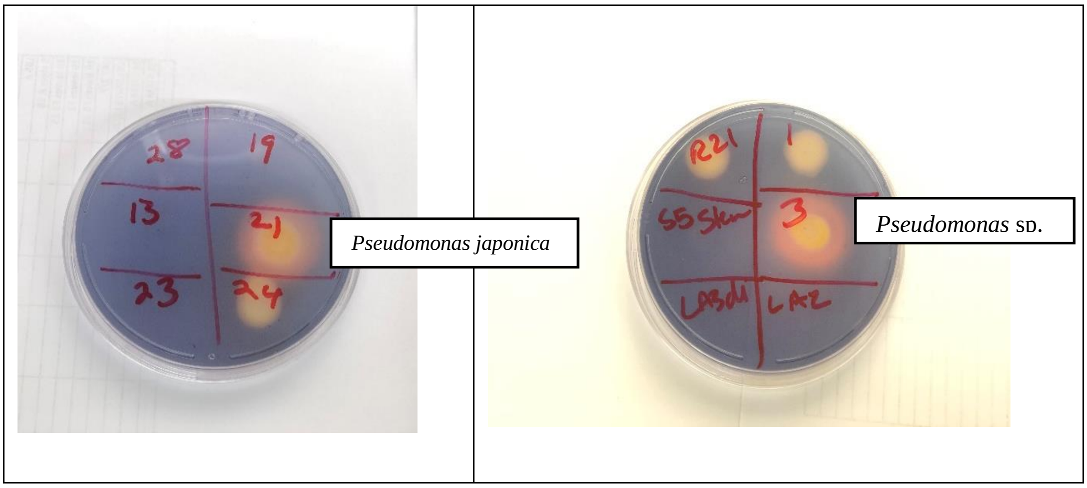
Isolate	**Specie**	**Sample origin**	**IAA µg/ml**
Control	Control	control	-
RzW	*Cupriavidus metallidurans*	Rhizosphere	-
LB1	*Cupriavidus metallidurans*	Leaves	-
LB3	*Staphylococcus hominis*	Leaves	-
Leaves A1	*Pseudomonas* sp.	Leaves	-
R15	*Bacillus cereus*	Roots	-
LB21	*Bacillus licheniformis*	Leaves	-
Stem B S10	*Cellulosimicrobium funkei*	Stem	-
R5	*Kosakonia* sp.	Roots	39.35 ± 2.39
LB7	*Cupriavidus metallidurans*	Leaves	37.41 ± 1.55
R8	*Bacillus* sp.	Roots	5.02 ± 0.83
R4	*Kosakonia cowanii*	Roots	-
R21	*Klebsiella aerogenes*	Roots	17.47 ± 0.75
R14	*Pseudomonas neuropathica*	Roots	-
Stem B S2	*Bacillus pumilus*	Stem	-
R13	*Bacillus licheniformis*	Roots	-
LA7	*Cupriavidus metallidurans*	Leaves	-
6 Stem A	*Bacillus subtilis*	Stem	-
R18	*Enterobacter cloacae*	Roots	-
RzBO	*Pseudomonas japonica*	Rhizosphere	247.29 ± 3.30

Table 3.4 (continued).

R3	*Kosakonia* sp.	Roots	65.98 ± 2.53
S8 stem A	*Pseudomonas lactis*	Stem	2.08 ± 0.23
LA3	*Enterobacteriaceae*	Leaves	-
R7	*Bacillus licheniformis*	Roots	-
R2	*Agrobacterium pusense*	Roots	-
R19	*Bacillus safensis*	Roots	-

3.3.8 Siderophore production

Out of 29 isolates, 51.7% of the isolates were able to grow on CAS agar. However, only (4) 13.8% of isolates were able to produce the orange halo zone for siderophore production as indicated below in (**Figure 3.7**). These isolates were isolated from the roots (*Klebsiella aerogenes*), rhizosphere (*Pseudomonas japonica),* stem (*Pseudomonas lactis*) and the leaves *Cupriavidus metallidurans*). Fifty percent of these isolates belonged to *Pseudomonas*, followed by *Klebsiella* and *Cupriavidus* with 25% each.



Pseudomonas japonica

Pseudomonas sp.

Figure 3.7: Confirmation of siderophore production by zone of orange halo on CAS-blue agar.

3.4 DISCUSSION

The PGP potential of rhizospheric and endophytic bacteria isolated from *Myrothamnus flabellifolius* was explored. A total of 33 isolates were isolated and identified. In this study, based on 16S rRNA, there was a >99% similarity on most isolates with *Bacillus* being the most abundant genera with 36.4% followed by *Pseudomonas* with 18.2%. *Bacilli* species are known to predominantly inhabit agricultural soils (Mahapatra, Yadav and Ramakrishna, 2022) and are the predominant culturable bacterial endophytes (Li, Cheng and An, 2017). Some studies have indicated that bacteria belonging to the genera *Bacillus*, *Pseudomonas, Enterobacter*, and *Agrobacterium* have previously been isolated from environments exposed to extreme conditions such as drought stress and still retained their PGP traits (Naderi *et al.*, 2022). *Bacillus* sp. were previously reported as the predominant endophytic bacteria in multiple crops and as the predominant genera in various plant tissues (Borah *et al.,* 2019). Furthermore, *Bacilli* bacteria have been known to have potential as PGPB in contrast to other bacterial genera, which could be due to their ubiquitous nature and presence in extreme environmental conditions (Orozco-Mosqueda, Glick and Santoyo, 2020).

Relative abundance showed that 94% of the isolates were endophytic, comprising of bacterial isolates from the roots, stems and leaves, whereas 6% of the isolates where from the rhizosphere. A study by Anjum and Chandra (2015) highlighted that there is a higher diversity of endophytic bacteria in roots, and further stated that plants generally have an abundance of endophytes in their roots in contrast to aboveground tissues such as stems and leaves. A study by Harrison and Griffin (2020) stated that relative tissue abundance of microbial endophytes could be dependent on host growth habits, age, and the resources available for the microbes within the tissues. To support this, previous studies have demonstrated that roots, which are long-lived tissues may harbour richer microbial assemblages than younger tissues, presumably because of greater exposure to microbial inoculum from the surrounding soil matrix (Harrison and Griffin, 2020). However, there was no notable separation in species found in the rhizosphere and above ground tissues. This was evident as species belonging to both *Cupriavidus* and *Pseudomonas,* were isolated from the rhizosphere, leaves, stems, and roots. The rhizosphere's microbiome structure may be determined by root exudates, environmental conditions and plant genotype (Kawasaki *et al.*, 2016; Reverchon *et al.*, 2023). The plant's interior is colonized by a range of bacterial endophytes, which may have crossed over from the rhizosphere (Compant *et al.*, 2021), or vertically transmitted from seeds, with most of the bacterial taxa harbouring the seeds similar to common soil isolates (Truyens *et al.*, 2014).

ACC deaminase activity was quantitatively determined by measuring the amount of α-ketobutyrate produced. Out of 29 isolates tested, only 20 isolates were able to produce detectable amounts of α-ketobutyrate while the remaining 9 isolates were unable to produce amount that were within the standard curve range of 0.0001-0.001 mM. This could imply that the hydrolysis by ACC deaminase enzyme may have variable potential depending on the type of isolate and their associated tissue of isolations, resulting in variable levels of α-ketobutyrate and ammonia as demonstrated in the results between 0.0005 mM and 0.0013 mM α-ketobutyrate in *Enterobacteriacea* (R11) and *Bacillus licheniformis* (R13), respectively.

Plant ethylene is inhibited by the production of ACC deaminase, which cleaves ACC into α-ketobutyrate and ammonia (Gupta and Pandey, 2019), consequently facilitating plant growth and development in harsh environmental conditions and thereby enabling plant tolerance to environmental stress such as drought (Saikia *et al.*, 2018; Ojuederie and Olanrewaju, 2019). According to Torbaghan *et al*. (2017) and Singh *et al*. (2022), it is stated that ACC deaminase activity of more than 0.00002 mM α-ketobutyrate per mg per hour is sufficient for the enhancement of plant growth under stress conditions. In this study, it was notable that *Bacillus licheniformis* (R13) exhibited the highest value of ACC deamination, producing 0.0013 mM of α-ketobutyrate, followed by *Staphylococcus hominis* (LB3) and *Enterobacteriaceae* (LA3). A study on *Vitis vinifera* endophytes by Barrado (2018) previously detected ACC deaminase activity in *Staphylococcus hominis*. *Enterobacter cloacae* isolated from the rhizosphere of the desert plant *Ziziphus nummularia*, were previously investigated for their ACC deaminase effectiveness and potential to mitigate stress-induced plant growth inhibition (Singh *et al.*, 2022).

IAA acts as a suitable marker for bacterial effectiveness particularly under osmotic stress (Sandhya *et al.*, 2010). Rhizospheric microbes are known to be efficient auxin producers in contrast to microbes isolated from soil (Maharana, 2019). The results in the study show that the highest concentration of IAA produced in the presence and absence of Tryptophan was exhibited by rhizospheric *Pseudomonas japonica* (RzBO) with 247.29 µg/ml and 32.32 µg/ml, respectively. Contrastingly, the highest IAA produced by endophytic bacteria in the presence of Tryptophan was exhibited by *Kosakonia* sp. (R3) with 65.98 µg/ml, *Kosakonia* sp. (R5) 39.35 µg/ml and *Cupriavidus metallidurans* (LB7) with 37.41 µg/ml. These endophytic bacteria were isolated from the roots and leaves respectively. A recent study by (Majeed *et al.*, 2015) stated that various strains from the rhizosphere and root-endosphere were indeed able to produce high levels of IAA, as demonstrated in our results.

According to Maharana (2019), rhizospheric soil can provide a rich source of IAA producing bacteria and can produce a significant amount of IAA in a tryptophan-supplemented medium, which resonates with the findings in our study. This could be indicative of a substantial variability among the microbes, which may be due to the various biosynthetic pathways, bacterial species gene location, and presence of enzymes to convert active free IAA into adjoined form (Duca *et al.*, 2014).

In the present study, the majority (50%) of phosphate solubilizers were isolated from the roots. This study further reveals that *Bacillus* was the most abundant genera of solubilizing bacteria with 50%, and *Agrobacterium pusense* with 8.5%. Additionally, *Bacillus* and *Rhizobium* are known to solubilize phosphate through the secretion of various reactions (Ullah *et al.*, 2019). Most of the isolates able to solubilise phosphate belong to the *Bacillus* genera, a true reflection of phosphate solubilizers as reported in a previous study by Gupta and Pandey (2019).

The highest phosphate solubilising activity observed in the present study were from isolate *Cupriavidus metallidurans* (LB7) with PSI of 4, followed by *Agrobacterium* (R2) and *Bacillus* sp. (R8) with PSI of 2.1 and 2, respectively. Previous studies show isolates from *Pseudomonas stutzeri* and *Pseudomonas veronii* species exhibiting index values of 3.5 and 2.8, respectively, with *Bacillus megaterium* producing an index value of 1.4 (Reinhold-hurek, Haiyambo and Chimwamurombe, 2015). Presence of these microbes is a positive indicator of these microbes to serve as potential biofertilizers for crops.

Isolates from the *Bacillus* genera were among the producers of ammonia with 42.1%. Previous studies from Borah *et al.* (2019) and Kandjimi, Uzabakiriho and Chimwamurombe (2015) have shown isolates from *Bacillus* genera to be ammonia producers, which support our results. Ammonia is known to influence plant growth by making Nitrogen available to the plant (Kandjimi, Uzabakiriho and Chimwamurombe, 2015). Hence, ammonium-producing isolates can provide Nitrogen to the plant for growth promotion. Furthermore, production of ammonia by PGPB is known to indirectly promote plant growth by suppressing pathogens (Minaxi *et al.,* 2012). The suppression of some phytopathogens due to the excretion of ammonia by PGPB is beneficial to the plants as the accumulation of ammonia in soil has detrimental effects due to its potent inhibition effect (Borah *et al.*, 2019; Gupta and Pandey, 2019).

Production of HCN has been considered to have biological control of phytopathogens by inducing systemic resistance of plant (Kandjimi, Uzabakiriho and Chimwamurombe, 2015; Priyanka *et al.*, 2017). This study reveals that isolates from *Agrobacterium*, *Bacillus*, *Cellulosimicrobium*, and *Pseudomonas* genera were able to produce HCN, with *Pseudomonas japonica* (RzBO) producing the most distinct orange pigment in contrast to other isolates. This is consistent with a study by Abd El-Rahman *et al*. (2019), who reported that just like phosphate solubilizers, PGPB such as *Pseudomonas japonica*, *Rhizobium*, and *Bacillus* are among a few species known to produce HCN with the ability to inhibit growth of phytopathogens. The isolated microbes may play a role in protecting *Myrothamnus flabellifolius* from its harsh and arid native habitat. Despite HCN's major role in biocontrol of pathogens, previous studies have suggested that its production may indirectly increase phosphorus availability via metal chelation (Lotfi *et al.*, 2022), which may explain the correlation between phosphate solubilizers and HCN producers.

In our study, a qualitative estimation of siderophores was determined with bacteria isolated from the roots (*Klebsiella aerogenes*), rhizosphere (*Pseudomonas japonica),* stem (*Pseudomonas lactis*) and the leaves (*Cupriavidus metallidurans*). This demonstrates 50% of siderophore producers to belong to *Pseudomonas*. A study by Priyanka *et al.* (2017) and Srivastava *et al.* (2022) has shown *Pseudomonas* species to be remarkable siderophore producers. However, no production of Siderophores was observed in *Bacillus* species, despite isolates from this specie known to be significant siderophore producers as reported by (Borah *et al.*, 2019).

Bacterial nitrogen fixation is usually correlated with expression of *nif*H gene according to Masood, Zhao and Shen (2020). This study revealed majority (94%) of isolates were putative nitrogen fixing bacteria with 2 isolates, *Pseudomonas lactis* and *Cupriavidus metallidurans* testing negative for the presence of *nif*H gene. This is indicative of nitrogen fixation. Furthermore, the high number of potential nitrogen fixers could be an indication of symbiosis to the poor nutrient soils, where the plant grows. Interestingly, these 2 isolates were isolated from the stems. Expression of *nif*H gene is known to increase in the presence of *Bacillus pumilus* (Masood, Zhao and Shen, 2020). This is evident as the gene was expressed by isolate S2 stem B (*Bacillus pumilus*).

Phylogenetic analysis based on 16S rRNA gene sequence suggested that only isolates *Agrobacterium pusense* (R2), *Pseudomonas* sp. (Leaves A1), *Staphylococcus hominis* (LB3), *Pseudomonas japonica* (RzBO), *Kosakonia* sp. (R3), and *Kosakonia* sp. (R5) clustered with the strains they showed high similarity to in BLAST with bootstrap value of 100%.

Isolates (R3, R5) *Kosakonia* sp., (LB7, LB1, LA7) *Cupriavidus metallidurans,* and (R10, R16) *Pseudomonas* sp. exhibited a bootstrap value of 100% each, indicating that these isolates could be the same species. Furthermore, the remaining isolates did not cluster with their NCBI references, suggesting a requirement for more data to determine the relationship between the isolates and their reference strains. This was observed with isolates from the *Cupriavidus* genera, which are understudied and under explored due to less species identified (Arroyo-Herrera *et al.*, 2020).

Cupriavidus metallidurans species have previously been isolated from various types of environments, specifically the rhizosphere with more discoveries expected in the future (Arroyo-Herrera *et al.*, 2020). In this study, *C. metallidurans* was isolated from leaves, stems, and rhizosphere and this is validated by Janssen *et al.* (2010) who reported that *Cupriavidus* species are diverse and are found in a wide range of habitats.

Furthermore, *Kosakonia* has been highlighted as an important novel genus that is involved in plant-bacteria interaction (Mosquito *et al.*, 2020), colonizing different ecological niches, enabling the isolation and characterization of novel strains of non-pathogenic *Kosakonia cowanii* (González Espinosa *et al.*, 2023). Moreover, according to Han *et al.* (2023), *Kosakonia cowanii* is known to be closely related to *Enterobacter*. However, based on our results, the one identified is not related to any of the reference *Enterobacter* strains, neither is there any relation with the *Kosakonia* species identified in our study, thus making it a novel specie. Additionally, *Pseudomonas* species in our study did not cluster with their reference strains. This is consistent with a study by Girard *et al.* (2021) that reported that new *Pseudomonas* species are regularly identified and the number of species within the genus are ever evolving.

3.5 CONCLUSION

Myrothamnus flabellifolius plant that inhabit stressful environments harbour ACC deaminase producing bacteria endowed with PGP traits, including production of IAA, siderophores, HCN, ammonia, and phosphate solubilisation. These findings suggest that these bacteria have the potential to improve plant growth and enhancing drought tolerance in arid regions.

Most isolates did not cluster with their reference strains, which could merit identification of more gene regions from this drought-tolerant plant. *Myrothamnus flabellifolius* have been unexplored and hence identification of new species could serve as a promising tool for bioprospecting microbes for use in sustainable agriculture. However, further exploration of the plant and its associated microbial community are necessary to develop formulations such as biofertilizers that can be integrated in sustainable agricultural practices to reduce the use of chemical fertilizers.

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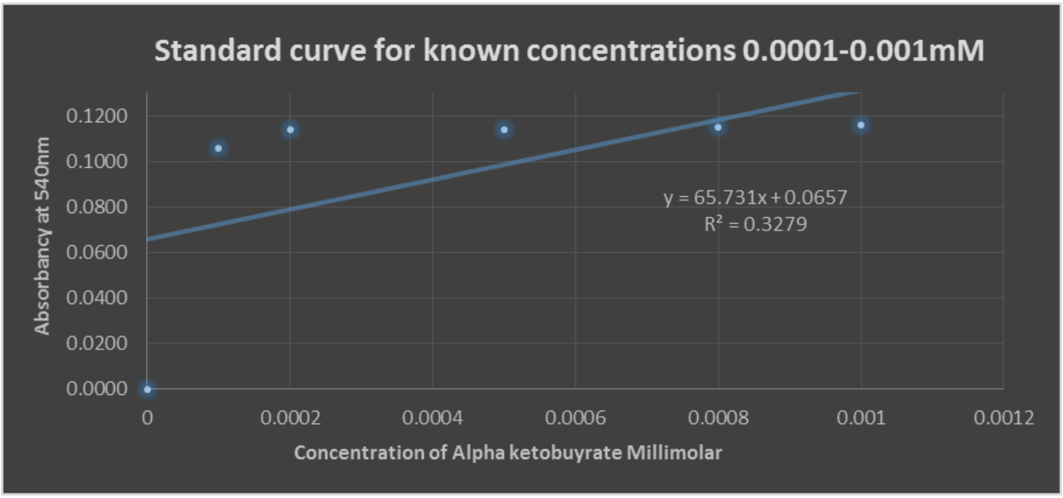
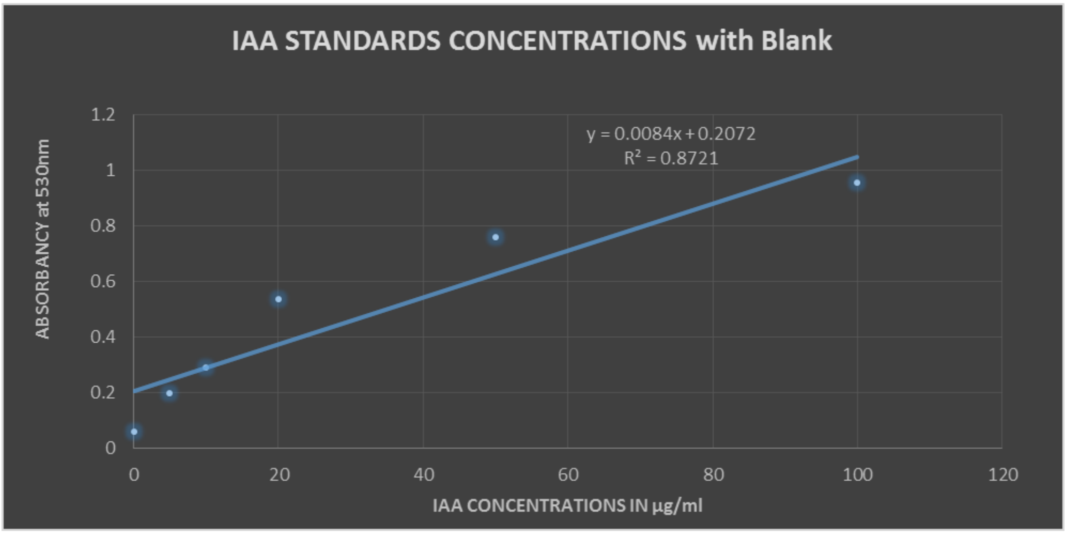
APPENDIX

Appendix Table 3.1: Reference sequences used in phylogenetic alignment and their accession numbers.

Sample Reference	**Reference Sequence**	**NCBI Accession Number**
R2	*Agrobacterium tumifaciens*	MH131676.1
R2	*Agrobacterium pusense*	MW513440.1
R2	*Rhizobium* sp.	MN211548.1
R3	*Enterobacter* sp.	MT270802.1
R3	*Kosakonia* sp.	MT341776.1
R4	*Escherichia hermanii*	JQ311994.1
R4	*Kosakonia cowanii*	MG517427.1
R5	*Enterobacter* sp.	MT270802.1
R6	*Bacillus licheniformis*	OR335072.1
R7	*Bacillus licheniformis*	OR335072.1
R8	*Bacillus* sp.	MN606168.1
R11	*Klebsiella aerogenes*	MT436841.1
R13	*Bacillus licheniformis*	OR335072.1
R14	*Pseudomonas neuropathica*	OM971251.1
R15	*Bacillus albus*	MH197001.1
R15	*Bacillus cereus*	OR243903.1
R16	*Pseudomonas putida*	MT515799.1
R17	*Bacillus safensis*	OQ979024.1
R21	*Klebsiella aerogenes*	MT436840.1
LA3	*Klebsiella*	OK614026.1
LA3	*Enterobacteriaceae*	MH512313.1
LA7	*Cupriavidus metallidurans*	MN543867.1
Leaves A1	*Pseudomonas putida*	MT626818.1
6 Stem A	*Bacillus subtilis*	OR161815.1
S8 stem A	*Pseudomonas gessardi*	MT605328.1
LB1	*Cupriavidus metallidurans*	MN543867.1
LB3	*Staphylococcus haemolyticus*	OQ652562.1
LB7	*Cupriavidus metallidurans*	MN543867.1
LB21	*Bacillus haynesii*	OR144806.1
9 stem B	*Cupriavidus metallidurans*	MN543867.1
S2 stem B	*Bacillus pumilus*	KX896754.1
Stem B S10	*Cellulosimicrobium funkei*	MT527539.1
Stem B S10	*Cellulosimicrobium cellulans*	MT549101.1
RzBO	*Pseudomonas japonica*	MK629783.1
RzW	*Cupriavidus metallidurans*	MN543867.1
R12	*Bacillus amyloliquefaciens*	MT626036.1



Appendix Figure 3.1: Graph of standard curve for known concentration of α-ketobutyrate in Millimolar.



Appendix Figure 3.2: Graph of standard curve for known concentration of IAA 0-100 µg/ml.

CHAPTER 4: EFFECTS OF ACC DEAMINASE-PLANT GROWTH PROMOTING BACTERIA (PGPB) ON SEED GERMINATION AND EARLY SEEDLING GROWTH OF WHEAT UNDER SIMULATED DROUGHT CONDITIONS

ABSTRACT

Drought stress triggers a delay in various developmental stages in plants specifically during germination. Therefore, the ability of seeds to germinate under low water availability during extreme weather conditions is thus an essential physiological trait for successful crop establishment and survival in semi-arid regions by use of plant growth-promoting bacteria to mitigate drought stress in crops. In this present study, three 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing bacteria, *Cupriavidus metallidurans*, *Kosakonia* sp., and *Pseudomonas* sp. were isolated from *Myrothamnus flabellifolius,* a desert-adapted plant. Their effect on germination and early seedling growth of *Triticum aestivum* (wheat) was evaluated by simulating drought using various levels (0%, 10%, 15%, and 30%) of polyethylene glycol (PEG) 6000 solutions with each treatment replicated five times and arranged using a completely randomized block design. Morphological and physiological parameters were measured. Out of 26 isolates, the top two isolates that were able to grow at minimum osmotic potential (30% PEG 6000) were *Staphylococcus hominis* (LB3) 0.52 ± 0.37 and *Bacillus licheniformis* (R13) 0.35 ± 0.18. Inoculation of seeds with *Cupriavidus metallidurans* significantly ($P<0.05$) reduced germination, mean germination time, seedling weight, seedling vigour index, radicle length, shoot length, and number of radicles in contrast to other treatments. Seeds treated under 30% PEG 6000 had significantly ($P<0.05$) reduced shoot length, number of radicles, number of shoots, seedling weight, germination percentage (GP), and seedling vigour index (SVI). In addition, proline levels were significantly reduced by treatment of seeds with *Pseudomonas* sp. Enzymatic activity was significantly influenced by treatment of bacterial isolates, with the control significantly reducing enzymatic activity in contrast to other treatments.

Overall, inoculation of wheat seedlings with *C. metallidurans* did not influence growth of wheat in contrast to the control and other treatments. However, further research is recommended to evaluate the effectiveness of the bacterial treatments in mitigating effects of drought in wheat and other crops under actual field conditions.

Keywords: PEG 6000, germination, proline, plant growth, wheat, *Myrothamnus flabellifolius*

4.1 INTRODUCTION

Agricultural sustainability faces challenges due to the adverse impact of climate change, with particular emphasis on the detrimental effects of drought stress. Notably, drought stress has the potential to significantly diminish the global production of essential staple crops, including maize, wheat, and rice, as highlighted in the study by Yadav, Bhagat and Sharma (2022). Namibia predominantly comprises semi-arid landscapes and is one of the driest countries in sub-Saharan Africa (Barnes, MacGregor and Alberts, 2012). The production of staple crops including maize and pearl millet that ensure food security are adversely negatively affected by consistent recurrent droughts (Shifiona, Dongyang and Zhiquan, 2016). Drought stress usually triggers a delay in various developmental stages in plants such as germination (Camaille *et al.*, 2021). Therefore, the ability of seeds to germinate under low water availability is imperative to establish successful crops more importantly in semi-arid regions (Muscolo *et al.*, 2014). In addition, the availability of water is very critical for enzymatic reactions, as well as for the hydrolytic degradation of molecules in germinating seeds (Muscolo *et al.*, 2014).

Seed germination is regulated by various factors such as phytohormones and environmental conditions, including temperature and light (Liu *et al.*, 2018). During germination, gibberellic acid (GA), produced in the seed embryo, induces the expression of the α-amylase synthesis, which is then secreted into the endosperm to hydrolyse stored starch (Liu *et al.*, 2018). Degradation of this stored starch serves as the major source for seed germination in the endosperm (Damaris *et al.*, 2019; Yuan *et al.*, 2020), as well as for growth of shoots and roots (Yuan *et al.*, 2020).

During stress, proline acts as a metal chelator, signalling molecule, and an oxidative defence molecule (Ashry *et al.*, 2022), and together with antioxidant activities assist plants to withstand drought (Ilyas *et al.*, 2020). During drought stress, protein structures could be denatured, further compromising membrane integrity, thus interaction between proline and enzymes are needed to maintain protein structure and activity (Ashry *et al.*, 2022). Consequently, when proline metabolism is affected by stress such as water deficits within the plant, physiological conditions such as fresh weight, and seed development become affected (Yadav, Bhagat and Sharma, 2022). Therefore, proline could serve as an evaluating parameter for scheduling of irrigated plants and for selecting drought-resistant varieties due to its role during drought stress (Jain *et al.*, 2013).

Wheat (*Triticum aestivum* L.) is one of the most highly-nutritional and cultivated cereal grain in the world (NAB, 2021). However, the crop remains subjected to a variety of biotic and abiotic stresses, which impose deleterious effects to the plant (Dutilloy *et al.*, 2022). The use of beneficial soil microbes with the potential to alleviate stress and promote plant growth could be a useful alternative to improve plant fitness under stressful conditions (Saikia *et al.*, 2018), and achieve sustainable agriculture and increase productivity (Camaille *et al.*, 2021).

Most soil inhabiting microbes are known to interact with plants symbiotically, enhancing their growth via direct and indirect mechanisms (Khan and Bano, 2019). IAA, a phytohormone that enhances root growth and development (Singh and Jha, 2015), is commonly found in bacteria possessing plant growth promoting abilities such as root development and nutrient uptake (Islam *et al.*, 2016). Additionally, IAA-producing bacteria mediate plant growth enhancement from seed germination to maturity (Asaf *et al.*, 2017), as well as enhancing plant growth, and increasing root surface area (Majeed *et al.*, 2015). On the other hand, ACC deaminase-producing bacteria help reduce the deleterious effects of environmental stressors and promote recovery of plants during drought stress (Glick *et al.*, 2018). Therefore, the use of bacteria with multifarious traits could be a potent alternative for ameliorating stress conditions in the plant (Singh and Jha, 2015). Furthermore, positive results have been observed from the use of PGPB on various crops such as wheat, rice, maize and barley (Asaf *et al.*, 2017). However, the utilisation of beneficial microbes from native *Myrothamnus flabellifolius* as bio-inoculants in agricultural crops specifically wheat have not been explored. Inoculation of plants with PGPB have resulted in increased germination rate, root growth, yield, tolerance to drought, shoot and root weight as well as delayed leaf senescence (Minaxi *et al.*, 2012). Wheat has previously been inoculated with PGPB to mitigate the negative impacts of drought stress (Ilyas *et al.*, 2020).

Bacterial isolates belonging to the genera *Pseudomonas* are dominant in the rhizosphere and endosphere of plants under stressful and non-stressful conditions, and are commonly associated with plants with multiple PGP traits (Naderi *et al.*, 2022). These bacteria are also known to produce auxins, which help stimulate plant growth (Barnawal, Singh and Singh, 2019). *Pseudomonas putida* strains have previously been studied as ACC deaminase producers with the ability to impose stress tolerance on plants (Nordstedt and Jones, 2020). Furthermore, *Pseudomonas fluorescens* bacterial isolates have been used as bioinoculants in plants, demonstrating significant increases in various growth parameters (Majeed *et al.*, 2015). The use of PGPB in the form of bioinoculants has widely enhanced growth and yield in crops such as wheat (Majeed *et al.*, 2015). Therefore, the utilisation of PGPB as a tool to promote drought tolerance in vulnerable plants has thus become a promising intervention as global climate change continues to impact the agricultural industry (Yadav, Bhagat and Sharma, 2022). However, there is little to no literature on the utilisation of PGPB from *Myrothamnus fllabellifolius* Welw. with bio-inoculating abilities.

Myrothamnus fllabellifolius generally occurs in arid regions experiencing irregular rainfall patterns (Moore *et al.*, 2007). In Namibia, it occurs in mountainous regions such as the Khomas Hochland and the Spitzkoppe (Moore *et al.*, 2007). Arid plants that survive in drought-prone areas are known to harbour microbes that could confer beneficial effects to cultivated plants (Eke *et al.*, 2019). Hence, the microbiome composition of plants inhabiting arid or drought-prone environments could enhance the drought tolerance ability of crops (Hone *et al.*, 2021).

In this study, three ACC deaminase-producing bacteria namely *Cupriavidus metallidurans* (LB7), *Kosakonia* sp. (R3), and *Pseudomonas* sp. (Leaves A1) were isolated from *M. flabellifolius* and their effect on germination and early seedling growth of wheat cultivar (SST 884) were assessed by simulating drought stress using PEG 6000.

4.2 MATERIALS AND METHODS

4.2.1 Seed surface sterilisation and evaluation of seed viability

Seeds of wheat variety SST 884 (599 local D) produced by Syngenta were obtained from a local market in South Africa and 100 seeds were tested for viability, using 0.1% tetrazolium solution by soaking them in distilled water for 24 hours (Anghinoni *et al.*, 2019). Eight hundred (800) seeds of wheat were soaked in 10% sodium hypochlorite for 5 minutes and washed with sterile water (Wang and Ouyang, 2014). Finally, seeds were washed twice with 70% ethanol for 3 minutes (Khan and Bano, 2019). then rinsed with sterile distilled water 5 times (Omara and Elbagory, 2018). To determine the seed sterility, a 100 µl aliquot was spread onto nutrient agar (NA) and incubated at 28 °C for 24-72 hour.

4.2.2 Screening selected bacterial strains for drought tolerance

Isolates were screened and evaluated for their drought stress tolerance using PEG 6000 at different osmotic levels (5%, 10%, 15% and 30%) along with 0% PEG 6000 as the control (Gupta and Pandey, 2019) amended with 5 ml tryptic soy broth (TSB) (Glick *et al.*, 2018; Niu *et al.*, 2018) in 15 ml tubes via sterile filtration using a 0.22 µm filter. 100 µl of overnight bacterial inoculum was inoculated into the tubes and incubated on a shaker at 28 °C for 24 hours at 120 rpm. Bacterial growth was measured at optical density (OD) 600 nm) using a spectrophotometer (Spectro UV-11, China) (Ali, Sandhya and Rao, 2014; Glick *et al.*, 2018; Niu *et al.*, 2018) and isolates with higher OD under drought simulated conditions were considered to be drought tolerant (Nadeem and Ahmad, 2021).

Three bacterial strains with multifarious growth promoting traits as previously described in chapter 3 (**Table 3.2**) were selected to determine their growth promotion effects on wheat seedlings under various PEG-induced drought stress conditions.

4.2.3 Seed priming and germination test under induced drought conditions

Selected three endophytic bacterial strains endowed with multi-PGP traits, LB7 (*Cupriavidus metallidurans*), R3 (*Kosakonia* sp.), and Leaves A1 (*Pseudomonas* sp.) were isolated from *M. flabellifolius* and used to determine their impact on seed germination, vigour index, shoot and radicle length, seedling weight, proline and α-amylase activity.

The bacterial strains previously kept at -80 degree Celsius in 20% glycerol were cultured in Luria-Bertani (LB) broth and incubated at 28 °C for 24-48 hours at 110 rpm in a shaking incubator (Ilyas *et al.*, 2020) and centrifuged at 4000 rpm for 10 minutes. Their OD was then determined using the Spectro UV-11 machine at 535 nm and adjusted to 0.5 OD with 0.85% (w/v) Sodium chloride (NaCl) (Yadav, Bhagat and Sharma, 2022).

Sterile seeds were soaked in LB broth to allow for seed coating in a shaking incubator at 26-28 °C for 4 hours at 110 rpm (Hone *et al.*, 2021). Four level of drought induced by PEG 6000 solutions, (0%) control, 10%, 15%, 30%, were determined as described by Abido and Zsombik (2020).

During wheat germination under simulated drought conditions, each bacterial treatment was coated on 10 seeds and placed on a sterile Whatman filter paper in 90 mm petri dishes saturated with a volume of 3 ml of PEG 6000 of the respective concentration treatments (Muscolo *et al.*, 2014). The experiment was conducted in a complete randomized design (CRD) and each treatment was replicated five times across four PEG 6000 levels.

The petri dishes were sealed with parafilm wax and incubated at 25 °C in the dark for 8 days (Omara and Elbagory, 2018). Seeds were considered to have germinated when the radicle extended by 2 mm in length from the seed coat (Topacoglu, Sevik and Akkuzu, 2016). Various growth parameters including germination percentage and other related parameters were measured, according to Muscolo *et al*. (2014) at 8 days post inoculation.

The number of germinated seeds after 8 days of incubation was determined according to the method by, Yuan *et al*. (2020).

$$\text{Germination percentage (GP)} = \sum\left(\frac{G}{T}\right) \times 100$$

Where G = total number of germinated seeds and T = total number of seeds per petri dish.

The time it takes for seeds to germinate was determined by mean germination time (MGT). This was used to evaluate emergence of seedlings according to the formulation in Omara and Elbagory (2018).

$$MGT = \sum\left(\frac{n_i \cdot xt_i}{n_i}\right)$$

Where, n_i is the number of germinated seeds on germination day, and t_i is the number of days during the germination period e.g., 0-8 days.

Length of shoot and radicles was measured with a ruler, from the root base to the tip of the shoot, and from the root base to the root tip respectively, in millimetres (mm) for each treatment and replicate after 8 days of germination (Ghosh, Shahed and Robin, 2020).

4.2.4 Seedling vigour index

The rate at which seeds emerged was determined by the SVI. This was determined as the combination of shoot and root length divided by germination percentage in a formula according to Kandasamy *et al.* (2020).

Seedling vigour index (SVI) = Seedling length x Germination %

4.2.5 Determining enzyme activity (α-amylase) and proline content determination (stress indicator)

Alpha amylase was determined by crude extract of the seedlings by the homogenisation of the seedlings in a chilled mortar with distilled water by 1:4 (w/v). The method was performed as described by Muscolo *et al.* (2014) with minor modifications (3 ml soluble starch (2% v/v) and 3 ml crude extract heated in water bath at 37 °C for 15 minutes).

After incubation, an equal volume of alkaline colour reagent 3,5-dinitrosalicylic acid (DNS) was then added to 1 ml of incubation mixture, mixed and heated for five minutes in a boiling water bath. The reaction was terminated by cooling mixture to room temperature by placing tubes in cool water.

The absorbance at 540 nm was measured against a blank (1 ml H_2O plus 1 ml DNS) and the standard curve obtained using different concentrations of maltose in the range of 0-1.0 mg/ml. Determining the Enzyme activity was done according to the formula below by Islam, Aktar and Rahman, (2014).

$$= \frac{\mu g\ maltose\ released\ x\ 1000}{MW\ maltose\ x\ Incubation\ time\ 15min}$$

$$= \mu mol/min$$

About 0.3 g of *Myrothamnus flabellifolius* leaf sample was homogenized in 5 ml of 3% sulphosalicylic acid, and the residue removed by centrifugation at 3000 rpm for 20 minutes. Extract was filtered using straining method instead of the prescribed muslin cloth. This was followed by (1:1:1) reaction of 2 ml extract (supernatant) with 2 ml glacial acetic acid and 2 ml acid ninhydrin and heating mixture for an hour at 100 °C. The reaction was then terminated by immersing in an ice bath, followed by extraction of the chromophore with 4 ml toluene. The absorbance of the chromophore-toluene reaction was then measured at 520 nm and the amount of proline determined from a standard curve prepared from proline (Muscolo *et al.*, 2014).

Proline levels were expressed as micromole per gram (µmole/g) of fresh weight (Ábrahám, Hourton-cabassa and Erdei, 2010).

Proline content was calculated according as per formula by Sharma *et al.* (2022):

Proline µmoles/g Fresh weight = 36.2311 x OD x V/2 x F

Where, 36.2311 is the standard curve value of proline, OD = optical density at 520 nm, V = total volume of extract in mL, F = milligram of fresh weight of leaf taken for one proline estimation, 2 = volume of aliquot taken for proline estimation.

4.2.6 Statistical analysis

All experiments were performed in triplicate and data were tested for normality using the Shapiro-Wilk test. Since, all data were non-parametric, they were analysed using the Kruskal-Wallis *H* test at significance level of $P<0.05$ using the statistical package for the social sciences (SPSS) software version 28.0. Pair-wise comparisons test was used to determine differences among the groups.

4.3 RESULTS

4.3.1 Drought tolerance assay

Among the 29 ACC deaminase producing isolates, only 5 were able to tolerate drought stress at the highest percentage level of PEG 6000 (30%); *Staphylococcus hominis* (LB3) **(0.52 ± 0.37)**, *Bacillus licheniformis* (R13) (0.35 ± 0.18), *Pseudomonas japonica* (RzBO) (0.34 ± 0.13), *Bacillus licheniformis* (R7) (0.31 ± 0.03) and *Cellulosimicrobium funkei* (Stem B S10) (0.30 ± 0.04). These 5 isolates were able to grow at minimum osmotic potential with *Staphylococcus hominis* and *Bacillus licheniformis* showing significant growth under severe drought stress as shown in **Table 4.1** below.

Table 4.1: Growth (OD_{600}) of plant growth-promoting bacteria (PGPB) at different concentrations of polyethylene glycol (PEG 6000) (Drought tolerance test). Error bars are mean of ±SD (n=3).

			PEG-6000 Concentration					
Isolates	**Specie**	**Sample origin**	**0%**	**5%**	**10%**	**15%**	**20%**	**30%**
Control	*Control*		0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01
R14	*Pseudomonas neuropathica*	Roots	0.94 ± 0.00	0.39 ± 0.24	0.50 ± 0.04	0.21 ± 0.05	0.53 ± 0.28	0.22 ± 0.04
LB21	*Bacillus licheniformis*	Leaves	0.22 ± 0.61	0.23 ± 0.11	0.17 ± 0.05	0.18 ± 0.10	0.25 ± 0.20	0.12 ± 0.00
Leaves A1	*Pseudomonas* sp.	Leaves	0.32 ± 0.41	0.41 ± 0.23	0.27 ± 0.06	0.39 ± 0.13	0.50 ± 0.23	0.14 ± 0.02
RzW	*Cupriavidus metallidurans*	Rhizobacteria	1.08 ± 0.04	0.58 ± 0.04	0.42 ± 0.13	0.41 ± 0.21	0.29 ± 0.10	0.22 ± 0.04
R3	*Kosakonia* sp.	Roots	0.93 ± 0.10	0.50 ± 0.06	0.43 ± 0.10	0.33 ± 0.08	0.22 ± 0.03	0.29 ± 0.18
RzBO	*Pseudomonas japonica*	Rhizobacteria	1.27 ± 0.02	0.59 ± 0.05	0.64 ± 0.05	0.45 ± 0.17	0.45 ± 0.27	0.34 ± 0.13
R5	*Kosakonia* sp.	Roots	0.99 ± 0.13	0.55 ± 0.13	0.31 ± 0.10	0.50 ± 0.15	0.42 ± 0.16	0.29 ± 0.12
R15	*Bacillus cereus*	Roots	0.04 ± 0.00	0.04 ± 0.00	0.19 ± 0.18	0.33 ± 0.30	0.06 ± 0.04	0.03 ± 0.00
S8 STEM A	*Pseudomonas lactis*	Stem	3.03 ± 0.36	0.73 ± 0.29	0.89 ± 0.11	0.34 ± 0.05	0.24 ± 0.02	0.21 ± 0.00
R2	*Agrobacterium pusense*	Roots	1.89 ± 0.17	0.37 ± 0.05	0.36 ± 0.03	0.24 ± 0.03	0.20 ± 0.04	0.17 ± 0.01
R8	*Bacillus* sp.	Roots	1.67 ± 0.47	0.57 ± 0.03	1.26 ± 1.22	0.40 ± 0.12	0.26 ± 0.01	0.27 ± 0.05
R21	*Klebsiella aerogenes*	Roots	10.16 ± 1.52	1.02 ± 0.06	0.84 ± 0.03	0.35 ± 0.02	0.37 ± 0.04	0.29 ± 0.02
LB7	*Cupriavidus metallidurans*	Leaves	3.95 ± 3.18	0.67 ± 0.04	0.94 ± 0.18	0.36 ± 0.00	0.31 ± 0.05	0.30 ± 0.06
STEM B S10	*Cellulosimicrobium funkei*	Stem	2.04 ± 0.24	0.43 ± 0.04	0.53 ± 0.19	0.43 ± 0.08	0.39 ± 0.03	0.30 ± 0.04
R6	*Bacillus licheniformis*	Roots	1.36 ± 0.19	1.24 ± 0.37	1.00 ± 0.72	0.64 ± 0.66	0.19 ± 0.09	0.16 ± 0.01
R7	*Bacillus licheniformis*	Roots	2.12 ± 0.22	0.42 ± 0.03	0.42 ± 0.00	0.41 ± 0.03	0.33 ± 0.03	0.31 ± 0.03
R19	*Bacillus safensis*	Roots	1.49 ± 1.34	0.42 ± 0.03	0.35 ± 0.03	0.40 ± 0.09	0.35 ± 0.06	0.26 ± 0.02
R4	*Kosakonia cowanii*	Roots	3.44 ± 1.39	0.56 ± 0.12	0.56 ± 0.41	0.68 ± 0.69	0.28 ± 0.06	0.21 ± 0.09
R17	*Bacillus safensis*	Roots	2.81 ± 0.38	0.47 ± 0.06	0.37 ± 0.02	0.40 ± 0.04	0.35 ± 0.02	0.29 ± 0.03
R18	*Providencia* sp.	Roots	1.72 ± 0.99	0.54 ± 0.28	0.62 ± 0.28	0.32 ± 0.12	0.37 ± 0.14	0.24 ± 0.02

Table 4.1 (continued).

STEM B S2	*Bacillus pumilis*	Stem	0.62 ± 0.23	0.14 ± 0.02	0.19 ± 0.07	0.18 ± 0.05	0.12 ± 0.04	0.14 ± 0.01
STEM A 6	*Bacillus subtilis*	Stem	0.40 ± 0.12	0.18 ± 0.07	0.27 ± 0.13	0.21 ± 0.12	0.12 ± 0.01	0.09 ± 0.01
LB1	*Cupriavidus metallidurans*	Leaves	0.78 ± 0.26	0.31 ± 0.06	0.17 ± 0.09	0.18 ± 0.06	0.14 ± 0.02	0.13 ± 0.02
R13	*Bacillus licheniformis*	Roots	1.04 ± 0.52	0.69 ± 0.43	0.75 ± 0.50	1.02 ± 0.59	0.48 ± 0.25	0.35 ± 0.18
LA7	*Cupriavidus metallidurans*	Leaves	1.60 ± 0.06	0.90 ± 0.20	0.54 ± 0.23	0.43 ± 0.14	0.44 ± 0.32	0.23 ± 0.01
LB3	*Staphylococcus hominis*	Leaves	3.39 ± 1.03	0.41 ± 0.39	1.10 ± 0.46	0.55 ± 0.23	0.39 ± 0.28	0.52 ± 0.37

4.3.2 Effects of bacterized and non-bacterized treatments on seeds that germinated under simulated drought conditions using PEG 6000

The effect of water stress on seed germination for the period of the experiment was assessed by the germination percentage across all treatments. The number of seeds that germinated under simulated drought conditions across treatments over a period of 8 days differed significantly ($P<0.05$) as shown in (**Appendix Figure 4.1**).

4.3.2.1 Effects on seeds that germinated under control

The number of seeds that germinated under control differed significantly ($P<0.05$) (H=18.882, df=3). Pair-wise comparison showed that *C. metallidurans* significantly decreased germination in contrast to control ($P=0.011$, $H=11.813$, df=3), and *Kosakonia* sp. ($P<0.001$, $H=-19.312$, df=3) as seen in (**Table 4.2**) below. Additionally, pair-wise comparison showed that *Pseudomonas* sp. significantly decreased germination in compared to *Kosakonia* sp. ($P=0.004$), $H=13.438$, df=3). However, no significant differences ($P>0.05$) were observed in the number of seeds that germinated upon inoculation with *C. metallidurans* and *Pseudomonas* sp. ($P=0.207$, $H=-5.875$, df=3), *Pseudomonas* sp. and the control ($P=0.202$, $H=5.938$, df=3), as well as the control and *Kosakonia* sp. ($P=0.107$, $H=-7.500$, df=3).

4.3.2.2 Effects on seeds that germinated under 10% PEG 6000

The number of seeds that germinated under 10% PEG 6000 across treatments significantly differed ($P<0.05$) ($H=21.965$, df=3). Pair-wise comparison showed that *C. metallidurans* significantly decreased germination in contrast to *Kosakonia* sp. ($P=0.020$, $H=-10.875$, df=3), *Pseudomonas* sp. ($P=0.008$, $H=-12.312$, df=3), and the control ($P<0.001$, $H=21.813$, df=3). Additionally, pair-wise comparison showed that inoculation of seeds with the control (non-bacterized) significantly increased germination compared to *Kosakonia* sp. ($P=0.019$, $H=10.938$, df=3), and

Pseudomonas sp. (*P*=0.042, *H*=9.500, df=3). However, no significant difference (*P*>0.05) was observed in the number of seeds that germinated upon inoculation with *Kosakonia* sp. and *Pseudomonas* sp. (*P*=0.758, *H*=-1.437, df=3). The study further highlights that the control was more effective in germination of seeds upon the induction of drought in contrast to *Pseudomonas* sp., *Kosakonia* sp., and *C. metallidurans*.

4.3.2.3 Effects on seeds that germinated under 15% PEG 6000

The number of seeds that germinated under 15% PEG 6000 across treatments differed significantly (*P*<0.05) (*H*=25.734, df=3). Pair-wise comparison showed that *C. metallidurans* significantly decreased germination in contrast to *Kosakonia* sp. (*P*=0.048, *H*=-9.250, df=3), *Pseudomonas* sp. (*P*<0.001, *H*=-16.125, df=3), and the control (*P*<0.001, H=22.625, df=3). Pair-wise comparison further showed that *Kosakonia* sp. significantly decreased germination compared to the control (*P*=0.004, *H*=13.375, df=3). However, no significant differences (*P*>0.05) were observed in the number of seeds that germinated upon inoculation of seeds with *Kosakonia* sp. and *Pseudomonas* sp. (*P*=0.142, *H*=-6.875, df=3), as well as *Pseudomonas* sp. and the control (*P*=0.165, *H*=6.500, df=3).

4.3.2.4 Effects on seeds that germinated under 30% PEG 6000

The number of seeds that germinated under 30% PEG 6000 across treatments differed significantly (*P*<0.05) (*H*=18.102, df=3). Pair-wise comparison showed that *C. metallidurans* significantly decreased germination compared to *Kosakonia* sp. (*P*=0.009, *H*=-12.125, df=3), *Pseudomonas* sp. (*P*<0.01), *H*=-19.437, df=3) and the control (*P*=0.006, *H*=12.938, df=3). However, no significant differences (*P*>0.05) were observed in the number of seeds that germinated upon inoculation with *Kosakonia* sp. and the control (*H*=0.813, df=3), *Kosakonia* sp. and *Pseudomonas* sp.

(*H*=-7.312, df=3), as well as the control and *Pseudomonas* sp. (*H*=-6.500, df=3). This suggests that *Kosakonia* sp., *Pseudomonas* sp., and the control exhibited the same effect.

4.3.3 Effect of bacterial isolates on mean germination time (number of seeds germinated per day)

The effects of endophytic bacterial inoculation on wheat seeds germination were assessed. The number of seeds that germinated daily significantly differed across bacterial isolates

(*P*<0.05, *H*=214.937, df=3) (**Figure 4.1**).

Pair-wise comparison showed that inoculation with *C. metallidurans* significantly decreased the number of seeds that germinated daily in contrast to *Kosakonia* sp. (*P*=0.000, *H*=-214.206, df=3), *Pseudomonas* sp. (*P*=0.000, *H*=-223.331, df=3) and the control (*P*=0.000, *H*=278.725, df=3).

Moreover, pair-wise comparison showed that non-bacterized seeds (control) had a significantly higher number of seeds that germinated in contrast to *Kosakonia* sp. and *Pseudomonas* sp. (*P*=0.002, *H*=64.519, df=3 and *P*=0.007, *H*=55.394, df=3) respectively. However, inoculation of *Kosakonia* sp. and *Pseudomonas* sp. did not significantly enhance seed germination (*P*=0.656, *H*=-9.125, df=3). Overall, the results suggest that the inoculants did not exhibit a favourable impact on number of seeds that germinated compared to the control as shown in (**Figure 4.1**) below. Furthermore, germination time (per day) did not differ significantly across the different bacterial isolates (*P*=0.922, *H*=0.485, df=3) for control and simulated drought.

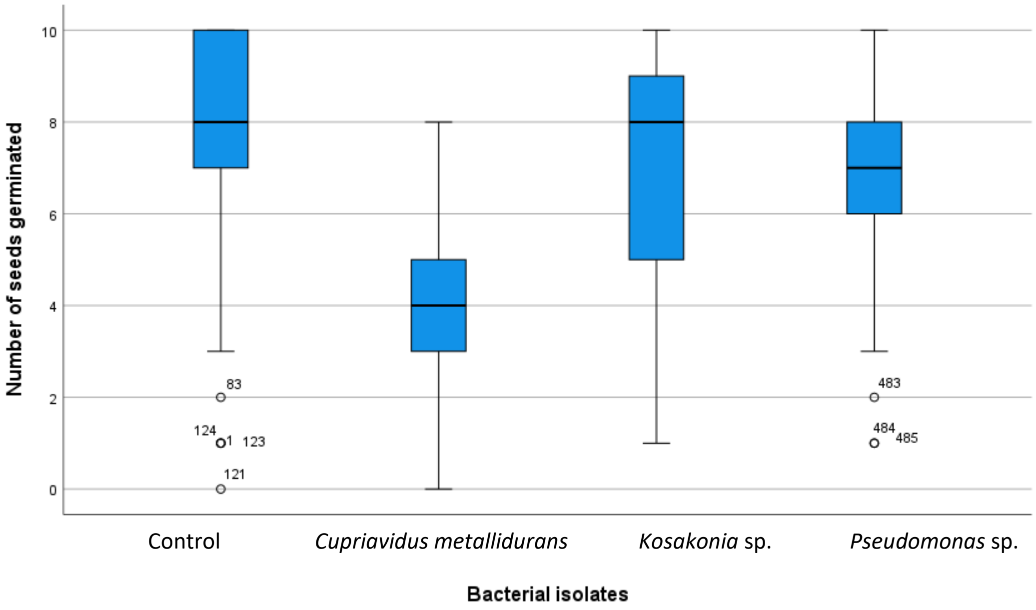


Figure 4.1: Effect of bacterization on germination of wheat seeds.

Table 4.2: Mean germination time indicating number of seeds germinated per day. Error bars are mean of ±SD (n=5, for Germination %) (n=8, number of seeds germinated per day).

Treatments	PEG 6000 (%)	Germination percentage (%)	MGT (number of seeds germinated per day)
Control	0	80 ± 7.07	4 ± 2.57
	10	96 ± 8.94	5 ± 3.03
	15	92 ± 10.95	5 ± 2.91
	30	70 ± 21.21	4 ± 2.40
C. metallidurans	0	62 ± 10.95	3 ± 2.00
	10	56 ± 13.42	3 ± 1.85
	15	38 ± 13.04	2 ± 1.20
	30	26 ± 5.48	1 ± 0.82
***Kosakonia* sp.**	0	90 ± 7.07	5 ± 2.78
	10	82 ± 13.04	4 ± 2.73
	15	80 ± 18.71	4 ± 2.63
	30	64 ± 27.02	3 ± 2.12
***Pseudomonas* sp.**	0	72 ± 10.95	4 ± 2.45
	10	82 ± 8.37	4 ± 2.64
	15	84 ± 11.40	5 ± 2.66
	30	70 ± 12.25	4 ± 2.24

4.3.4 Effect of PEG 6000 on mean germination time (number of seeds germinated per day)

There was a significant difference in the number of seeds that germinated under various PEG 6000 drought treatments ($P<0.05$, $H=73.546$) (**Figure 4.2**). Pair-wise comparison test revealed that 30% PEG 6000 significantly decreased the number of seeds that germinated in contrast to 15% PEG 6000 ($H=126.238$, df=3), 10% PEG 6000 ($H=154.756$, df=3), and 0% PEG 6000 (control) ($H=143.956$, df=3). However, there was no significant difference ($P>0.05$) in number of seeds that germinated between seeds treated under PEG concentrations 15%, 10% and 0% (control), 15% PEG 6000 and 0% PEG 6000 ($P=0.388$, $H=17.719$, df=3), 15% PEG 6000 and 10% PEG 6000 ($P=0.164$, $H=28.519$, df=3), and 0% PEG 6000 and 10% PEG 6000 ($P=0.598$, $H=-10.800$, df=3).

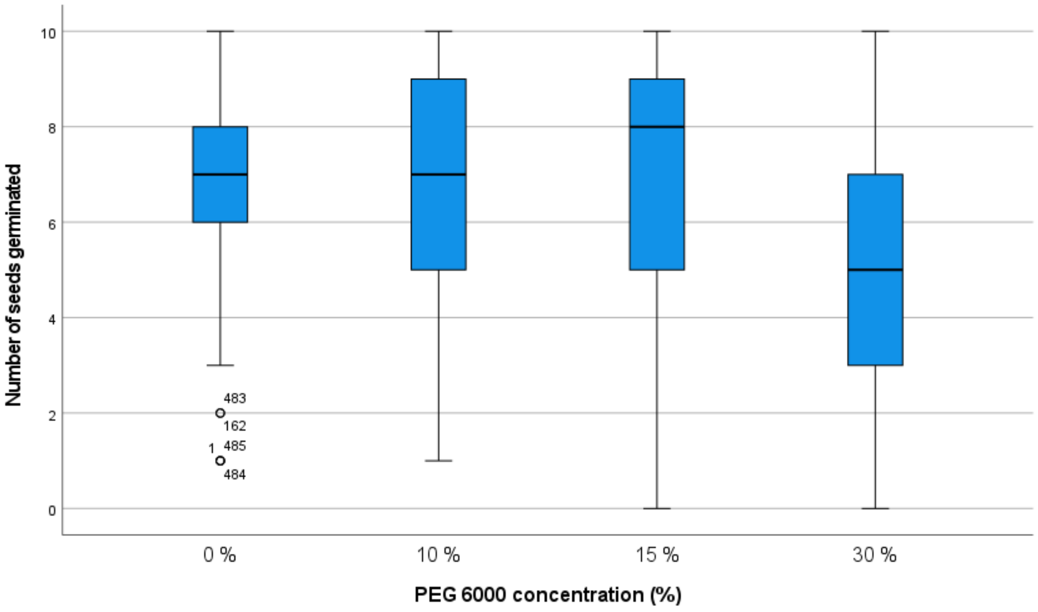


Figure 4.2: Number of germinated wheat seeds treated under various PEG 6000 drought levels.

4.3.5 Effect of bacterization on selected growth parameters of wheat

Inoculation of bacterial isolates on wheat seeds significantly influenced shoot length, radicle length, number of radicles, seedling weight, germination percentage and seedling vigour (*P*<0.05) (**Appendix Figures 4.2**) except for the number of shoots (*P*=0.077, *H*=6.853, df=3).

Pair-wise comparison showed that seeds treated with *C. metallidurans* significantly decreased seedling weight in contrast to *Pseudomonas* sp. (*P*=0.009, *H*=-19.150, df=3), *Kosakonia* sp. (*P*=0.000, *H*=-28.900, df=3), and the control (*P*=0.001, *H*= 23.350, df=3).

However, seedling weight did not significantly differ between seeds inoculated with *Pseudomonas* sp. and the control (*P*=0.568, *H*=4.200, df=3), *Pseudomonas* sp. and *Kosakonia* sp. treated seeds (*P*=0.185, *H*=9.750, df=3) as well as between the control and *Kosakonia* sp. treated seeds (*P*=0.450, *H*=-5.550, df=3).

Overall, inoculating seeds with *C. metallidurans* was less effective in increasing seedling weight compared to other treatments.

Pair-wise comparison showed that inoculating seeds with *C. metallidurans* significantly decreased germination percentage in contrast to *Pseudomonas* sp. treated seeds (*P*=0.000, *H*=-27.600, df=3), *Kosakonia* sp. (*P*=0.000, *H*=32.557, df=3) and the control (*P*=0.000, *H*=38.375, df=3). However, germination percentage did not differ significantly (*P*>0.05) between *Pseudomonas* sp. and *Kosakonia* sp. (*P*=0.542, *H*=4.425, df=3), *Pseudomonas* sp. and the control (*P*=0.138, *H*=10.775, df=3), as well as between *Kosakonia* sp. and the control (*P*=0.382, *H*=6.350, df=3). Suggesting that *C. metallidurans* treated seeds showed the least influence on germination percentage.

Cupriavidus metallidurans treated seeds significantly decreased the SVI in contrast to *Pseudomonas* sp. treated seeds (*P*=0.000, *H*=-29.400, df=3), *Kosakonia* sp. treated seeds (*P*=0.000, *H*=-35.225, df=3) and the control (*P*=0.000, *H*=47.275, df=3).

Pair-wise comparison further demonstrated that SVI did not differ significantly (*P*>0.05) in seeds inoculated with *Pseudomonas* sp. and *Kosakonia* sp., (*H*=5.825, df=3) as well as between *Kosakonia* sp. and the control (*H*=12.050, df=3). Furthermore, the results demonstrate that treatment of seeds with *C. metallidurans* significantly showed the least effect on SVI.

Radicle length significantly differed across treatments (*P*<0.005, *H*=58.887, df=3) except between *Kosakonia* sp. and *Pseudomonas* sp. (*P*=0.876, *H*=1.150, df=3).

Pair-wise comparison showed that seeds treated with *C. metallidurans* significantly decreased radicle length in contrast to *Pseudomonas* sp. treated seeds (*P*=0.000, *H*=-28.850, df=3), *Kosakonia* sp. treated seeds (*P*=0.000, *H*=-30.00, df=3) and the control (*P*=0.000, *H*=56.350, df=3). Seeds treated with the control significantly increased radicle length in contrast to *Pseudomonas* sp. (*P*=0.000, *H*=27.500, df=3), and *Kosakonia* sp. treated seeds (*P*=0.000, *H*=26.350, df=3).

The results suggest that non-bacterized seeds (control) had the highest effect on radicle length in contrast to all bacterized seeds, with *C. metallidurans* treated seeds showing the least effect on radicle length among all the treatments.

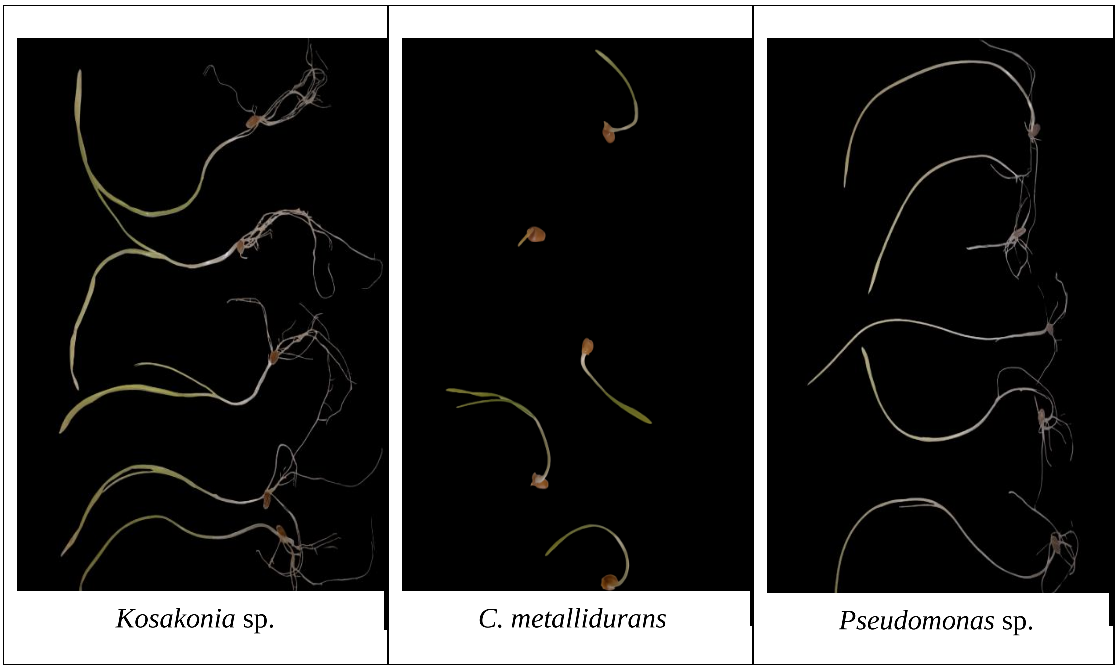
Cupriavidus metallidurans treated seeds significantly decreased shoot length in contrast to the control (*P*=0.006, *H*=20.350, df=3), *Pseudomonas* sp. (*P*=0.003, *H*=-22.175, df=3), and *Kosakonia* sp. (*P*=0.000, *H*=-31.075, df=3). However, pair-wise comparison showed that shoot length did not significantly differ between seeds treated with the control and *Pseudomonas* sp. (*P*=0.804, *H*=-1.825, df=3), control and *Kosakonia* sp. (*P*=0.144, *H*=-10.725, df=3), as well as *Pseudomonas* sp. and *Kosakonia* sp. (*P*=0.226, *H*=8.900, df=3). The results further suggest that treatment of seeds with *C. metallidurans* significantly showed the least effect in shoot length of seeds. This can be seen in **Figures 4.3 and 4.4** below.

Pair-wise comparison showed that *C. metallidurans* significantly decreased the number of radicles in contrast to the control (*P*=0.002, H=22.300, df=3), *Pseudomonas* sp. treated seeds (*P*=0.000, *H*=-34.800, df=3) and *Kosakonia* sp. treated seeds (*P*=0.006, *H*=-40.500, df=3). However, pair-wise comparison showed that the number of radicles did not significantly differ between seeds treated with the control and *Pseudomonas* sp. (*P*=0.088, *H*=-12.500, df=3), the control and *Kosakonia* sp. (*P*=0.013, *H*=-18.200, df=3), as well as *Pseudomonas* sp. and *Kosakonia* sp. (*P*=0.437, *H*=5.700, df=3). Furthermore, the study suggests that seeds treated with *C. metallidurans* showed the least influence on number of radicles, in contrast to other treatments as seen in **Figure 4.4** below.

Control | *Kosakonia* sp. | *C. metallidurans* | *Pseudomonas* sp.

Figure 4.3: Response of wheat seeds treated with (*Kosakonia* sp., *C. metallidurans* and *Pseudomonas* sp.) under stressed conditions.

Figure 4.4 below shows the effects of wheat seeds under various treatments, with growth parameters such as number of radicles, radicle length, and shoot length significantly increased and decreased upon inoculation with *Kosakonia* sp. and *C. metallidurans*, respectively.



Kosakonia sp. *C. metallidurans* *Pseudomonas* sp.

Figure 4.4: Response of wheat seeds treated with (*Kosakonia* sp., *C. metallidurans* and *Pseudomonas* sp.) under non-stressed conditions.

The results above show that the effects of bacterization on wheat seeds varied across all growth parameters with *C. metallidurans* treated seeds exhibiting the least effect as shown in (**Appendix Table 4.1**).

4.3.6 Effects of PEG induced drought treatment on selected growth parameters of wheat

PEG-induced drought stress significantly ($P<0.05$) influenced shoot length ($H=47.903$, df=3), number of shoots ($H=33.776$, df=3), number of radicles

(*H*=15.681, df=3), seedling weight (*H*=44.521, df=3), germination percentage (*H*=9.256, df=3), and SVI (*H*=21.160, df=3). However, no significant effect of PEG induced drought stress (*P*>0.05) was observed on the radicle length across the seedlings (**Appendix Figure 4.3**).

Pair-wise comparison demonstrated that seeds treated under 30% PEG 6000 concentrations significantly showed less effect on shoot length in contrast to 15% PEG 6000 (*P*=0.000, *H*=27.450. df=3), 10% PEG 6000 (*P*=0,000, *H*=43.100, df=3) and 0% PEG 6000 (*P*=0.000, *H*=44.850, df=3).

Furthermore, seeds treated with 15% PEG 6000 significantly decreased shoot length in contrast to seeds treated under 10% PEG 6000 (*P*=0.033, *H*=15.650, df=3) as well as 0% PEG 6000 (*P*=0.018, *H*=17.400, df=3). However, pair-wise comparison showed that there was no significant difference in shoot length of seeds treated with 10% PEG 6000 and 0% PEG 6000 (*P*=0.812, *H*=1.750, df=3). The results suggest that seeds treated under 30% PEG 6000 showed the least effect on shoot length in contrast to other treatments.

Pair-wise comparison showed that seeds treated under 30% PEG 6000 significantly reduced the number of shoots in contrast to seeds treated under 10% PEG 6000 (*P*=0.001, *H*=20.950, df=3), and 0% PEG 6000 (*P*=0.000, *H*=34.950, df=3).

Pair-wise comparison showed that seeds treated under 15% PEG 6000 significantly reduced the number of shoots in contrast to seeds treated under 10% PEG 6000 (*P*=0.049, *H*=12.650, df=3), and under 0% PEG 6000 (*P*=0.000, *H*=26.650, df=3). Pair-wise comparison further showed that treatment of seeds under 10% PEG 6000 significantly reduced the number of shoots compared to seeds treated under 0% PEG 6000 (*P*=0.030, *H*=14.000, df=3).

However, seeds treated with 30% PEG 6000 and 15% PEG 6000 showed no significant difference (*P*=0.197, *H*=8.300, df=3) on the number of shoots. The results therefore suggest that treatment of seeds with 0% PEG 6000 (control) showed the highest effect on number of shoots in contrast to all treatments.

Seeds treated under 30% PEG 6000 had significantly reduced the number of radicles in contrast to seeds treated under 0% PEG 6000 (*P*=0.046, *H*=14.600, df=3), 15% PEG 6000 (*P*=0.008, *H*=19.350, df=3) and 10% PEG 6000 (*P*=0.000, *H*=28.350, df=3).

However, radicles did not significantly differ (*P*>0.05) between seeds treated with 0% PEG 6000 and 15% PEG 6000 (*P*=0.517, *H*=-4.750, df=3), 0% PEG 6000 and 10% PEG 6000 (*P*=0.061, *H*=-13.750, df=3), and 15% PEG 6000 and 10% PEG 6000 (*P*=0.219, *H*=9.000, df=3). The results suggest that treatment of seeds with 30% PEG 6000 showed the least effect on number of radicles in contrast to all treatments.

Treatment of seeds with 30% PEG 6000 significantly reduced the seedling weight in contrast to seeds treated under 15% PEG 6000 (*P*=0.001, *H*=23.750, df=3), 10% PEG 6000 (*P*=0.000, *H*=41.250, df=3), and 0% PEG 6000 (*P*=0.000, *H*=43.100, df=3). Pair-wise comparison showed that seeds treated under 15% PEG 6000 significantly reduced the seedling weight in contrast to seeds treated under 10% PEG 6000 (*P*=0.017, *H*=17.500, df=3), and 0% PEG 6000 (*P*=0.008, *H*=19.350, df=3).

However, pair-wise comparison showed that seeds treated with 10% PEG 6000 and 0% PEG 6000 showed no significant difference (*P*=0.801, *H*=1.850, df=3) on seedling weight. Therefore, the results suggest that treatment of seeds with 30% PEG 6000 showed the least effect on seedling weight in contrast to all treatments.

Seeds treated under 30% PEG 6000 significantly reduced germination percentage in contrast to seeds treated under 0% PEG 6000 (*P*=0.032, *H*=15.550, df=3), 15% PEG 6000 (*P*=0.024, *H*=16.357, df=3) and 10% PEG 6000 (*P*=0.005, *H*=20.575, df=3).

However, pair-wise comparison showed that germination percentage did not significantly differ (*P*>0.05) between seeds treated with 0% PEG 6000 and 15% PEG 6000 (*P*=0.910, *H*=-0.825, df=3), 0% PEG 6000 and 10% PEG 6000 (*P*=0.489, *H*=-5.025, df=3), and 15% PEG 6000 and 10% PEG 6000 (*P*=0.563, *H*=4.200, df=3). The above results suggest that treatment of seeds with 30% PEG 6000 showed the least effect on germination in contrast to all treatments.

Treatment of seeds with 30% PEG 6000 significantly reduced SVI in contrast to seeds treated under 15% PEG 6000 (*P*=0.005, *H*=20.775, df=3), 0% PEG 6000 (*P*=0.001, *H*=25.575, df=3).and 10% PEG 6000 (*P*=0.000, *H*=31.850, df=3). However, pair-wise comparison showed that SVI did not significantly differ (*P*>0.05) between seeds treated with 15% PEG 6000 and 0% PEG 6000 (*P*=0.514, *H*=4.800, df=3), 15% PEG 6000 and 10% PEG 6000 (*P*=0.132, *H*=11.075, df=3), and 0% PEG 6000 and 10% PEG 6000 (*P*=0.393, *H*=6.275, df=3). Suggesting that treatment of seeds with 30% PEG 6000 showed the least effect on SVI in contrast to all treatments.

4.3.7 Effect of bacterial isolates and PEG 6000 concentrations on proline levels of wheat seedlings

Seeds under selected PEG 6000 concentrations showed no significant difference (*P*=0.982, *H*=0.174, df=3) in proline levels. However, treatment of seeds with selected bacterial isolates significantly differed (*P*<0.05, *H*=32.406, df=3) (**Appendix Figure 4.4**).

Pair-wise comparison showed that *Pseudomonas* sp. significantly reduced proline levels in contrast to seeds treated with the control (*P*=0.002, *H*=17.583, df=3), *Kosakonia* sp. (*P*=0.000, *H*=23.000, df=3), and *C. metallidurans* (*P*=0.000, *H*=31.417, df=3). Furthermore, pair-wise comparison showed that treatment of seeds with the control significantly reduced proline levels in contrast to seeds treated with *C. metallidurans* (*P*=0.015, *H*=-5.417, df=3).

However, proline levels did not significantly differ between seeds inoculated with the control and *Kosakonia* sp. (*P*=0.343, *H*=-5.417, df=3), as well as *Kosakonia* sp. and *C. metallidurans* treated seeds (*P*=0141, *H*=8.417, df=3).

The results further suggest that inoculating seeds with *Pseudomonas* sp. was less effective in increasing proline levels in contrast to other treatments (**Appendix Table 4.2**).

4.3.8 Effect of bacterial isolates and PEG 6000 concentrations on enzymatic activity (α-amylase) of wheat seedlings

Enzymatic activity (measured as µmoles/min maltose) significantly differed among different treatments of bacterial isolates (*P*=0.029, *H*=9.024, df=3) (**Appendix Figure 4.5**). However, the levels of enzymatic activity did not significantly differ across different concentrations of PEG 6000 (*P*=0.364, *H*=3.188, df=3).

Pair-wise comparison showed that seeds treated with the *C. metallidurans* significantly increased enzymatic activity in contrast to seeds treated with the control (*P*=0.005, *H*=-16.000, df=3), and *Pseudomonas* sp. (P=0.021, *H*=13.167, df=3). However, pair-wise comparison showed that enzymatic activity did not significantly differ between seeds treated with the control and *Pseudomonas* sp. (*P*=0.620, *H*=-

2.833, df=3), control and *Kosakonia* sp. (*P*=0.398, *H*=-4.833, df=3), *Pseudomonas* sp. and *Kosakonia* sp. (*P*=0.726, *H*=2.000, df=3), as well as *Kosakonia* sp. and *C. metallidurans* (*P*=0,051, *H*=11.167, df=3).

The results further suggest that inoculating seeds with the control showed the least effect in enzymatic activity in contrast to other treatments (**Appendix Table 4.3**).

4.4 DISCUSSION

The present study is the first report exploring the potential of PGPB isolated from *M. flabellifolius* with growth promoting abilities in wheat seeds under drought simulations. The study aimed at assessing germination and early growth of wheat seedlings tolerance to drought via seed bacterization by three bacteria, *Kosakonia* sp., *Pseudomonas* sp., and *Cupriavidus metallidurans* endowed with multifarious growth promoting traits.

Our findings indicated that drought tolerance was observed at the highest level of 30% PEG 6000 from *Bacillus licheniformis* (R13, R7), *Pseudomonas japonica* (RzBO), *Staphylococcus hominis* (LB3) and *Cellulosimicrobium funkei* (Stem B S10). These results are in accord with a study by Sati, Pande and Samant (2023), which suggested that bacterial strains belonging to genera *Bacillus*, *Pseudomonas* and *Staphylococcus* were endowed with the ability to mitigate drought stress in wheat plants when evaluated in varying concentrations of PEG 6000.

Additionally, Ali, Sandhya and Rao (2014) reported that *Pseudomonas* sp. are known ACC deaminase producers with the ability to tolerate high levels of drought stress. Thus, inoculation of PGPB endowed with ACC deaminase such as *Pseudomonas* sp. could significantly improve growth of wheat under water deficit conditions to alleviate drought stress as manifested in crops such as wheat (Munir *et al.*, 2008;

Chandra, Srivastava and Sharma, 2018). Both ACC and the phytohormone, IAA are known to work synergistically in promoting plant growth by lowering harmful ethylene levels during drought stress, further resulting in root elongation (Chandra, Srivastava and Sharma, 2018).

According to the study's results, seeds treated with *Kosakonia* sp. exhibited significant growth in number of radicles and shoot length. Plant assays for seed germination revealed that bacteria belonging to the *Kosakonia* genus could stimulate plant growth, particularly root elongation, given their ability to produce phytohormones and various PGP traits (similar to our study) (Haiyambo, Reinhold-Hurek and Chimwamurombe, 2015; Berger *et al.*, 2018; Eid *et al.*, 2021; Ali and El-kholy, 2022). This is in accord with a study by Berger *et al*. (2018) were *Kosakonia* sp. were previously isolated from wheat plants due to their growth promoting capacity of synthesizing IAA, an observation which was detected in the *Kosakonia* sp. that was previously isolated from *M. flabellifolius*.

Our results further revealed that non-bacterized wheat yielded high germination percentage in contrast to bacterized wheat, thus indicating that bacterization did not improve germination. However, *Pseudomonas* sp. treated seeds significantly exhibited the highest number of germinated seeds across 8 days. To support this, both *Pseudomonas* sp. and *Bacillus* sp. endowed with ACC deaminase have been known to have the ability to increase seed germination as a mechanism to tolerate drought stress according to Abdelaal *et al.* (2021). Furthermore, there is evidence from previous studies suggesting reduced germination by bacterial inoculation in contrast to the control (Naderi *et al.*, 2022), and our observations are in accord with this report.

In addition, Naderi *et al.* (2022) further reported that *Pseudomonas* isolates with IAA producing traits have decreased germination in wheat due to a decrease in enzymatic activity as a result of insufficient water in the soil, which consequently inhibits the breakdown of starch into soluble sugar e.g., maltose, further decreasing germination (Joshi, 2018). However, this study demonstrated that *Pseudomonas* sp. treated seeds showed a higher influence on germination percentage across 8 days in contrast to other bacterial treatments, despite the isolate exhibiting the least effect on enzymatic activity, an observation which could be attributed to various factors such as type of cereal, enzyme, and germination conditions (Guzmán-Ortiz *et al.*, 2019). This has therefore made *Pseudomonas* sp. the most efficient isolate in influencing germination under stressed conditions.

Enhanced radicle and shoot have been previously regarded as vital parameters for drought tolerance (Loutfy, Hassanein and Salem, 2021). IAA-bacteria are known to enhance radicle length and number of radicles (Kasim *et al.*, 2021), as evidently observed in *Pseudomonas* sp. and *Kosakonia* sp., known to stimulate root growth, root initiation, cell elongation and ultimate growth. The findings of this study are in agreement with the study by (Barnawal, Singh and Singh, 2019; Pandey and Gupta, 2020) who's results suggested that ACC deaminase-containing *Pseudomonas* sp. induced better root health, leading to an improved water uptake during drought stress. In addition, wheat is known to respond well to inoculation with *Kosakonia* with significant increases in growth and yield in both greenhouse and field trials (Berger *et al.*, 2018).

Furthermore, several studies state that growth parameters such as shoot and radicle length are significantly affected by water deficit simulated by PEG 6000 (Belay and Zhang, 2021). During drought stress, shoot growth is limited as a mechanism to reduce evaporation, resulting in loss of turgor, which consequently inhibits cell elongation and expansion, further enhancing plant survival (Kasim *et al.*, 2021). Based on the results of the effects of PEG induced drought stress on shoot and radicle growth, seedlings exhibited relatively low tolerance to high concentrations of PEG 6000 and high tolerance to low PEG 6000 concentrations. This can be morphologically observed in the seedlings, as illustrated in **Figure 4.3** above.

However, high radicle and shoot length in seedlings treated with *Kosakonia* sp. is indicative of drought tolerance by the bacterial strain as reported by Belay and Zhang (2021). Similarly, the SVI was equally affected by drought, hence the decrease in SVI with increasing PEG 6000 concentrations. However, according to Hatzig *et al*. (2018), higher SVI was expected develop under drought stress conditions to improve plant density and have a direct positive effect on crop production and growth.

Cupriavidus metallidurans showed the least significant effect on growth promotion (shoot length, number of radicles, seedling weight, SVI) in contrast to control, *Pseudomonas putida* and *Kosakonia.* Additionally, seeds treated with *C. metallidurans* and control exhibited the lowest and highest seedling vigour index, respectively.

Seedling weight was enhanced by the inoculation of wheat seeds with *Kosakonia* sp., which is attributed to the potential of *Kosakonia* sp. producing PGP traits such as IAA, ACC and Siderophores, which have the potential to improve plant biomass, according to (Kasim *et al.*, 2021).

Proline is produced during drought stress as a response to osmotic stress (Ilyas *et al.*, 2020) and according to Camaille *et al.* (2021), proline levels may increase up to 90% in wheat after periods of drought stress. Hence, application of drought stress on wheat seeds is expected to induce proline accumulation (Miranda *et al.*, 2023). According to Belay and Zhang (2021), high proline accumulation is associated with drought tolerance, with proline levels higher in stress tolerant plants in contrast to stress sensitive plants (Abdelaal *et al.*, 2021). It is further assumed that proline accumulation results from dehydration, and in cereals such as wheat it may be a feature of stress-tolerant varieties (Persic *et al.*, 2022).

As a response to drought stress, proline levels accumulate in plants to help protect cells from oxidative damage during drought (Abdelaal *et al.*, 2021). In this study, the amount of proline produced upon inoculation of wheat with *Pseudomonas* sp. was significantly lower in contrast to treatment with the control, *C. metallidurans* and *Kosakonia* sp. Seedlings treated with *Pseudomonas* sp. exhibited low amounts of proline, which demonstrated less dehydration within the plant (Abdelaal *et al.*, 2021). This could suggest that treatment of seeds with *Pseudomonas* sp. has the ability to lower stress levels within the plant, thereby reducing the levels of proline, which is usually correlated to water deficit and drought tolerance according to a study by Jain *et al.* (2013). In addition, *C. metallidurans* treated seeds exhibited the highest level of proline accumulation in contrast to the control, demonstrating a possibility of *C. metallidurans* to have an effect on drought tolerance on wheat due to high dehydration. In addition, Abdelaal *et al.* (2021) stated that the effectiveness of PGPB on plant drought tolerance is determined by the accumulation of proline.

Accumulation of proline in *C. metallidurans* treated wheat was supposed to facilitate growth and allow the seedlings to thrive under drought conditions by accumulating more proline. However, this was contrary to the data obtained as *C. metallidurans* treated wheat showed the least effect on growth despite high proline levels. This could be related to the fact that, proline accumulation for the adaptation of plants to drought remains uncertain (Loutfy, Hassanein and Salem, 2021; Persic *et al.*, 2022). Accumulation of proline enhances drought tolerance to the plant however proline accumulation could be a sign of stress rather than an indicator of stress tolerance (Loutfy, Hassanein and Salem, 2021). Inoculation of wheat with *Pseudomonas* sp. did not enhance proline accumulation. This may be supported by recent work by Naderi *et al.* (2022), which reported that plants inoculated with *Pseudomonas* spp. showed a decrease in proline under water stress.

Previous studies have also shown that inoculation of IAA-producing bacteria onto wheat lowered proline accumulation as seen in maize plants due to their potential to reduce proline in the leaves of maize under stress (Ali and El-kholy, 2022). Contrary to the effects of bacterial isolates on proline, the levels of proline increased with increasing PEG 6000 concentrations, usually during stress.

Alpha-amylase plays a major role in the degradation of carbohydrates and is an important factor during seed germination (Li, Yang and Zhang, 2019; Naderi *et al.*, 2022). However, it's activity is reduced by water stress, and under PEG induced drought stress (Muscolo *et al.*, 2014). Additionally, it is known that the activity of α-amylase could be triggered by high levels of phytohormones (Minaxi *et al.*, 2012). In this study, the highest level of enzymatic activity was exhibited by wheat treated with *C. metallidurans* with the least amount produced by untreated seeds (control). In this study, enzymatic activity (α-amylase) demonstrated a decreasing trend as PEG 6000 concentrations decreased, with low activity exhibited during non-stressed conditions and high activity produced during stressed conditions. This is shown in the study as seedlings subjected to drought stress demonstrated increased levels of enzymatic activity. However, this is in contradiction to previous studies by Muscolo *et al.* (2014) and García *et al.* (2021), which reported that enzymatic activity is likely reduced by osmotic stress. This was further observed in lentil seeds, which had a decreased enzymatic activity under PEG induced drought stress (Muscolo *et al.*, 2014). According to Guzmán-Ortiz *et al.* (2019), amylase is usually produced during germination, therefore, high levels of the enzyme during drought stress could indicate better germination of wheat seeds in contrast to non-stressed conditions.

Variation in agro-ecological environments could affect the efficacy and potential commercialisation application of bioinoculants. According to Orozco-Mosqueda *et al.* (2021), there has been some inconsistency observed in the performance of bioinoculants in field, in contrast to greenhouse trials, resulting in bioformulation constraint. This could be due to having bioinoculants that are incompatible with the environment and soil conditions in which they will be inoculated, thus, selection and application of highly competitive bioinoculants that are compatible with the environment is essential (Orozco-Mosqueda *et al.*, 2021).

4.5 CONCLUSION

To reduce the effects of drought stress on agricultural crops, there is a need to develop eco-friendly bio-inoculants in the form of PGPB. This is due to treatment of seeds with PGPB having the potential to improve seed germination and promote drought tolerance.

The study further showed that drought stress majorly impacts growth parameters, as seen in the reduction when PEG 6000 concentrations increased. Proline levels also decreased as drought stress levels increased. Hence, proline content may be used as a determining factor for drought resistant plants such as wheat (Sharma *et al.*, 2022). Treatment of seeds with IAA-producing *Kosakonia* as established from the above discussion has shown an increase in various growth parameters, in contrast to other isolates, thus making it the most effective isolate.

However, it can be concluded that in future, the potential of these isolates with multiple PGP traits can be assessed in consortium both *in vitro* and under field conditions to determine the effectiveness in drought mitigation. Additionally, further studies in the greenhouse and the field can be recommended to validate the results.

Lastly, inoculation of *Kosakonia* sp. on wheat seeds improved number of radicles, shoot length, number of shoots, and seedling weight in contrast to non-bacterized (control) and other treatments, proving that *Kosakonia* sp. was the most effective isolate in the study in contrast to *Pseudomonas* sp. and *C. metallidurans*.

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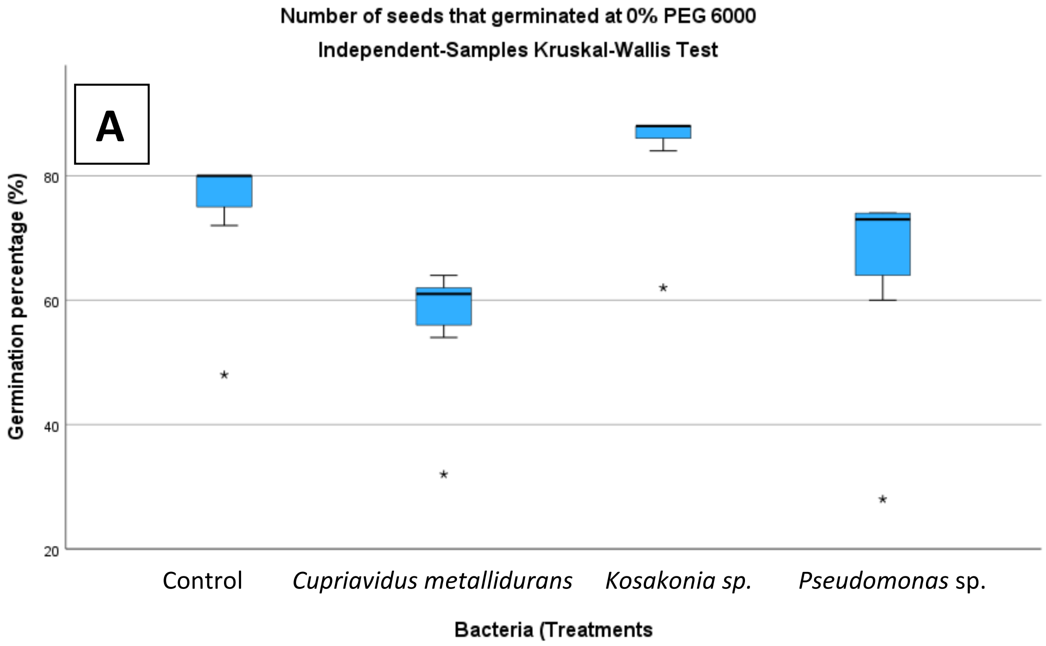
APPENDIX

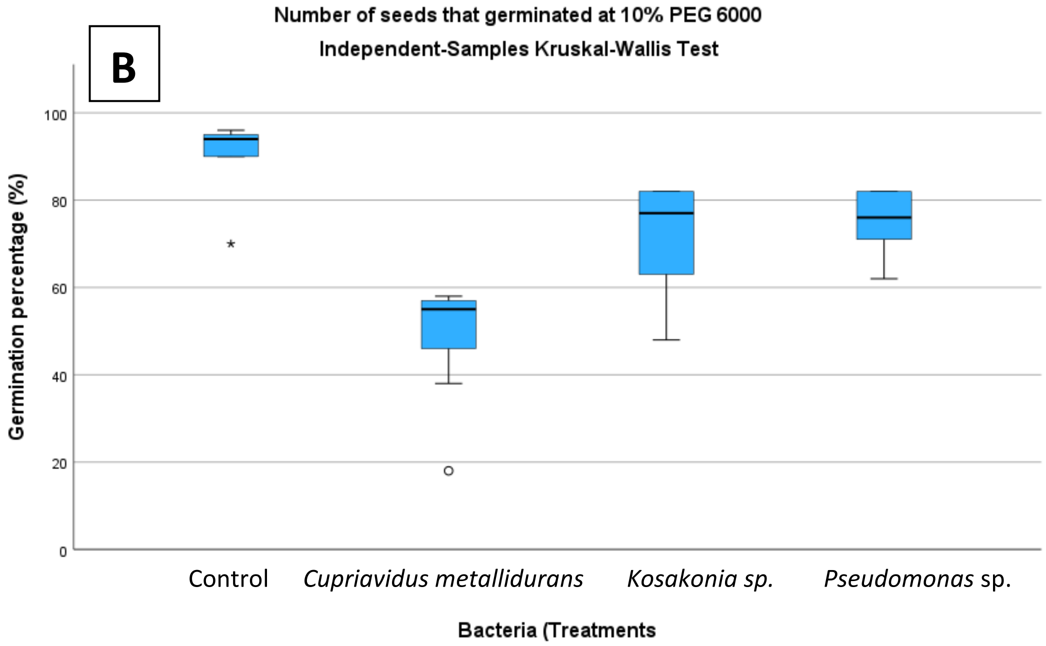
Appendix Table 4.1: Growth parameters of wheat seedlings treated with plant growth-promoting bacteria under various PEG-induced drought conditions.

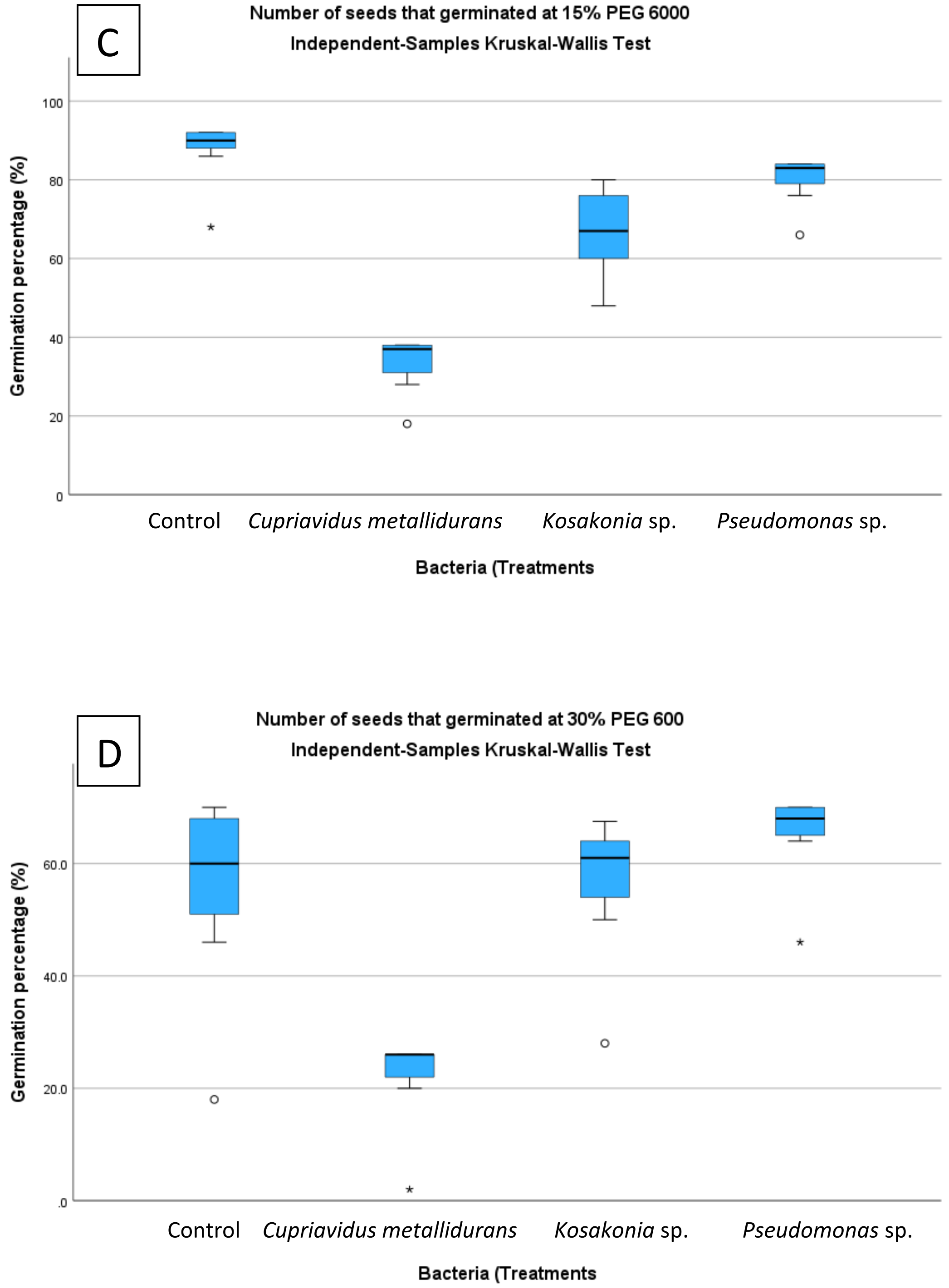
Treatments	**Shoot length (mm)**	**No. of shoots**	**Radicle length (mm)**	**No. of radicles**	**Seedling weight (g)**	**GP %**	**SVI**
Bacterial isolates							
Control	42.45	42.75	68.05	38.40	46.00	54.38	59.80
C.metallidurans	22.10	33.00	11.70	16.10	22.65	16.00	12.53
***Kosakonia* sp.**	53.18	48.90	41.70	56.60	51.55	48.03	47.75
***Pseudomonas* sp.**	44.28	37.35	40.55	50.90	41.80	43.60	41.93
MEAN	69.79	1.15	81.71	4.64	0.13	70.87	1232.94
PEG 6000 (%)							
0	56.50	59.40	37.40	39.53	56.58	42.93	46.53
10	54.75	45.40	48.88	53.28	54.73	47.95	52.80
15	39.10	32.75	43.70	44.28	37.23	43.75	41.73
30	11.65	24.45	32.03	24.93	13.48	27.38	20.95
MEAN	69.79	1.15	81.71	4.64	0.13	70.87	1232.94

Appendix Table 4.2: Proline levels and enzymatic activities (α-amylase) of wheat seedlings treated with plant growth-promoting bacteria under various PEG-induced drought conditions.

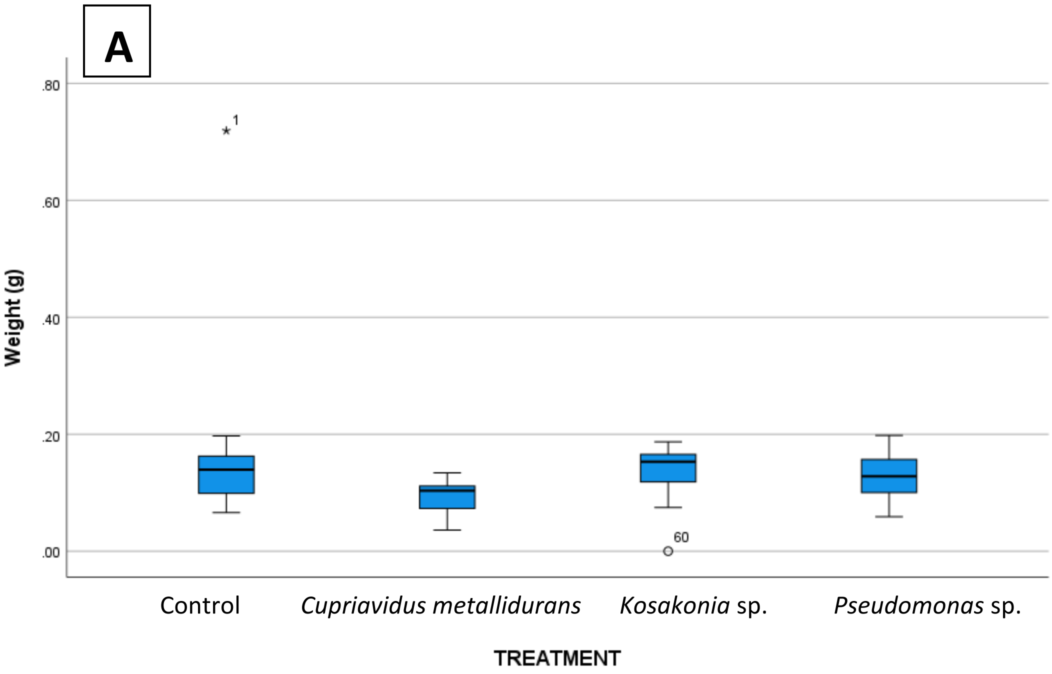
Treatments	**Proline µmoles/g fresh weight**	**Enzymatic activity µmoles/min maltose**
Bacterial isolates		
Control	24.08	18.58
C. metallidurans	37.92	34.58
***Kosakonia* sp.**	29.50	23.42
***Pseudomonas* sp.**	6.50	21.42
MEAN	148.22	1892.85
PEG 6000 Concentration (%)	**Proline µmoles/g fresh weight**	**Enzymatic activity µmoles/min maltose**
0	23.58	18.33
10	23.75	26.17
15	25.17	26.00
30	25.50	27.50
MEAN	148.22	1892.85

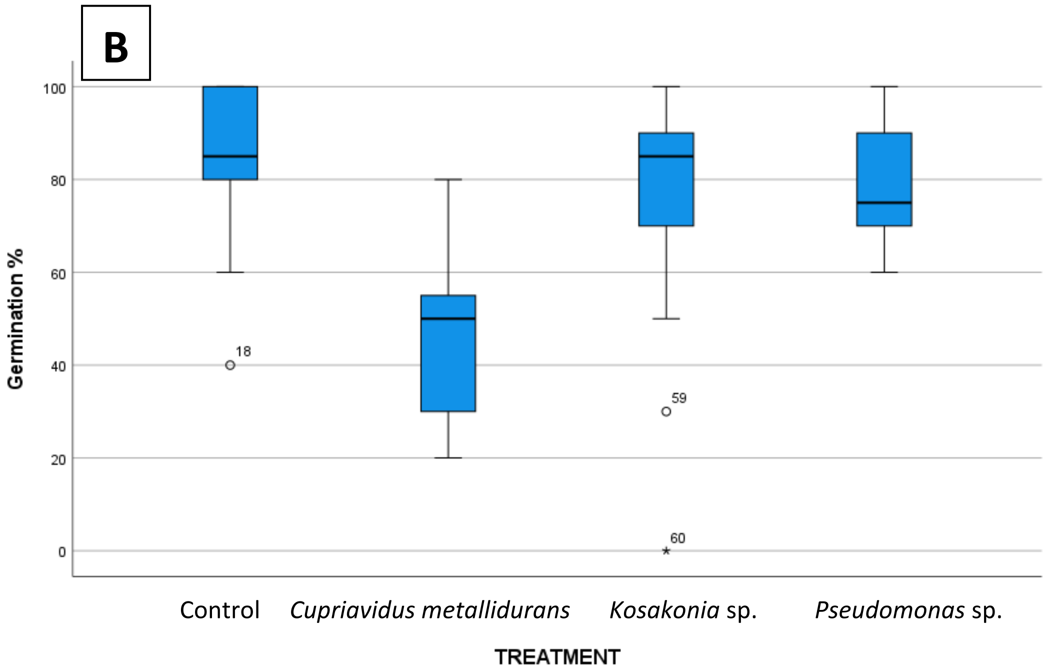

A
Number of seeds that germinated at 0% PEG 6000
Independent-Samples Kruskal-Wallis Test
Germination percentage (%)
80
60
40
20
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
Bacteria (Treatments

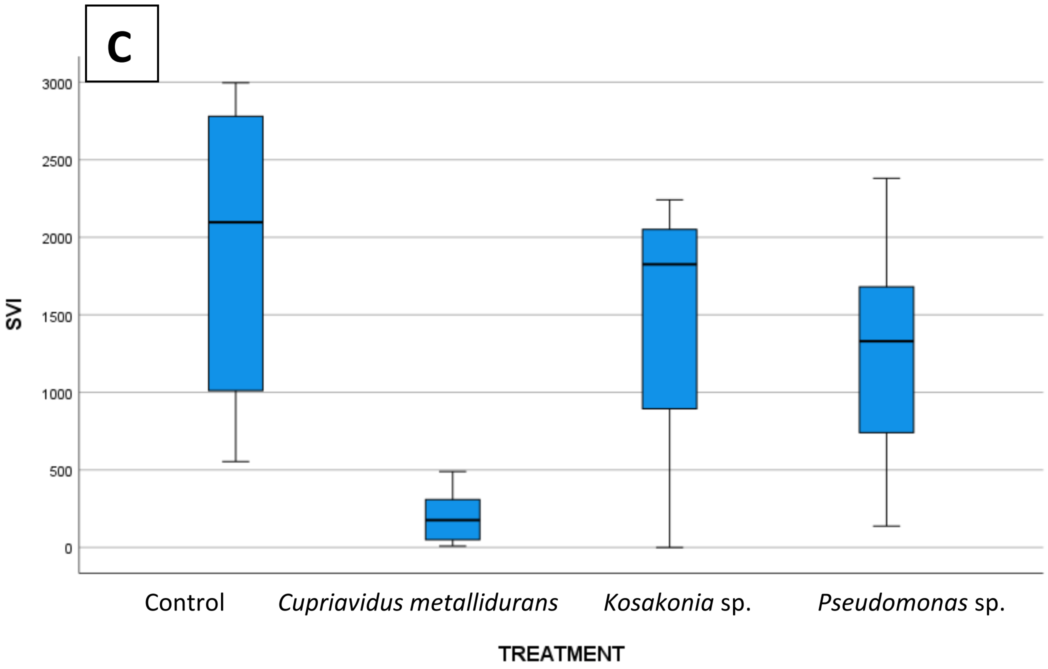

B
Number of seeds that germinated at 10% PEG 6000
Independent-Samples Kruskal-Wallis Test
Germination percentage (%)
100
80
60
40
20
0
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
Bacteria (Treatments

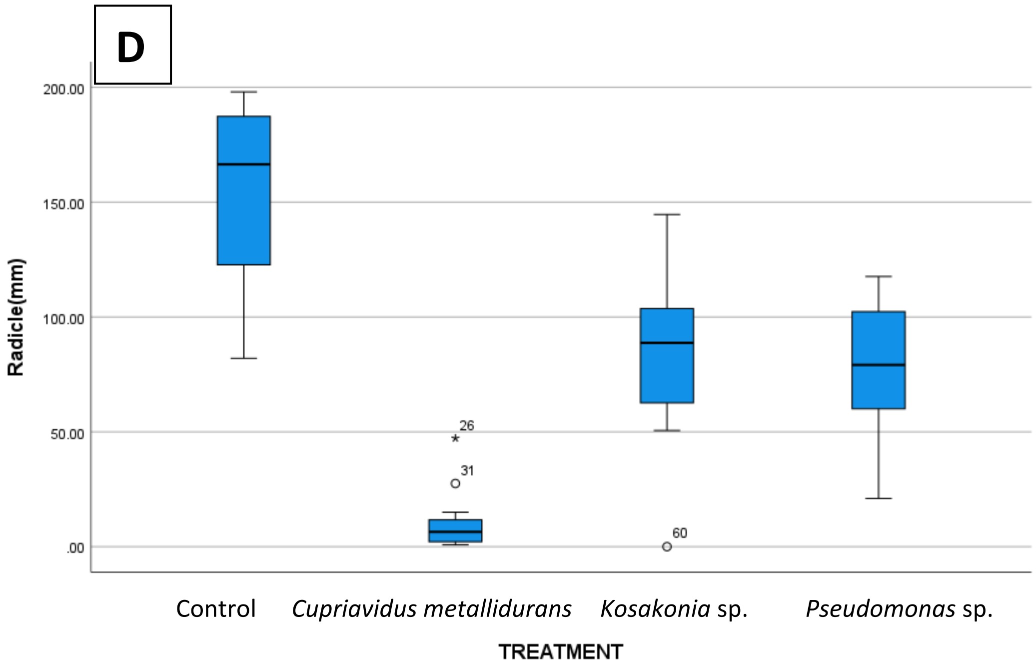


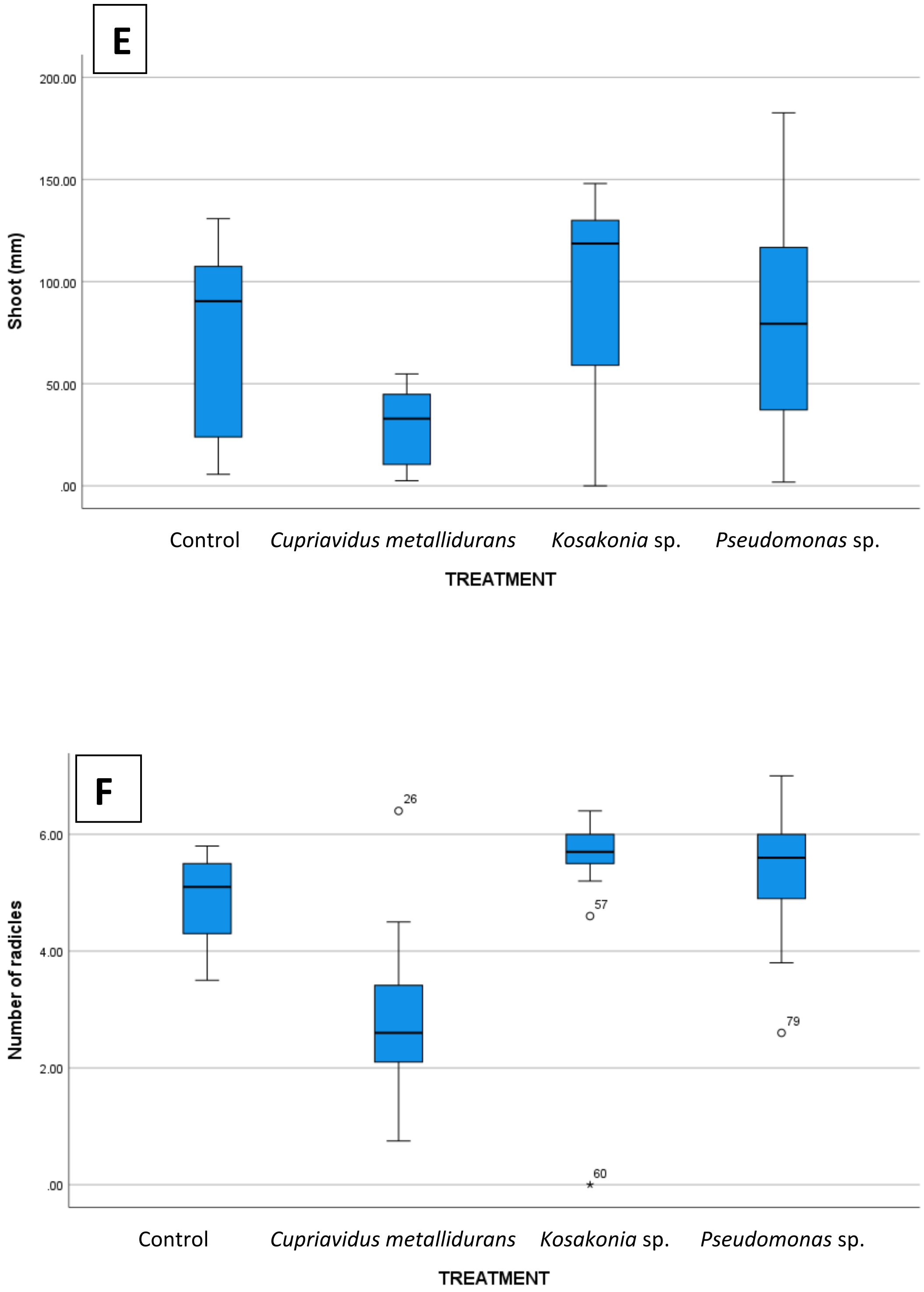
Appendix Figure 4.1: Effect of bacterized and non-bacterized treatments on seeds that germinated under simulated drought conditions (PEG 600) **(A)** 0%, **(B)** 10%, **(C)** 15%, **(D) 30%**.


A
Weight (g)
.80
.60
.40
.20
.00
1
60
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
TREATMENT

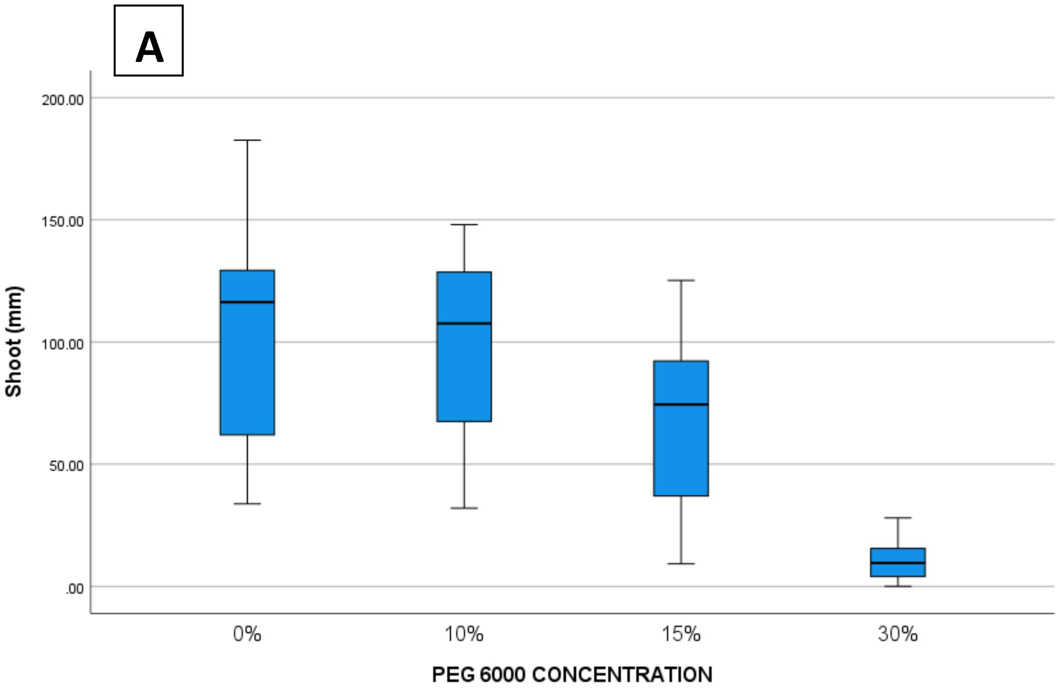

B
Germination %
100
80
60
40
20
0
18
59
60
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
TREATMENT

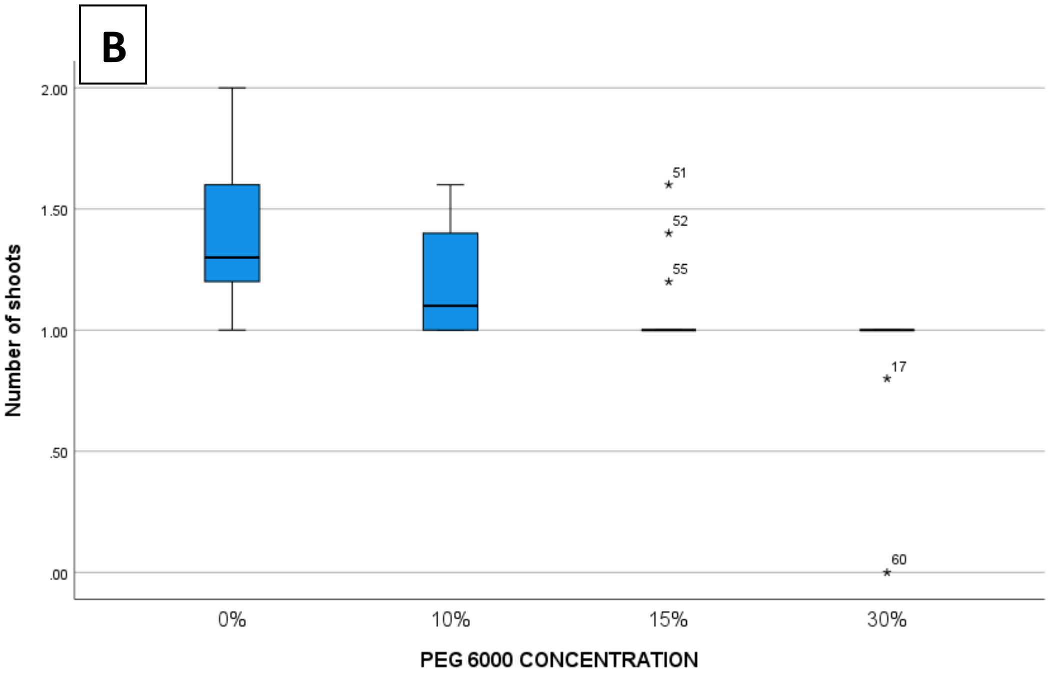

C
3000
2500
2000
1500
1000
500
0
SVI
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
TREATMENT

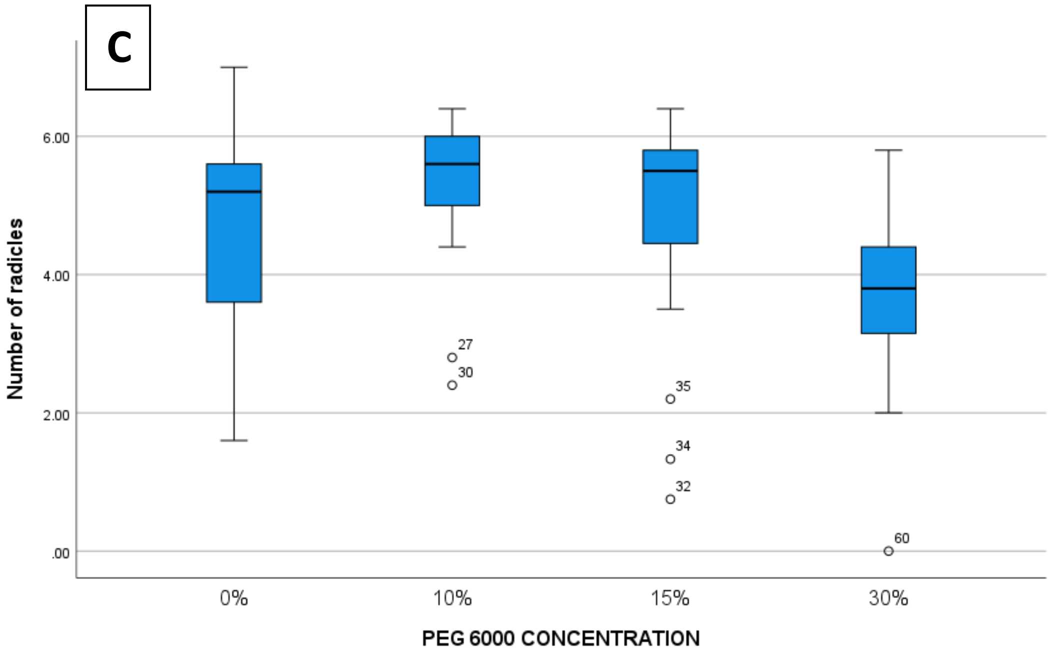

D
200.00
150.00
100.00
50.00
.00
Radicle(mm)
26
31
60
Control
Cupriavidus metallidurans
Kosakonia sp.
Pseudomonas sp.
TREATMENT

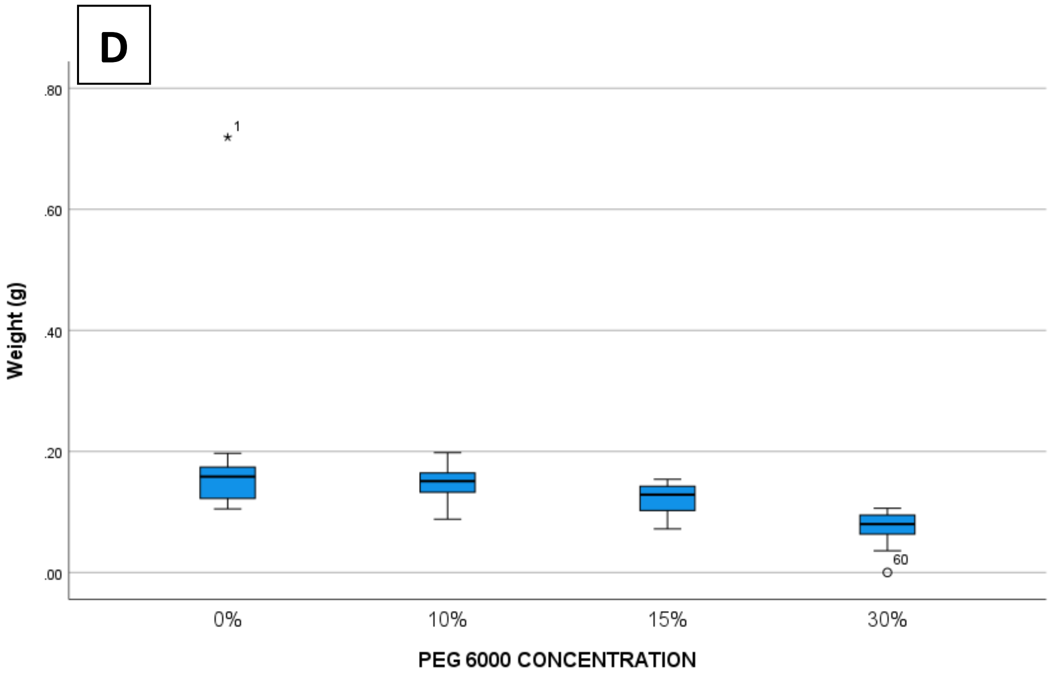


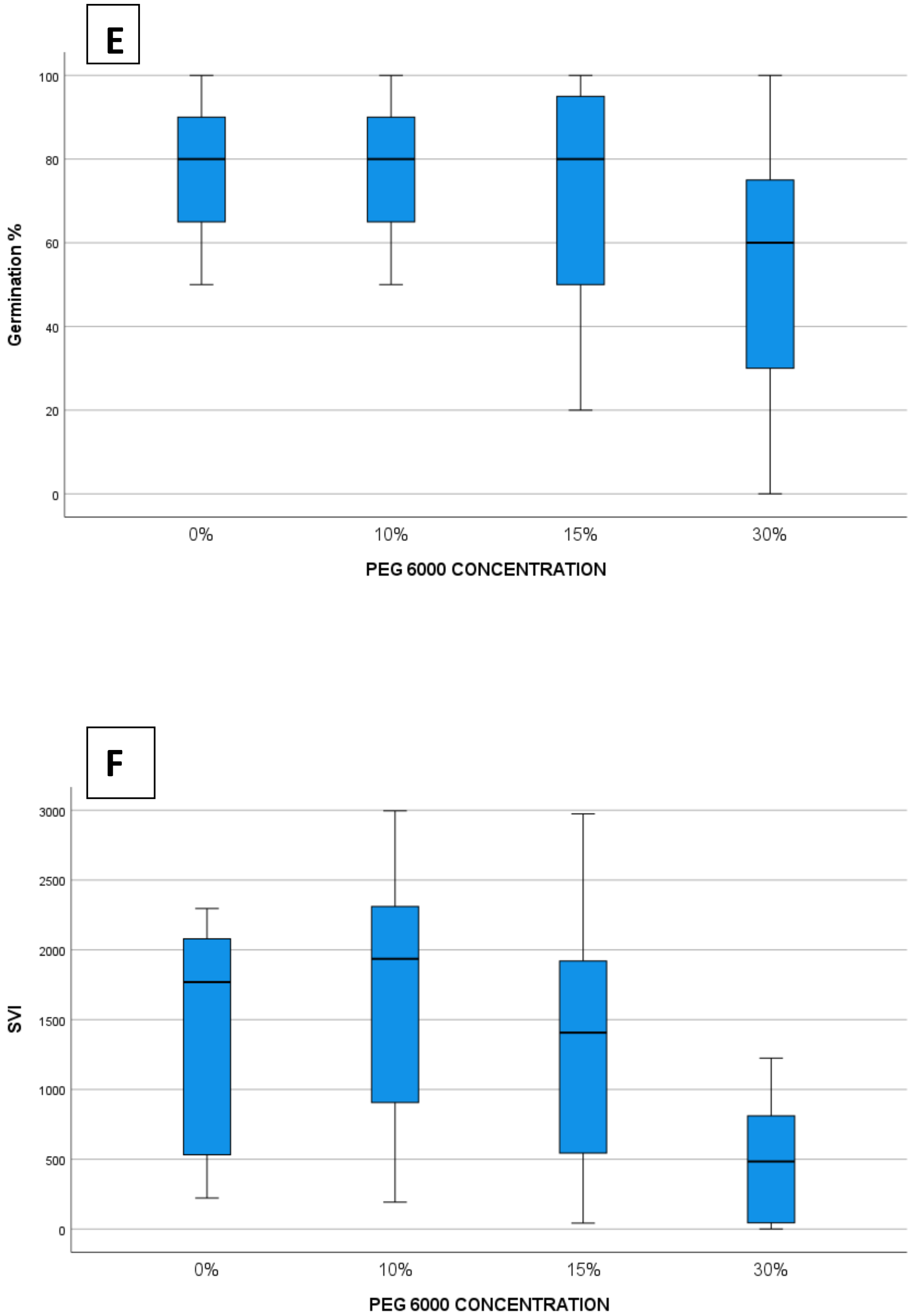
Appendix Figure 4.2: Effect of bacterization on selected growth parameters of wheat **(A)** seedling weight, **(B)** germination percentage, **(C)** seedling vigour, **(D)** radicle length, **(E)** shoot length, and **(F)** number of radicles.


A
Shoot (mm)
200.00
150.00
100.00
50.00
.00
0%
10%
15%
30%
PEG 6000 CONCENTRATION

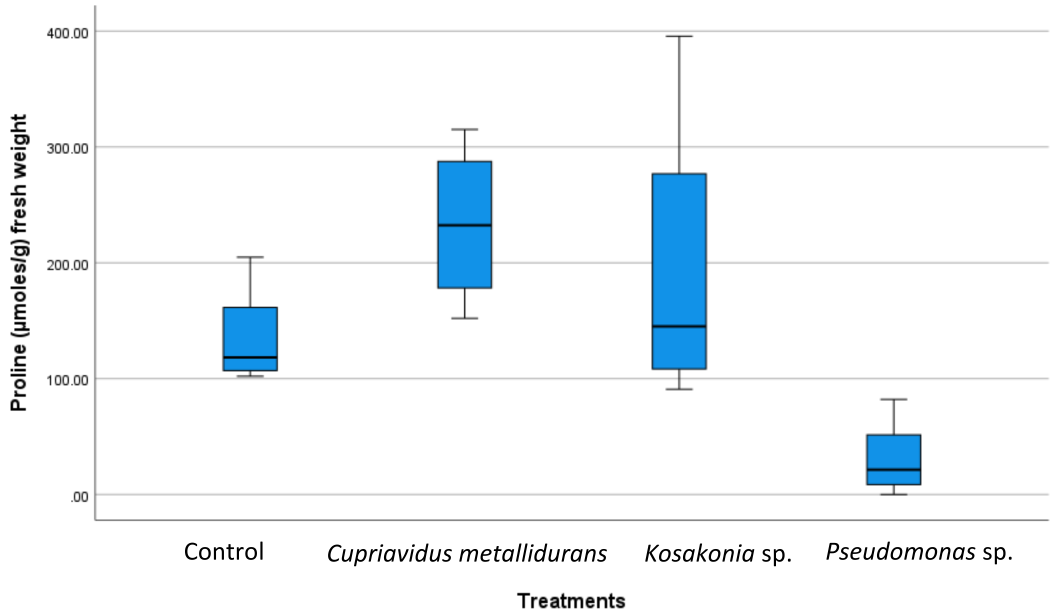

B
Number of shoots
2.00
1.50
1.00
.50
.00
51
52
55
17
60
0%
10%
15%
30%
PEG 6000 CONCENTRATION


C
6.00
4.00
2.00
.00
Number of radicles
27
30
35
34
32
60
0%
10%
15%
30%
PEG 6000 CONCENTRATION

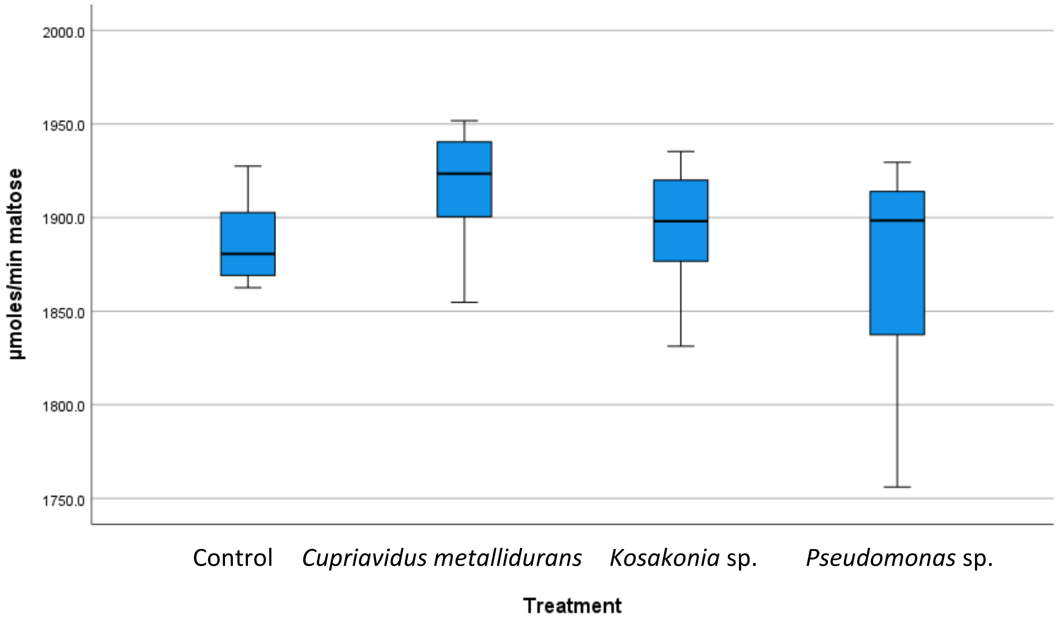

D
.80
.60
.40
.20
.00
Weight (g)
1
60
0%
10%
15%
30%
PEG 6000 CONCENTRATION



Appendix Figure 4.3: Effect of PEG-induced drought treatment on selected growth parameters of wheat **(A)** shoot length, **(B)** number of shoots, **(C)** number of radicles, **(D)** seedling weight, **(E)** germination percentage, and **(F)** seedling vigour.



Appendix Figure 4.4: Effect of bacterial isolates on proline levels of wheat seedlings.



Appendix Figure 4.5: Effect of bacterial isolates on enzymatic activity (α-amylase) of wheat seedlings.

CHAPTER 5: GENERAL CONCLUSIONS AND RECOMMENDATIONS

This study is the first of its kind to explore the diversity and characterization of beneficial drought tolerant PGPB from native *Myrothamnus flabellifolius*. It is also the first to explore the plants' potential use as bioinoculants to ameliorate drought stress in agricultural crops, specifically *Triticum aestivum* (wheat).

A total of 10 genera were isolated and identified, with *Bacillus* being the most abundant genera. The isolated strains: *Staphylococcus hominis, Bacillus licheniformis*, *Pseudomonas japonica*, and *Cellulosimicrobium funkei* showed tolerance to drought 30% PEG 6000, indicating ability to adapt to drought stress, therefore, use of these bacteria with additional PGP traits could be ideal as potential bioinoculants to ameliorate drought, specifically in arid environments.

The isolated bacterial strains demonstrated a potential to enhance growth in wheat as single bio-inoculum or as consortium. This was demonstrated by the use of *Kosakonia* sp., which led to an increase in the growth indices of wheat seeds compared to the control. Treatment of wheat seeds with ACC deaminase producing bacteria resulted in increased accumulation of proline, which in turn helped the plants to handle the adverse effects of drought stress.

It was observed that seed bacterization was effective in inducing drought tolerance as noticed through enhanced plant growth parameters compared to untreated control plants including the higher relative water content (RWC) in leaves, which confirms the effectiveness of the treatment. The findings from this study further suggest a great possibility of bioinoculant development as an eco-friendly and inexpensive alternative for a sustainable agricultural crop production. Furthermore, more studies should focus on the use of native drought-tolerant plant-associated microbes for plant growth.