ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *CLEOME GYNANDRA* L. BACTERIAL SEED ENDOPHYTES FROM NORTHERN NAMIBIA

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ABSTRACT

Endophytic microorganisms are symbionts found inside host tissue without eliciting disease, symptoms or causing any damage. They play an integral role in spermatophyte life cycles and are endowed with multi-functional traits that promote the host's health, growth and yield. *Cleome gynandra* L. (*C. gynandra*), is an unexplored "orphan" plant that has a diverse array of such endophytic microbial populations. *C. gynandra* (Ombidi in Oshiwambo; Ombowa in Herero; Gomabeb in Damara/Nama) is a neglected drought-tolerant wild annual crop spread in semi-arid regions of Northern Namibia. This plant has shown great potential in agronomy, climate adaptation, economy and traditional medicine, making it stand out as a prominent contender among climate-adapted future crops. Little, however, is known about *C. gynandra* plant growth promoting (PGP) seed endophytes. The study aimed to isolate and identify endophytic bacteria associated with *C. gynandra* spermatophyte and characterize their functional plant growth promoting potential which could be used to improve plant growth and yield. Twenty morphologically distinct isolates were assessed. They exhibited various abilities to produce growth regulators that contribute to ammonia production (85%), atmospheric nitrogen fixation (40%), fluorescence production (10%), hydrogen cyanide production (30%), indole acetic acid (IAA) production (100%), phosphate solubilization (15%) and siderophore production (80%). Phylogenetic analysis showed that all the isolates (excluding *Phytobacter ursingii*) belonged to the *Proteobacteria* phylum. The application of endophytic plant growth promoting bacteria (PGPB) is considered to have the potential for improving plant growth in extreme environments. Three best PGP producing isolates, namely *Enterobacter cloacae, Klebsiella pneumoniae* and *Kluyvera cryocrescens* were selected for greenhouse trials using *Brassica napus* L. (Rape English Giant) seeds. In comparison to the control groups, there was a statistically significant difference for root length ($p=0.024$), plant height ($p=0.037$) wet ($p=0.000$) and dry ($p=0.000$) mass in the microbial experimental group. This indicated that the microbe treatments had an effect on growth parameters. From this study, it is concluded that *C. gynandra* seeds harbour a reservoir of endophytic bacteria with the ability to improve plant growth and with the potential for use as inoculants (biofertilizers) to establish sustainable crop production systems.

Keywords: agriculture, *C. gynandra*, endophytes, plant growth-promoting, seed bacteria, seed inoculants.

List of Publications

The work, or parts thereof, presented in this thesis have been published as part of the citations below:

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Shipoh, **P**.**M**., Chimwamurombe, P.M. and J.D Uzabakiriho (2020). *Cleome gynandra* L. Seed Endophytes Induce Rape (*Brassica napus* L.) Seedling Germination Rate and Promote Rape Plant Growth and Leaf Yield. Paper in preparation of journal submission.

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Dedication

This thesis is dedicated to my parents Etuna and Peingeondjabi Shipoh, my brother Twaaluka Shipoh and my best friend Hiskia Petrus. I am extremely grateful for all the prayers, words of encouragement, love, and support you have provided throughout this journey. I will forever be indebted to all of you for all your efforts.

Declaration

I, **Panduleni Mwahepekange Shipoh**, hereby declare that this study is a true reflection of my research, and that this work, or part thereof has not been submitted for a degree at any institution.

…….. **……** **……**

Name of Student **Signature** **Date**

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Agriculture plays a vital role in most low income and middle-income countries where most households depend on smallholder agriculture for their livelihoods (Ng'etich, Aguyoh and Ogweno, 2012; Singh, 2013). As of 2018, 19.71% of the Namibian population were employed in the agriculture sector which contributed 974.80 USD Million to the GDP in the fourth quarter of 2018 (National Planning Commission, 2018). Modern agricultural systems have been focusing on a very limited number of high-input and high-yielding crop species for food production throughout the world (Chivenge *et al.*, 2015). With the advent of climate change, coupled with the rapid increase in population and urbanization, it has been predicted that the survival of these systems in the 21st century will face daunting challenges (Masuka, Goss and Mazarura, 2012; Mabhaudhi *et al.*, 2017).

The agriculture sector should undergo imperative metamorphosis to reduce the environmental impacts while increasing productivity and lead to better livelihoods and poverty reduction (Padulosi, Thompson and Rudebjer, 2013; Wangolo *et al.*, 2015). The development of resilient crops that can yield under uncertain and extreme climatic and poor soil growing conditions would significantly play a prominent role in mitigating these problems. Diversifying production and consumption habits to include underutilized crops can influence and contribute to improved nutrition and household food security, and elevate relegated indigenous crops to the status of neglected and underutilized crop species (Chivenge *et al.*, 2015; Mabhaudhi *et al.*, 2017). African indigenous crops can play a pivotal role in this endeavour (Ng'etich, Aguyoh and Ogweno, 2012). These species of plant are known to better withstand adverse weather conditions via increased hardiness

and reduced external inputs compared to the cultivated crops (Masuka, Goss and Mazarura, 2012). The production of African indigenous vegetables has been constrained by limited information on fertilization requirements coupled with inadequate scientific knowledge (Cruz-Garcia and Price, 2012; Ng'etich, Aguyoh and Ogweno, 2012; Gido, Bett and Bokelmann, 2016).

Some African indigenous leaf vegetables, which are also categorized as "orphan crops" are adapted to local environments in which they are grown and play an integral role in African agricultural and food systems (Adebooye and Opabode, 2004; Chivenge *et al.*, 2015). They are also known to have adequate amounts of essential dietary micronutrients and being a valuable source of nutrition in rural communities (Gido, Bett and Bokelmann, 2016). Available information on African leafy vegetables is limited, making commercialization, crop development and sustainable conservation difficult (Agbo *et al.*, 2009; Masuka, Goss and Mazarura, 2012).

During the past decade, microorganisms have garnered importance in sustainable agriculture due to the benefits they have on soil and crop productivity (Ramakrishna, Yadav and Li, 2019). Endophytes are microorganisms that dwell inside plants without causing any harm (Schulz and Boyle, 2006; Truyens *et al.*, 2014). For many decades, endophytes have been known for their growth benefits and health promotion (Ryan *et al.*, 2008; Reinhold-Hurek and Hurek, 2011). They are ubiquitous to the majority of plant tissue. Seed endophytes are of particular interest as they are vertically transmitted between plant generations through seedlings (Barea, 2015; Abbamondi *et al.*, 2016; Shahzad *et al.*, 2018). However, seed-borne endophytic microbes have not been fully explored yet (Shahzad *et al.*, 2018). They are potential producers of various phytohormones that can improve biomass, yield and stress mitigation, and are easily

selected by plants for mutually beneficial interactions because they are naturally occurring (Rosenblueth and Martínez-romero, 2006; Ryan *et al.*, 2008; Maheswari, Anbukkarasi and Hemalatha, 2014; Shahzad *et al.*, 2018). While previous studies (Shilla *et al.*, 2019) have centred around *Cleome gynandra* L. (Cat's Whisker) origin, morphology and taxonomy, none have focused on their seed endophytes nature. There are currently no structured agricultural practices that can effectively lead to their sustainable harvesting (Masuka, Goss and Mazarura, 2012; Onyango, Onwonga and Kimenju, 2016). The use of plant growth promoting endophytes as seed inoculants have shown promising prospects to increase crop yield, through the plants' mechanisms and in the most environmentally friendly manner as possible (Golding, 2012; O'Callaghan, 2016). The diversity of *C. gynandra* seed endophytes and their potential for agricultural practices in Namibia has not been explored. This study aimed to isolate endophytic bacteria from *Cleome gynandra* seeds and explore their potential and use in the Namibian agriculture landscape. To our knowledge, this study is the first report on the diversity of culturable endophytic seed bacteria associated with *Cleome gynandra* growing in Namibia. The outcome of this study will form the basis of knowledge on these endophytic seed bacteria and how they can be selected for utilization of plant growth promotion.

1.2 Statement of the problem

C. gynandra forms a substantial part of the diets of the Aawambo community in various parts of the country (Kolberg, 2001; Omondi *et al.*, 2017). It has great nutritional (high levels of calcium, iron, magnesium and zinc), economic and agricultural potential (Omondi *et al.*, 2017). Despite these exciting traits, very little attention has been given to it because it is a neglected and underutilized crop (Padulosi, Thompson and Rudebjer,

2013). There are currently no sustainable agronomic systems in place that can be used to successfully harvest and domesticate *C. gynandra* (Masuka, Goss and Mazarura, 2012) in Namibia. Research has shown that seed plant growth promoting endophytes have been linked to plant growth and yield (Chaturvedi, Singh and Gupta, 2016). This study aimed to determine whether or not *C. gynandra* seeds harbour endophytic bacteria that could contribute to its's growth and yield, which can potentially be used as a bio-inoculant on domesticated crops and increase their horticultural potential.

1.3 Objectives of the study

The specific objectives of this study were to:

(i) isolate, identify and characterize putative PGP *C. gynandra* seed endophytes from 4 geographical locations;

(ii) determine the phylogenetic classification of *C. gynandra* seed endophytes;

(iii) determine the effect of PGP isolates from *C. gynandra* on germination rates, plant growth and yield of *Brassica napus* (Rape English Giant) crops.

1.4 Significance of the study

C. gynandra endophytes stand out as a prominent contender for climate-adaptive agriculture. The outcome of this study will provide a greater understanding of its seed endophyte bacterial population. This knowledge might improve its sustainable agricultural production in Namibia, with potential benefits of providing an alternative source of income for local communities and in turn, providing affordable agricultural practices to local farmers that will contribute to the countries growing population.

1.5 Limitations of the study

Since a culture-dependent method was used, it is possible that available endophytes were not all isolated as not all strains could be cultured. This could have reduced the chance of genetic diversity among the isolates identified.

1.6 Delimitation of the study

The study was conducted within the boundaries of the Omusati, Oshana, Ohangwena and Oshikoto regions. Regions have artificial lines as boundaries between them, inevitably meaning that they share features such as soil composition and have identical physical conditions (Mendelsohn, 2007). This indicates that more diverse variations of the seed endophytes might not be found.

REFERENCES

Abbamondi, G. R. *et al.* (2016) 'Plant Growth-Promoting Effects of Rhizospheric and Endophytic Bacteria Associated with Different Tomato Cultivars and New Tomato Hybrids', *Chemical and Biological Technologies in Agriculture*. Springer International Publishing, 3(1).

Adebooye, O. C. and Opabode, J. T. (2004) 'Status of Conservation of the Indigenous Leaf Vegetables and Fruits of Africa', *African Journal of Biotechnology*, 3(12), pp. 700–705.

Agbo, A. E. *et al.* (2009) 'Nutritional Importance of Indigenous Leafy Vegetables in Côte d' Ivoire', *Acta Horticulturae*, 806(45), pp. 361-366.

Barea, J. M. (2015) 'Future Challenges and Perspectives for Applying Microbial Biotechnology in Sustainable Agriculture Based on a Better Understanding of Plant-Microbiome Interactions', *Journal of Soil Science and Plant Nutrition*, 15(2), pp. 261–282.

Chataika, B. *et al.* (2020) 'Diversity and Domestication Status of Spider Plant (*Gynandropsis gynandra*, L.) Amongst Sociolinguistic Groups of Northern Namibia', *Agronomy*, 10(1), p. 56.

Chaturvedi, H., Singh, V. and Gupta, G. (2016) 'Potential of Bacterial Endophytes as Plant Growth Promoting Factors', *Journal of Plant Pathology & Microbiology*, 7(9), p. 376.

Chivenge, P. *et al.* (2015) 'The Potential Role of Neglected and Underutilised Crop Species as Future Crops under Water Scarce Conditions in Sub-Saharan Africa', *International Journal of Environmental Research and Public Health*, 12, pp. 5685–5711.

Cruz-Garcia, G. S. and Price, L. (2012) 'Weeds as Important Vegetables for Farmers', *Acta Societatis Botanicorum Poloniae*, 18, pp. 397–403.

Gido, E. O., Bett, H. K. and Bokelmann, W. (2016) 'Importance of African Indigenous Vegetables in Food Systems', *African Journal of Horticultural Science*, 10, pp. 34–41.

Golding, A.-L. (2012) 'Plant Growth Promoting H2-Oxidizing Bacteria as Seed Inoculants for Cereal Crops', *Agricultural Sciences*, 3(4), pp. 510–516.

Kolberg, H. (2001) 'Indigenous Namibian Leafy Vegetables: A Literature Survey and Project Proposal', *Agricola*, 12, pp. 54–60.

Mabhaudhi, T. *et al.* (2017) 'Developing a Roadmap for Improving Neglected and Underutilized Crops: A Case Study of South Africa', *Frontiers in Plant Science*, 14(8), p. 2143.

Maheswari, T. U., Anbukkarasi, K. and Hemalatha, T. (2014) 'Bacterial Endophytes as Biofertilizer for Pulse Crops', *Popular Kheti*, 2(3), pp. 89–93.

Masuka, A., Goss, M. and Mazarura, U. (2012) 'Morphological Characterization of Four Selected Spider Plant (*Cleome gynandra* L.) Morphs from Zimbabwe and

Kenya', *Asian Journal of Agriculture and Rural Development*, 2(4), pp. 646–657.

Mendelsohn, J. (2007) *A Digest of Information on Key Aspects of Oshikoto, Ohangwena, Omusati and Oshana's Geography*. Windhoek.

National Planning Commission, Office of the President. (2018) *Status of the Namibian Economy*. Windhoek.

Ng'etich, O. K., Aguyoh, J. N. and Ogweno, J. O. (2012) 'Growth, Yield and Physiological Responses of Spider Plant (*Cleome gynandra* L.) to Calcium Ammonium Nitrate Rates', *International Journal of Agronomy and Plant Production*, 3(9), pp. 346–355.

O'Callaghan, M. (2016) 'Microbial Inoculation of Seed for Improved Crop Performance: Issues and Opportunities', *Applied Microbiology and Biotechnology*, 100(13), pp. 5729–5746.

Omondi, E. O. *et al.* (2017) 'Nutritional Compound Analysis and Morphological Characterization of Spider Plant (*Cleome gynandra* L.) - an African Indigenous Leafy Vegetable', *Food Research International*. Elsevier, 100(1), pp. 284–295.

Onyango, C. M., Onwonga, R. N. and Kimenju, J. W. (2016) 'Assessment of Spider Plant (*Cleome gynandra* L.) Germplasm for Agronomic Traits in Vegetable and Seed Production: A Green House Study', *American Journal of Experimental Agriculture*, 10(1), pp. 1–10.

Padulosi, S., Thompson, J. and Rudebjer, P. (2013) *Fighting Poverty, Hunger and Malnutrition with Neglected and Underutilized Species (NUS): Needs, Challenges and The Way Forward*. Rome: Bioversity International.

Ramakrishna, W., Yadav, R. and Li, K. (2019) 'Plant Growth Promoting Bacteria in Agriculture: Two Sides of a Coin', *Applied Soil Ecology*, 138(2019), pp. 10–18.

Reinhold-Hurek, B. and Hurek, T. (2011) 'Living Inside Plants: Bacterial Endophytes', *Current Opinion in Plant Biology*, 14(4), pp. 435–443.

Rosenblueth, M. and Martínez-romero, E. (2006) 'Bacterial Endophytes and Their Interactions with Hosts', *Molecular Plant-Microbe Interactions*, 19(8), pp. 827–837.

Ryan, R. P. *et al.* (2008) 'Bacterial Endophytes: Recent Developments and Applications', *FEMS Microbiology Letters*, 278(1), pp. 1–9.

Schulz, B. and Boyle, C. (2006) *Microbial Root Endophytes*. 5th edn. Edited by T. Sieber. Berlin, Germany: Springer-Verlag GmbH.

Shahzad, R. *et al.* (2018) 'What Is There in Seeds? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth', *Frontiers in Plant Science*, 9(24).

Shilla, O. *et al.* (2019) '*Cleome gynandra* L. Origin, Taxonomy and Morphology: A Review', *African Journal of Agricultural Research*, 14(32), pp. 1568–1583.

Singh, J. S. (2013) 'Potential Microbes for Sustainable Agriculture', *Resonance: Journal of Science Education*, (March), pp. 275–281.

Truyens, S. *et al.* (2014) 'Bacterial Seed Endophytes: Genera, Vertical Transmission and Interaction with Plants (Minireview)', *Environmental Microbiology Reports*, 7(1), pp. 40–50.

Wangolo, E. *et al.* (2015) 'Effects of Shoot Tip and Flower Removal on Growth and Yield of Spider Plant (*Cleome gynandra* L.) in Kenya', *American Journal of Experimental Agriculture*, 8(6), pp. 367–376.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Indigenous vegetables and endophytic bacteria have become a new focus in modern agriculture (Adebooye and Opabode, 2004; Bhattacharyya and Jha, 2012). Previous and current literature have reviewed the potential production of indigenous crops and the use of endophytic bacteria as biofertilizers (Hynes *et al.*, 2008; Agbo *et al.*, 2009; Gido, Bett and Bokelmann, 2016; Timmusk *et al.*, 2017; Kalayu, 2019). This review chapter will focus on the following themes: background of *Cleome gynandra*, seed endophytic bacteria interactions and importance, plant growth promoting endophytes modes of action and potential of endophytic bacteria as biofertilizers. Furthermore, this chapter will focus on the application of seed endophytic bacteria and its application to Namibian agriculture.

2.2 *Cleome gynandra* L. (*C. gynandra*)

Ombidi (in Oshikwanyama and Oshimbadja); ombivi (in Oshimbalanhu); omboga (in all other Oshiwambo language dialects); ombowayozondu, ombowa, ombowayozongombe (in Herero language), gomabeb (in Damara/Nama language); Cat's whisker, spider plant (English) (Kolberg, 2001; P.T Shipoh 2018, pers. comm., 12 December).

Cleome gynandra L. (**Figure 2.1**), synonym *Gynandropsis gynandra* (L.) Briq., is a wild annual leafy vegetable spread around Namibia (Kolberg, 2001). It is dominant in the northern Namibian regions (**Figure 2.2**) of Omusati, Oshana, Ohangwena and Oshikoto, which hosts 40% of the Namibian population (Starkey *et al.*, 2017), though, it is not restricted to these areas only (Kolberg, 2001). It belongs to the *Capparaceae* family (formerly *Capparidaceae*), *Cleomoideae* subfamily and is a phylogenetic near relative to

the *Brassicaceae* family (Chweya and Mnzava, 1997; Wu *et al.*, 2017), with 15 species native to Namibia (Craven, 1999) and *C. gynandra* being the most widely used species as a leafy vegetable (Kolberg, 2001). *C. gynandra* grows as a weed in Southern Africa and is characterized by its long taproots, with fewer secondary roots (Chweya and Mnzava, 1997).

C. gynandra has shown great agronomic, medicinal, economic and adaptive potential (Wangolo *et al.*, 2015). It has a C_4 photosynthetic pathway, an adaptation that allows it to survive arid environments (Chweya and Mnzava, 1997; Marshall *et al.*, 2007). Its seeds have high levels of polyunsaturated oil, allowing the oil to be edible (Mnzava, 1990), to make soap and used to kill lice (Burkill, 1985). The use of its oil is not practised in Namibia (P.T. Shipoh 2018, pers. comm., 12 December). The plant has a variety of medicinal properties such as the ability to induce childbirth labour (Kamatenesi-Mugisha and Oryem-Origa, 2007; Kujeke, Edziwa and Icishahayo, 2017), treat scorpion stings, snake bites and fevers (Manandhar, 2002) and displayed antifungal properties (Imanirampa and Alele, 2016). The African spider has been known to have pest repellent properties (Guarino, 1997; Opiro *et al.*, 2013) and through its sale, provide a source of income in some African countries (Chweya and Mnzava, 1997; Muhanji *et al.*, 2011).

Figure 2.1: *Cleome gynandra* L. plant. (Masuka, Goss and Mazarura, 2012).

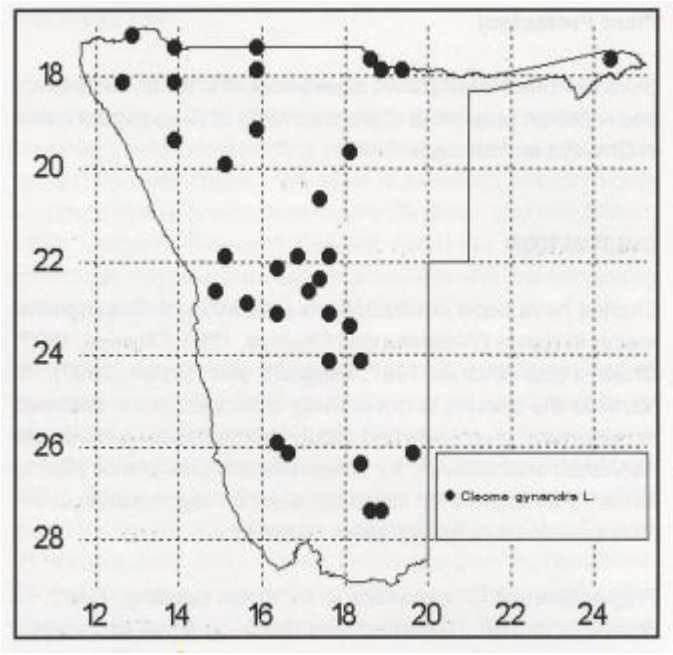


Figure 2.2: Geographical distribution of *Cleome gynandra* L. in Namibia (Kolberg, 2001).

C. gynandra is a wildflower with an origin that is not very clear (Lokesha, 2018). It is believed to have originated in sub-Saharan Africa and southern Asia, but to date, it is spread out worldwide in tropical, subtropical and pacific regions (Bremer and Wannorp, 1975; Lokesha, 2018; Shilla *et al.*, 2019). It is a herbaceous plant with edible leaves that can grow between 0.5 to 1.5m tall, depending on environmental conditions (Guarino, 1997; van Rensburg *et al.*, 2007). Leaf shape is compound and palmate (covered with glandular hair), with the plant bearing three to seven leaflets (Guarino, 1997; Masuka, Goss and Mazarura, 2012). It is an annual plant that grows best during summer (between December and March) when it is supplied with adequate amounts of water during the rainy season and when it has complete sunlight exposure at temperatures of 18˚C to 25˚C (Kakujaha-Matundu, 1996; Kolberg, 2001; van Rensburg *et al.*, 2007). *C. gynandra* has variable pigmentation in its stems and flowers. Stems vary from green, pink to purple while flowers come in shades of white, light pink or purple (Mishra, Moharana and Dash, 2011; Masuka, Goss and Mazarura, 2012). It has been reported that the purple stems are more nutritious while other reports have claimed that the green stems are more resistant to insects, but more susceptible to diseases (Silué, 2009). Although *C. gynandra* pollination mechanisms are not fully understood, it is believed that the plant has evolved strategies that are specific to self-pollinating plant species (Lokesha, 2018). The plants properties, coupled with its ability to grow in arid conditions make it versatile and suitable for various agricultural prospects (Onyango, Onwonga and Kimenju, 2016).

Human contribution does not dictate its growth as it grows wild (Rodin, 1985; Edmonds and Chweya, 1997). The plant is available in areas with an abundance of animal manure, noting that it is mostly found near cattle posts and cultivated fields (Rodin, 1985; Shackleton *et al.*, 1998). Its seeds are considered stubborn as they have a hard shell,

allowing for them to only be propagated by cattle, and can remain dormant for up to six months or longer depending on the region (Chweya and Mnzava, 1997). Dormancy can however be artificially disrupted by incubation of the seeds at 40 ˚C for one to five days (Lokesha, 2018). Cattle eat the flowers, seeds/seed pods which are then expelled into its dung, which is used as a fertilizer and spread out onto the field, allowing the plant to grow when conditions are deemed conducive. According to subsistence farmers in the area, the plant is only harvested for consumption during the months of November, December and January (P.T. Shipoh 2018, pers comm., 12 December; Kolberg, 2001; van Rensburg *et al.*, 2007). If it is harvested any time after that, it is known to have a bitter taste and considered a weed (Guarino, 1997; Kolberg, 2001; van Rensburg *et al.*, 2007; Silué, 2009) which is why it gets destroyed from then on as the farmers begin to prepare for the harvesting season.

There are very few genetic diversity studies that have been done on *Cleome gynandra* L., that are specific to Africa, where the species is widely distributed. Not much literature has reported on its breeding or harvesting. The plant is known to be widely distributed in tropic and sub-tropic areas, which can attribute to its diversity. Genetic variation can be attributed to edaphic factors, mutation load and morphological traits such as differences in aroma (Lokesha, 2018). Morphological variation is not much but can be narrowed down to aromatic and non-aromatic individual plants (Kolberg, 2001). Previous studies have attempted to assess genetic diversity by identifying species according to taxonomic descriptions and through the use of molecular markers such as allozymes and inter-simple sequence repeats (Omondi *et al.*, 2016).

2.3 *Cleome gynandra* nutritional content and its importance

There are several underutilized naturally occurring, green leafy vegetables with promising nutritional value (Kavitha and Ramadas, 2013; Gido, Bett and Bokelmann, 2016). *C. gynandra* is a nutritive vegetable that can retain most of its nutrients after cooking in comparison to other leafy vegetables in its category (Silué, 2009). Understanding this retainability will allow subsistence farmers to further improve the nutritional value of this plant (Silué, 2009; Muhanji *et al.*, 2011).

Table 2.1: Nutrient comparison of raw *C. gynandra* leaves to exotic vegetables (Adapted from Kolberg, 2001).

Vegetable	Vit. A mg/100g	Vit. C mg/100g	Iron mg/100g	Calcium mg/100g	Protein g/100g
Lettuce (*Lactuca sativa* L.)	0.2 - 7.8	3 - 33	0.5 - 4.0	17-107	0.8 - 1.6
Spinach (*Spinacia oleraecea*)	2.8 - 7.4	1 - 59	0.8 - 4.5	60 - 595	2.3 - 3.1
Cleome gynandra L.	6.7 - 18.9	127 - 484	1 - 18.8	213 - 434	3.1 - 7.7

Table 2.2: Activities of vitamins and minerals (Adapted from Hedges and Lister, 2005).

Name	Major Function
Vitamin A	Essential for eyesight and eye health
Vitamin C	Needed for healthy connective tissue
Iron	Component for haemoglobin in blood
Calcium	Structural component for teeth and bones
Protein	Essential for growth and maintenance

In comparison to lettuce and spinach, *C. gynandra* has a significantly higher overall nutrient content (**Table 2.1**). *C. gynandra* is an important vegetable in rural areas across several African countries as it is the main source of vitamins and minerals (Omondi *et al.*, 2017) (**Table 2.2**). Exotic vegetables have taken a permanent place in most households which have allowed modern agronomy to cater for and conserve their genetic resources

and boost their productivity (Ng'etich, Aguyoh and Ogweno, 2012). With leafy vegetables such as *C. gynandra* being nutritionally superior to exotic vegetables, knowledge of its overall agronomy, broadening food bases and domestication should be at the forefront of modern agricultural practices (Chivenge *et al.*, 2015; Wangolo *et al.*, 2015; Chataika *et al.*, 2020).

2.4 Seed Endophytes

Seed-borne endophytes are vertically transmitted and live in close relationships with their host throughout successive generations (Truyens *et al.*, 2014; Verma, Kharwar and White, 2019; Tyc *et al.*, 2020). Seeds play an integral role in spermatophyte life cycles: they can remain dormant until favourable conditions allow them to germinate and develop into a new plant (Nelson, 2004; Shahzad *et al.*, 2018). Seeds can benefit from seed-associated microbe interactions as they assist in seed preservation (Bednarek and Osborn, 2009; Geisen *et al.*, 2017). When seeds germinate, their intake of water leads to an exudate secretion that attracts endophytes, which in turn colonises the rhizosphere and spermosphere (Nelson, 2004). They allow seedlings to, directly and indirectly, promote their host's growth (**Figure 2.3**), as they are tolerant to abiotic stress and resistant to pests and pathogens (Nelson, 2004; Parsa *et al.*, 2016). Endophytes also produce secondary metabolites for plant environmental adaptation and crop yield improvement (Liu, Kloepper and Tuzun, 1995; Ramamoorthy *et al.*, 2001). Direct mechanisms include nitrogen (N_2) fixation, phosphate solubilization, indole-3-acetic acid (IAA) production, while indirect mechanisms include siderophore production and ammonia (NH_3) production (Goswami, Thakker and Dhandhukia, 2016; Premachandra, Hudek and Brau, 2016). Secondary metabolite production comes in the form of fluorescence production

and hydrogen cyanide (HCN) production (Allu, Kumar and Audipudi, 2014; Kamei, Dolai and Kamei, 2014). Seed endophytes have been of particular interest in recent studies as they are transmitted from generation to generation (Truyens *et al.*, 2014; Shahzad *et al.*, 2018). Seed-borne endophytes ensure that the next developing plant will be favoured by mutualism and grow to an optimum capacity (Rudgers *et al.*, 2009; Truyens *et al.*, 2014). To the best of my knowledge, no previous studies have been done on seed endophytes of *C. gynandra*.

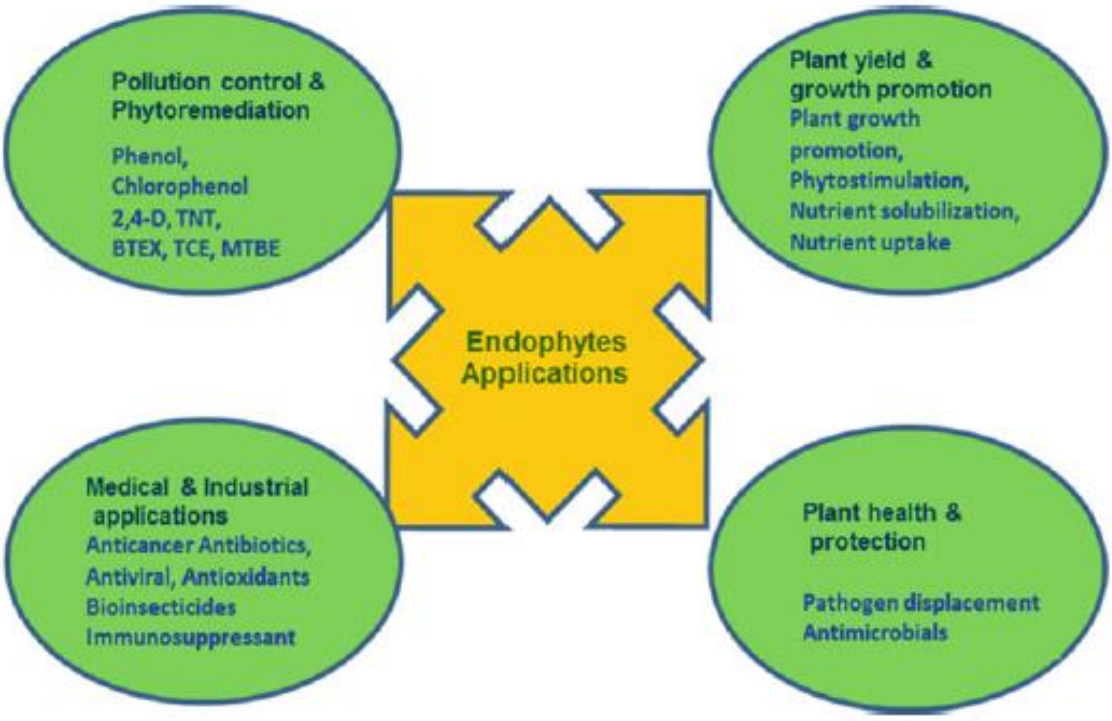


Figure 2.3: Application of endophytes in various fields (Arora and Ramawat, 2017).

2.5 Plant-Microbe Interactions: How Seeds Get Their Microbiome

Plant microbiomes are communities where endophytes dwell in plants (Hardoim *et al.*, 2012; Mitter *et al.*, 2017). Seeds were reported to be the initial source of endophytes or

pathogens in a plant (Baker and Smith, 1966). The internal seed microbiome is inherited via the seed from previous generations to survive conditions of seed storage and desiccation (Truyens *et al.*, 2014; Mitter *et al.*, 2017). Although studies report that seeds of most plant species harbour endophytes (Schardl, Leuchtmann and Spiering, 2004), seed-vectored endophytes have been poorly investigated (Marianela *et al.*, 2018; Verma, Kharwar and White, 2019). The concept of seeds being an important source of bacterial endophytes has only become accepted as of late (Mano and Morisaki, 2008). It has been revealed that a wide array of endophytes can be obtained from plant tissue (Hardoim *et al.*, 2012).

Studies have centred around the concept of root colonisation by beneficial endophytes in the rhizosphere. The rhizosphere is an area of soil that borders the root system of a plant (Barea *et al.*, 2005; Ahemad and Kibret, 2014). This area facilitates metabolic processes and serves as a vector for nutrient uptake (Bowen and Rovira, 1999; Bednarek and Osborn, 2009). For endophytes to grow optimally in the rhizosphere, the soil should have a neutral pH, a good supply of organic and inorganic nutrients, and lastly, good water holding capacity. In meeting the needs of the endophytes, the plant will release secondary metabolites (Stone and Williams, 1992; Demain, 1999; Kai, Effmert and Piechulla, 2016). These secondary metabolites will interact symbiotically with the endophytes to improve the plants' overall growth indirectly by inhibiting plant pathogens and directly by solubilizing nutrients (Whipps, 2001; Premachandra, Hudek and Brau, 2016). Root exudates attract beneficial bacteria and other soil microorganisms for interaction in the rhizosphere (Walker *et al.*, 2003). However, for the endophytes to be successful in colonising the root, they would have to compete against the other microbes in the surrounding area (Weller *et al.*, 2002; Compant, Clement and Sessitsch, 2010). Secondary

metabolites have been known to confer a competitive edge over other microorganisms, which in turn further contributes to root colonisation (Premachandra, Hudek and Brau, 2016). These metabolites are secreted by specific bacterial strains allowing for better competition among the natural flora in the root zone of the plant (Haas and Défago, 2005; Carvalhais *et al.*, 2013). This promotes their competitive capabilities. Some endophytes have shown the ability to colonise internal tissues in conjunction with rhizosphere colonisation, which has allowed them to display plant growth promoting traits (Bais *et al.*, 2006; Araujo *et al.*, 2008). Colonisation occurs when bacteria penetrate cracks occurring on the root surface or directly by the root tip (**Figure 2.4**; **Figure 2.5**). Some bacteria have developed further adaptations such as nodules or specific mechanisms for root penetration. In some interactions, colonisation can take place through fissures at the lateral root base and the cortical intercellular crack entry (Compant, Clement and Sessitsch, 2010). In other interactions, colonisation occurs in the interior of hairy root tissues and specialized organs called nodules (Garg and Geetanjali, 2007). Previous studies have indicated that endophytes make use of the various individual or combined mechanisms to allow complete root colonisation. Plant-microbe interactions can be classified according to the direction they occur in the rhizosphere: interaction with protozoa and metazoa (Ronn, Vestergard and Ekelund, 2012); interspecific and intraspecific communication in the bacterial community (Tyc *et al.*, 2014) and bacterial interaction with the plant (Bednarek and Osborn, 2009).

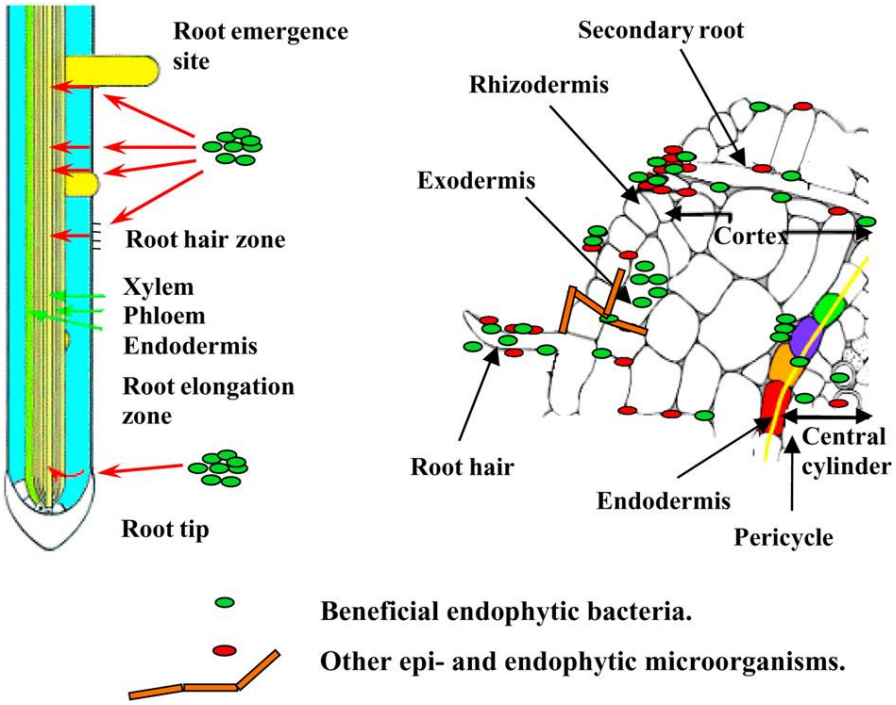


Figure 2.4: Sites of endophytic bacteria colonisation in plants (Compant, Clément and Sessitsch, 2010).

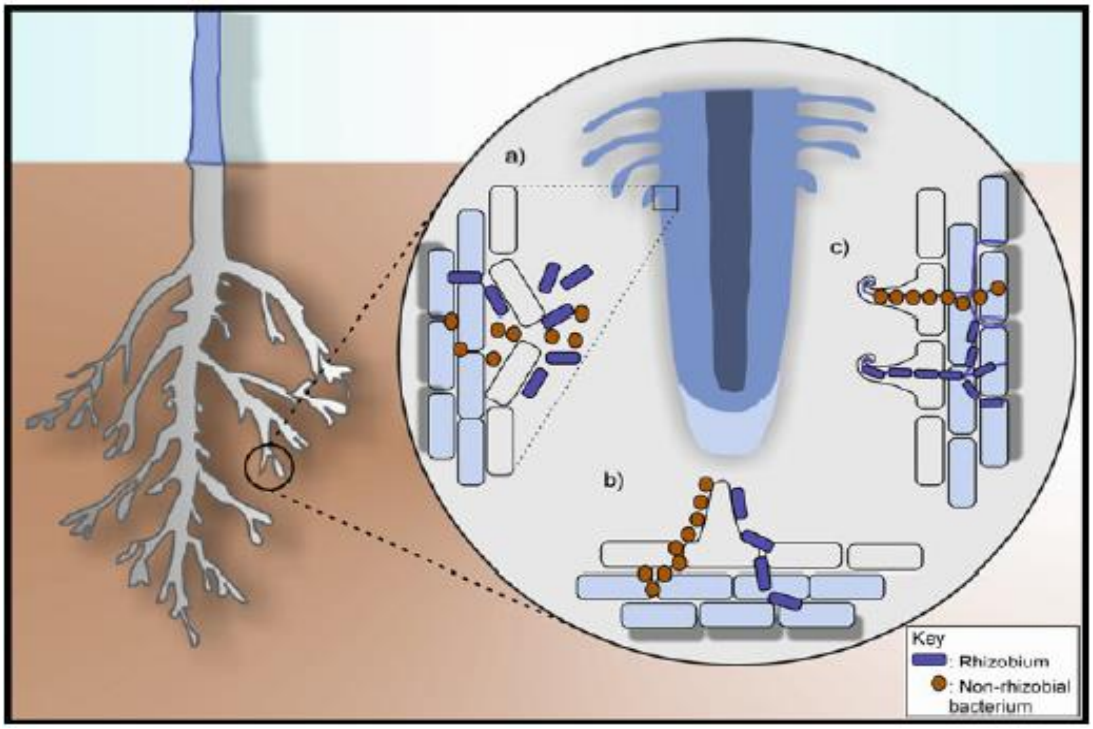


Figure 2.5: Mode of entry to plant roots as follows: a) Through epidermal cell layers; b) Through root hair colonisation; c) Through colonisation of infection threads (Ibanez *et al.*, 2017).

2.6 Plant growth promoting seed endophytes and their importance.

Endophytes are transferred to the seed from vegetative parts of the plant (lateral acquisition) and inherited from parent to offspring via gametes (vertical acquisition) (Shade, Jacques and Barret, 2017). Endophytes enter the seed through parent to offspring transmission where they are dispersed as the seed germinates. They get transferred to other seeds which they enter laterally and thus the cycle continues (Barea, 2015; Cope-Selby *et al.*, 2017; Shearin *et al.*, 2017). This ensures that the endophytes will be present in new plants to come (Truyens *et al.*, 2014). Endophytic bacteria are known to enhance plant growth in crops to improve their nutrition through nitrogen fixation and phosphate solubilization (Rodríguez and Fraga, 1999; Yadegari *et al.*, 2010; Ahemad and Kibret, 2014). Besides biofertilization, endophytic bacteria are also reported to promote plant growth and yield through the production of phytohormones like IAA, an auxin produced to stimulate root growth (Ahemad and Kibret, 2014; Chaturvedi, Singh and Gupta, 2016). As biopesticides, endophytes can indirectly control plant pests and diseases or induce resistance response like siderophores would (Goswami, Thakker and Dhandhukia, 2016). In return, the plant protects endophytes and provides them with nutrients in the form of photosynthates. Phytochemicals such as alkaloids, flavonoids and amino acids have also shown potential plant growth promoting functionality through auxin transport, seed dormancy and ion regulation, as well as defense against plant pathogens (Field, Jordán and Osbourn, 2006; Pérez-Montaño *et al.*, 2014; Vejan *et al.*, 2016). Endophytes are increasingly gaining scientific and commercial interest because of this potential to

improve plant quality and growth, and their close association with internal tissues of host plants (Ahemad and Kibret, 2014; Santoyo *et al.*, 2016). In general, the most commonly reported seed endophytes belong to the genera *Bacillus* and *Pseudomonas*, with *Acinetobacter, Paenibacillus, Staphylococcus, Micrococcus* and *Pantoea* (Sivasakthi, Usharani and Saranraj, 2014; Truyens *et al.*, 2014) (**Figure 2.6**).

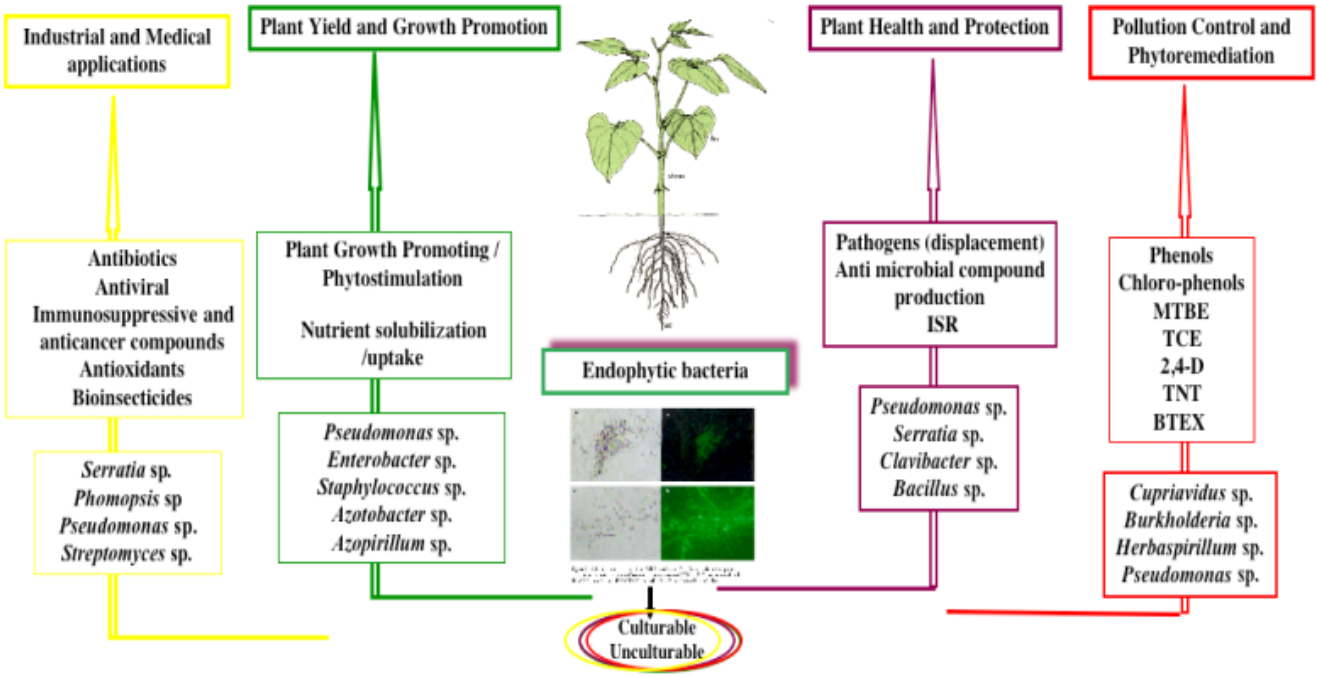


Figure 2.6: Shows a schematic diagram of various plant-bacterial endophyte interactions and their applications that have been previously studied (Ryan *et al.*, 2008).

2.7 Mode of action of Plant Growth Promoting Bacteria (PGPB) as biofertilizers

2.7.1 Direct mechanisms of action:

2.7.1.1 Nitrogen fixation

Nitrogen is an element that living organisms require in order to synthesize proteins and nucleic acids, making it essential for plant growth (Shridhar, 2012; de Souza, Ambrosini

and Passaglia, 2015). Biological nitrogen fixation (BNF) is the process where atmospheric nitrogen is converted into ammonia by nitrogen fixing bacteria using the nitrogenase enzyme to make it bioavailable (Egamberdieva and Kucharova, 2008; Franche, Lindström and Elmerich, 2009). Nitrogen fixing bacteria can be classified as symbiotic (forming a symbiotic relationship with a host) or non-symbiotic (free-living bacteria) (Shridhar, 2012). Nitrogenase (protein complex) is required to make the nitrogen fixation process possible in plants (Mahmud *et al.*, 2020). Nitrogenase is made up of enzymes and metal co-factors with the first one being dinitrogenase and the second being dinitrogenase reductase (Bulen and LeComte, 1966). Molybdenum nitrogenase is one of the three naturally occurring nitrogenases (Mahmud *et al.*, 2020). It is the most abundant nitrogenase as it has the most significance in nitrogen fixing bacteria and is produced by all natural diazotrophs (Dos Santos *et al.*, 2012; McGlynn *et al.*, 2013). The nitrogen fixing genes (*nif* genes) are found in symbionts and non-symbionts (Ahemad and Khan, 2011). These genes are required for the synthesis of nitrogenase by encoding enzyme complexes, nitrogen fixation functions and nodulation (Mahmud *et al.*, 2020). Nitrogen can serve as a limiting factor in crop production. The presence of these genes will regulate the function of nitrogenase, enable the plant to synthesize proteins and thus contribute to its growth (Dixon and Kahn, 2004). It has been reported over the years that there is a limited number of nitrogen fixing bacteria, but in more recent years, studies revealed that most microorganisms have nitrogen fixation as one of their traits (Franche, Lindström and Elmerich, 2009). Endophytic species belonging to the genera that have nitrogen fixing capabilities include *Rhizobium, Azotobacter, Bacillus, Enterobacter, Klebsiella, Pseudomonas, Arthrobacter* and *Azospirillum* (Dixon and Kahn, 2004; Tilak *et al.*, 2005; Sivasakthi, Usharani and Saranraj, 2014). *Rhizobium* are well-known symbiotic nitrogen

fixing bacteria that interact with leguminous plants by infecting their roots, forming nodules that serve as the site where endophytes fix nitrogen and make it readily available for the plant (Gonzalez-lopez and Pozo, 2005). Non-legume plants can fix nitrogen through plant organs other than the roots, intracellularly and extracellularly (Santi, Bogusz and Franche, 2013). *Azospirillum* (endophytic diazotroph) and *Azotobacter* are free-living nitrogen fixing bacteria that are linked to programs associated with non-legume plants worldwide (Shridhar, 2012). They develop and colonize the interior of the roots and surface of the host plant, with this interaction being considered the focal point of BNF (Gonzalez-lopez and Pozo, 2005).

2.7.1.2 Phosphate solubilization

Phosphorus is a key nutrient that is considered to limit plant growth (Ezawa, Smith and Smith, 2002; Vejan *et al.*, 2016). Microorganisms play an essential role in the natural phosphate cycle. This cycle takes place through the cyclic oxidation and reduction of phosphorous compounds, where phosphine is oxidized to phosphate (Rodríguez and Fraga, 1999). Soluble phosphorus is readily absorbed as HPO_4^{2-} and $H_2PO_4^-$ which will be used by the plant (Rodríguez and Fraga, 1999). It has an essential role in biochemical pathways which are particularly important for photosynthesis and BNF (Khan, Zaidi and Wani, 2007). Phosphate solubilizing endophytes are found in soil but are not abundantly spread (Mohammadi, 2012). This leads to competition for phosphate adsorption sites with other microbes that are more abundantly distributed throughout the rhizosphere (Mohammadi, 2012). This results in inadequate phosphate supply to the plant (Mohammadi, 2012). To overcome this, plants should be inoculated with a microbe that specifically solubilizes phosphate to allow the plant to receive it in a higher concentration

(Rodríguez and Fraga, 1999; Pradhan and Sukla, 2005). Studies show that the contribution phosphorus-solubilizing endophytes have towards plant nutrition has led to overall improvements in plant growth (Krasilinikov, 1957; Islam *et al.*, 2007). Phosphate solubilizing endophytes being used as inoculants allows for increased uptake of phosphorus, thus leading to crop yield increases (Rodríguez and Fraga, 1999; Walpola and Yoon, 2012). Previous studies indicated that bacteria able to express phosphate solubilization are among the *Klebsiella, Enterobacter, Pseudomonas, Bacillus, Agrobacterium, Rhizobium, Aereobacter, Achromobacter, Burkholderia, Micrococcus, Flavobacterium, Proteus, Serratia, Citrobacter* and *Erwinia* genera (Rodríguez and Fraga, 1999; Khan, Zaidi and Wani, 2007; Yousefi *et al.*, 2011; Walpola and Yoon, 2012; Singh *et al.*, 2014).

2.7.1.2.1 Mechanisms of Phosphate Solubilizing Microbes:

2.7.1.2.1.1 Inorganic phosphate solubilization

Inorganic phosphate solubilization is absorbed by lowering the soil or medium pH (**Figure 2.7**) and through chelation (Khan *et al.*, 2009). In alkaline soils, phosphates can precipitate and form insoluble calcium phosphate (Khan *et al.*, 2009). The solubility of calcium phosphate increases when the soil pH decreases and once the pH is lowered, phosphorus is made available (Fankem *et al.*, 2006; Khan *et al.*, 2009). Phosphate solubilizing microbes lower the soil pH through the secretion of organic acids (Ingle and Padole, 2017). Through metabolic processes such as oxidative respiration or fermentation, the microbes can produce organic acids, with the strength of the acid produced determining how efficient solubilization will be (Kalayu, 2019). Chelation enables phosphate solubilizing microbes to dissolve inorganic phosphate by competing with phosphate for absorption sites on the soil and through the chelation of cations bound

to phosphorous (Ingle and Padole, 2017). The chelation reaction involves the carboxyl and hydroxyl groups of acids chelating the cations bound to phosphorus which converts it to a more soluble form. These acids also compete for fixation sites on insoluble oxides such as Al and Fe. They react with them causing them to stabilize and thus allow them to be called ‘chelates’ (Walpola and Yoon, 2012).

2.7.1.2.1.2 Organic Phosphate Mineralization

Microorganisms need to mineralize (hydrolyse) substrates using the phosphatase enzyme (**Figure 2.7**) for them or the plant to utilize phosphorus in its organic form, with the process taking place at the expense of plant and animal remains (Khan *et al.*, 2009; Kalayu, 2019). This process plays an essential role in phosphorus cycling and makes use of a variety of enzymes that can either be alkaline or acidic (Alori, Glick and Babalola, 2017). Soil bacteria mineralize organic phosphorus by producing extracellular enzymes such as phytases, phospholipases and phosphoesterases (Walpola and Yoon, 2012).

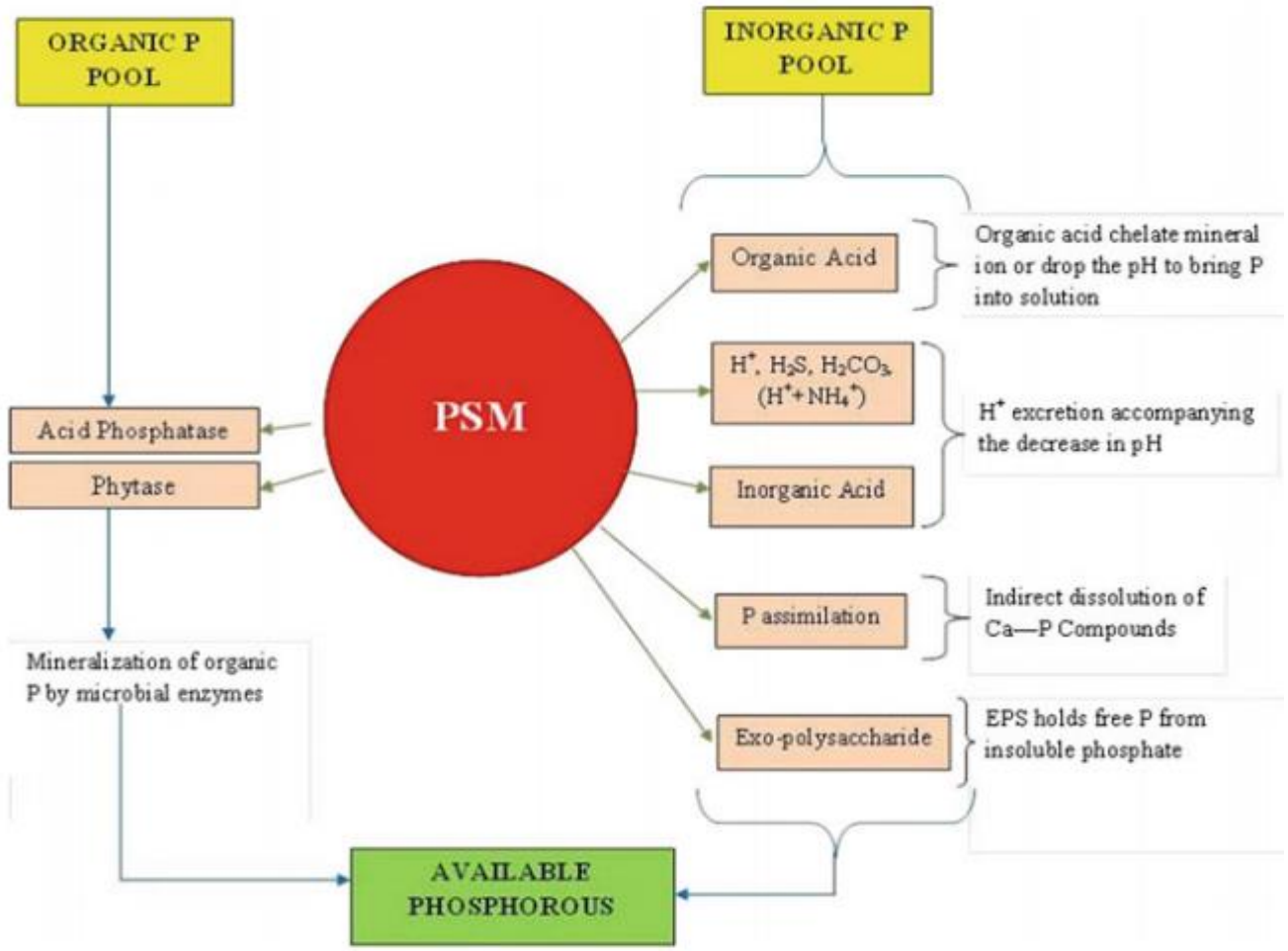


Figure 2.7: Mechanisms of phosphate solubilization/mineralization from organic/inorganic substances produced by phosphate solubilizing microorganisms (Walia *et al.*, 2017).

2.7.1.3 Indole-3-acetic acid (IAA) production

Indole-3-acetic acid (IAA) is an abundant naturally occurring auxin that belongs to a group of phytohormones, which are found in plants and stimulates root growth (**Figure 2.8**), with up to 80% of microorganisms being able to synthesize it (Vessey, 2003; Miransari and Smith, 2014; Gupta *et al.*, 2015). It influences an array of cellular functions which regulate plant growth and development. It is synthesized and secreted by PGPB, from which it gets absorbed directly on the seed or root surface of the plant by tryptophan

(an amino found in root exudates) available on root exudate or seed (Saleem *et al.*, 2007). Microorganisms inhabiting the rhizosphere synthesize and release IAA as a secondary metabolite because of the rich supply of substrates released from the roots (Shahab, Ahmed and Khan, 2009). IAA plays a vital role in plant-microbe interactions, varying from phytostimulation to pathogenesis. IAA also serves as a reciprocal signalling molecule that affects gene expression and regulates plant development through cellular responses in several microorganisms (Ahemad and Kibret, 2014). These responses include cell expansion, division and differentiation (Sivasakthi, Usharani and Saranraj, 2014). Studied *Pseudomonas* strains such as *P. fluorescens* and *P. putida* were able to synthesize indole-3-acetic acid (Khakipour *et al.*, 2008).

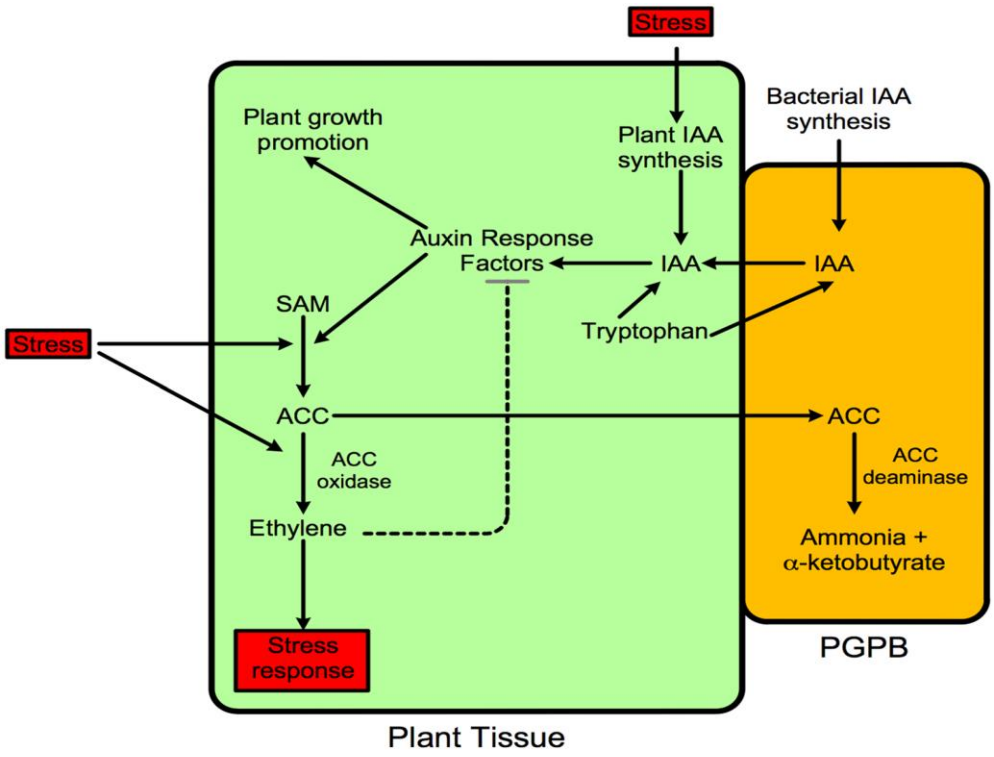


Figure 2.8: Schematic representation of plant growth stimulation by plant growth promoting phytohormones (Olanrewaju, Glick and Babalola, 2017).

As more bacterial species are analyzed, more precise routes for IAA bacterial biosynthesis pathways (**Figure 2.9**) are identified and classified according to intermittent compounds (Ljung *et al.*, 2002; Spaepen, Vanderleyden and Remans, 2007; Maheshwari, Dheeman and Agarwal, 2015). The tryptamine (TAM) pathway involves TAM being directly converted to indole-3-acetaldehyde (IAAld) by amine oxidase. Decarboxylation which is brought on with indole-3-pyruvate decarboxylase leads to the formation of IAA (Hartmann, Singh and Klingmuller, 1983). With the tryptophan side-chain oxidase (TSO) pathway, tryptophan is directly converted to IAAld by indole-3-pyruvate and is then oxidized to IAA simultaneously (Oberhansli, Defago and Haas, 1991). The main step of the indole-3-acetonitrile (IAN) pathway consists of the conversion of indole-3-acetamide via nitrilase where IAN is produced by tryptophan via indole-3-acetaldoxime (Patten and B. R. Glick, 1996; Zhao *et al.*, 2001). The indole-3-acetamide (IAM) pathway is considered the best pathway characterized by bacteria (Spaepen, Vanderleyden and Remans, 2007). It entails the conversion of tryptophan into IAA through two steps. The first step sees tryptophan being converted to IAM which is produced by the enzymatic action of tryptophan-2-monooxygenase. The second step sees IAA being obtained by the enzymatic hydrolysis of IAM by IAM hydrolase (Bar and Okon, 1993; Prinsen *et al.*, 1993; Spaepen, Vanderleyden and Remans, 2007; Maheshwari, Dheeman and Agarwal, 2015). The IAAld pathway centres around tryptophan being converted into indole-3-pyruvate by the aminotransferase enzyme and decarboxylated into IAAld by indole-pyruvate decarboxylase. The terminal step involves IAAld being oxidized into IAA (Ljung *et al.*, 2002; Maheshwari, Dheeman and Agarwal, 2015).

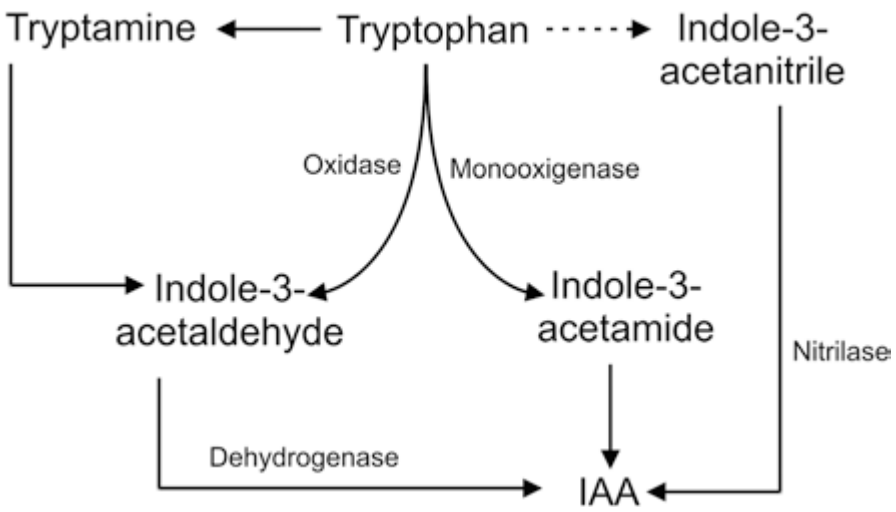


Figure 2.9: Indole-3-acetic acid biosynthesis pathways (Maheshwari, Dheeman and Agarwal, 2015).

2.7.1.4 Ammonia production

Ammonia is an important nutrient for plant growth and also serves as an anti-fungal metabolite (Whipps, 2001). Accumulation of ammonia in the soil creates alkaline conditions which suppress the growth of certain fungi (Bhattacharyya and Jha, 2012). Ammonia production is exhibited by microorganisms via metalloenzyme systems such as nitrogenase (Dixon and Kahn, 2004). Nitrogenases are enzymes that catalyze the biological reduction of atmospheric nitrogen to ammonia (Dixon and Kahn, 2004). The organism breaks down the complex nitrogenous material and releases ammonia in the soil which is taken up by the plant as a nutrient source (Bhattacharyya and Jha, 2012).

2.7.2 Indirect mechanisms of action:

2.7.2.1 Siderophore production

Iron (Fe) is one of the bulk minerals present in plenteous amounts on earth, yet it is unavailable in the soil for the plants (Rajkumar *et al.*, 2010). Iron is an important nutrient for microorganisms and plants as it plays a vital role in metabolic processes such as

photosynthesis, respiration and BNF (Kobayashi and Nishizawa, 2012). In instances where iron solubility in the soil is low, bacterial endophytes have been able to overcome this limitation by using a chelating agent known as a siderophore (Neilands and Leong, 1986; Krewulak and Vogel, 2008). The design of siderophores is intended to facilitate the formation of tight and stable complexes with ferric iron (Miethke and Marahiel, 2007). The mechanisms for Fe (II) uptake in endophytes have been elucidated. Fe (II) is widely available for living cells, however, it is oxidized to Fe (III) in the majority of microbial habitats (Hynes *et al.*, 2008). This is due to enzymatic oxidation as it is being assimilated by spontaneous reaction with molecular oxygen and circulation in a host organism, resulting in low concentrations or its lack of availability (Miethke and Marahiel, 2007; Premachandra, Hudek and Brau, 2016). As iron's bioavailability is relatively low, the role of siderophores has become essential in locating the iron environments and making it available to the plant (Chaitanya *et al.*, 2014). Siderophores have a high affinity to solubilize and bind iron together, allowing it to be transported. Bacterial endophytes belonging to the *Bacillus, Azotobacter, Pseudomonas* and *Enterobacter* genera have shown siderophore production capabilities, with the potential to promote plant growth and improve crop yield (Husen, 2003; Tian *et al.*, 2009). In the presence of siderophores, plant nutrition can improve and thus plant growth can increase.

2.7.2.2 Secondary Metabolite Production:

2.7.2.2.1 Fluorescence Production

Fluorescence producing bacteria have been considered the most promising PGPB as they can promote plant growth by the production of secondary metabolites such as phytohormones, hydrogen cyanide and antibiotics (Sivasakthi, Usharani and Saranraj, 2014). The proposed phytohormones produced are mainly, auxins, cytokinins,

gibberellins and ethylene which regulate plant growth and development in their respects (Kumar, Behl and Narula, 2001). Auxins regulate root growth, cytokinins and gibberellins inhibit plant pathogens and lastly, ethylene stimulates seed germination (Walsh, Morrissey and O'Gara, 2001). The most prominent fluorescence producing genus is *Pseudomonas* (Gupta *et al.*, 2015).

2.7.2.2.2 HCN production

HCN is a secondary metabolite that is produced naturally by a vast number of organisms including soil bacteria, plants, insects, algae and fungi and is known to serve as a biological control against pathogens as it has toxic properties, which however do not negatively affect the host plant (Haas and Défago, 2005; Kamei, Dolai and Kamei, 2014). Recent studies suggested that HCN could bind iron in the rhizosphere, indirectly making phosphate more readily available, directly increase other nutrient availability and by doing so, result in increased plant growth (Rijavec and Lapanje, 2016; Sagar *et al.*, 2018). *Pseudomonas* and *Bacillus* are genera of plant growth promoting bacterial strains that are noted for having HCN production as a common trait (Ahmad, Ahmad and Khan, 2008).

2.8 Endophytic bacteria as seed inoculants

Microbial inoculants have been used to serve as seed treatments as they deliver the microbe directly to the rhizosphere where plant-microbial activities take place. Seed inoculant treatments have been praised for their success to improve plant nutrient uptake and availability (O'Callaghan, 2016; Murphy, Doohan and Hodkinson, 2018). There are however factors that affect inoculant quality and effectiveness such as cost-effectiveness, time-consuming production and viable storage conditions (O'Callaghan, 2016). Studies

have focused on how to overcome these barriers. Endophytic PGPB have been of particular interest as an inoculant because their root colonisation ability has provided a favourable environment for plant function and development (de Souza, Ambrosini and Passaglia, 2015).

2.9 Endophytic bacteria and their potential role in Namibian agriculture

To date, there are no farming methods that can be used to successfully cultivate/domesticate *Cleome gynandra* L. in Namibia. To fully understand the farming methods of a specific crop, factors such as soil structure and fertility, sunlight concentration, temperature, climate and water management should be taken into account. Subsistence farmers do not have the means to look into these factors which is why any scientific contribution to farming methods would be in their interest. Namibia is a country that has farming as one of its most prominent economic activities (Mendelsohn, 2006). With this in mind, providing a reliable agricultural system that can easily be replicated is a necessity. The understanding of endophytes and their mechanisms can bring us one step closer to determining how they can be applied as effective treatments for the cultivation of indigenous vegetables. This will in turn improve the horticultural capability of indigenous plant species in Namibia.

REFERENCES

Adebooye, O. C. and Opabode, J. T. (2004) 'Status of Conservation of the Indigenous Leaf Vegetables and Fruits of Africa', *African Journal of Biotechnology*, 3(12), pp. 700–705.

Agbo, A. E. *et al.* (2009) 'Nutritional Importance of Indigenous Leafy Vegetables in Côte d' Ivoire', *Acta Horticulturae*, 806(45), pp. 361–366.

Ahemad, M. and Khan, M. S. (2011) 'Effect of Pesticides on Plant Growth Promoting Traits of *Mesorhizobium* strain MRC4', *Journal of the Saudi Society of Agricultural Sciences*. King Saud University & Saudi Society of Agricultural Sciences, 11(1), pp. 63–71.

Ahemad, M. and Kibret, M. (2014) 'Mechanisms and Applications of Plant Growth Promoting Rhizobacteria: Current Perspective', *Journal of King Saud University - Science*. King Saud University, 26(1), pp. 1–20.

Ahmad, F., Iqbal Ahmad and Khan, M. S. (2008) 'Screening of Free-Living Rhizospheric Bacteria for Their Multiple Plant Growth Promoting Activities', *Microbiological Research*, 163(2), pp. 173–181.

Allu, S., Kumar, N. P. and Audipudi, A. V (2014) 'Isolation, Biochemical and PGP Characterization of Endophytic *Pseudomonas aeruginosa* Isolated from Chilli Red Fruit Antagonistic Against Chilli *Anthracnose* Disease', *International Journal of Microbial Application Science*, 3(2), pp. 318–329.

Alori, E. T., Glick, B. R. and Babalola, O. O. (2017) 'Microbial Phosphorus Solubilization and It's Potential for Use in Sustainable Agriculture', *Frontiers in Microbiology*, 8(971).

Araujo, W. L. *et al.* (2008) 'Interaction Between Endophytes and Plant Host: Biotechnological Aspects', in Barka, E.A. and Clement, C. (eds.) *Plant-Microbe Interactions*. Research Signpost, pp. 95–115.

Arora, J. and Ramawat, K. G. (2017) 'An Introduction to Endophytes', in Maheshwari, D. K. (ed.) *Endophytes: Biology and Biotechnology*. Springer International Publishing, pp. 1–23.

Bais, H., *et al.* (2006) 'The Role of Root Exudates in Rhizosphere Interactions with Plants and other Organisms', *Annual Review of Plant Biology*, 57, pp. 233–266.

Baker, K. F. and Smith, S. H. (1966) 'Dynamics of Seed Transmission of Plant Pathogens', *Annual Review of Phytopathology*, 14, pp. 311–334.

Bar, T. and Okon, Y. (1993) 'Tryptophan Conversation to Indole-3-Acetic via Indole-3-Acetamide in *Azospirillum brasilense* Sp7', *Canadian Journal of Microbiology*, 39(1), pp. 81–86.

Barea, J.-M. *et al.* (2005) 'Microbial Co-Operation in the Rhizosphere', *Journal of Experimental Botany*, 56(417), pp. 1761–1778.

Barea, J. M. (2015) 'Future Challenges and Perspectives for Applying Microbial Biotechnology in Sustainable Agriculture Based on a Better Understanding of Plant-Microbiome Interactions', *Journal of Soil Science and Plant Nutrition*, 15(2), pp. 261–282.

Bednarek, P. and Osborn, A. (2009) 'Plant-Microbe Interactions: Chemical Diversity in Plant Defense', *Science*, pp. 746–748.

Bhattacharyya, P. N. and Jha, D. K. (2012) 'Plant Growth-Promoting Rhizobacteria (PGPR): Emergence in Agriculture', *World Journal of Microbiology and Biotechnology*, 28(4), pp. 1327–1350.

Bowen, G. D. and Rovira, A. D. (1999) 'The Rhizosphere and its Management to Improve Plant Growth', *Advances in Agronomy*, 66, p. 102.

Bremer, R. and Wannorp, H. E. (1975) 'Phylogenetic Systematics in Botany', *Taxon*, 27, pp. 317–329.

Bulen, W. and LeComte, J. J. (1966) 'The Nitrogenase System from *Azotobacter*: Two-Enzyme Requirement for N_2 Reduction, ATP-dependant H_2 Evolution, and ATP Hydrolysis', *Proceedings of the National Academy of Sciences of the United States of America*, 56, p. 979.

Burkill, H. M. (1985) *The Useful Plants of West Tropical Africa*. 2nd edn. Kew, UK: Royal Botanic Gardens.

Carvalhais, L. C. *et al.* (2013) 'Linking Plant Nutritional Status to Plant-Microbe Interactions', *PLoS ONE*, 8(7).

Chaitanya, K. *et al.* (2014) 'Polymer Producing Bacteria Showing Siderophore Activity with Chrome Azurol S (CAS) Agar Plate Assay', *International Journal of Scientific and Research Publications*, 4(12).

Chataika, B. *et al.* (2020) 'Diversity and Domestication Status of Spider Plant (*Gynandropsis gynandra* L.) amongst Sociolinguistic Groups of Northern Namibia', *Agronomy*, 10(1), p. 56.

Chaturvedi, H., Singh, V. and Gupta, G. (2016) 'Potential of Bacterial Endophytes as Plant Growth Promoting Factors', *Journal of Plant Pathology & Microbiology*, 7(9), p. 376.

Chivenge, P. *et al.* (2015) 'The Potential Role of Neglected and Underutilised Crop Species as Future Crops under Water Scarce Conditions in Sub-Saharan Africa',

International Journal of Environmental Research and Public Health, 12, pp. 5685–5711.

Chweya, J. A. and Mnzava, N. A. (1997) *Cat's Whiskers: Cleome gynandra L.*, *Biodiversity International*. Rome: Italy: International Plant Genetic Resources Institute.

Compant, S., Clement, C. and Sessitsch, A. (2010) 'Plant Growth-Promoting Bacteria in the Rhizo- and Endosphere of Plants: Their Role, Colonization, Mechanisms Involved and Prospects for Utilization', *Soil Biology & Biochemistry*, 42(5), pp. 669–678.

Cope-Selby, N. *et al.* (2017) 'Endophytic Bacteria in *Miscanthus* Seed: Implications for Germination, Vertical Inheritance of Endophytes, Plant Evolution and Breeding', *GCB Bioenergy*, 9(1), pp. 57–77.

Craven, P. (1999) *A Checklist of Namibian Plant Species.* Windhoek, Namibia.

Demain, A. (1999) 'Pharmaceutically Active Secondary Metabolites of Microorganisms', *Applied Microbiology and Biotechnology*, 52(4), pp. 455–463.

Dixon, R. and Kahn, D. (2004) 'Genetic Regulation of Biological Nitrogen Fixation', *Nature Reviews Microbiology*, 2(8), pp. 621–631.

Edmonds, J. M. and Chweya, J. A. (1997) *Black Nightshades: Solanum nigrum L . and Related Species.* Rome: Italy: International Plant Genetic Resources Institute.

Egamberdieva, D. and Kucharova, Z. (2008) 'Cropping Effects on Microbial Population and Nitrogenase Activity in Saline Arid Soil', *Turkish Journal of Biology*, 32(2), pp. 85–90.

Ezawa, T., Smith, S. E. and Smith, F. A. (2002) 'P Metabolism and Transport in AM Fungi', *Plant and Soil*, 244(1), pp. 221–230.

Fankem, H. *et al.* (2006) 'Occurrence and Functioning of Phosphate Solubilizing Microorganisms from Oil Palm Tree (*Elaeis guineensis*) Rhizosphere in Cameroon', *African Journal of Biotechnology*, 5(24), pp. 2450–2460.

Field, B., Jordán, F. and Osbourn, A. (2006) 'First Encounters – Deployment of Defence-Related Natural Products by Plants', *New Phytologist*, 172(2), pp. 193–207.

Franche, C., Lindström, K. and Elmerich, C. (2009) 'Nitrogen-Fixing Bacteria Associated with Leguminous and Non-Leguminous Plants', *Plant and Soil*, 321(1), pp. 35–59.

Garg, N. and Geetanjali (2007) 'Symbiotic Nitrogen Fixation in Legume Nodules: Process and Signaling. A Review', *Agronomy for Sustainable Development*, 27(1), pp. 59–68.

Geisen, S. *et al.* (2017) 'Seed and Root Endophytic Fungi in a Range Expanding and a Related Plant Species', *Frontiers in Microbiology*, 8(1645), pp. 1–11.

Gido, E. O., Bett, H. K. and Bokelmann, W. (2016) 'Importance of African Indigenous Vegetables in Food Systems', *African Journal of Horticultural Science*, 10, pp. 34–41.

Gonzalez-lopez, J. and Pozo, C. (2005) 'Liberation of Amino Acids by Heterotrophic Nitrogen Fixing Bacteria: Minireview Article', *Amino Acids*, 28(4), pp. 363–367.

Goswami, D., Thakker, J. N. and Dhandhukia, P. C. (2016) 'Portraying Mechanics of Plant Growth Promoting Rhizobacteria (PGPR): A Review', *Cogent Food & Agriculture*, 2(1), pp. 1–19.

Guarino, L. (1997) 'Local Leafy Vegetables', in *Traditional African Vegetables*. Rome (Italy): International Plant Genetic Resources Institute, pp. 75–81.

Gupta, G. *et al.* (2015) 'Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture', *Journal of Microbial & Biochemical Technology*, 7(2), pp. 96–102.

Haas, D. and Défago, G. (2005) 'Biological Control of Soil-borne Pathogens by Fluorescent *Pseudomonads*', *Nature Reviews Microbiology*, 3(4), pp. 307–319.

Hardoim, P. R. *et al.* (2012) 'Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages', *PLoS ONE*, 7(2).

Hartmann, A., Singh, M. and Klingmuller, W. (1983) 'Isolation and Characterization of *Azospirillum* Mutants Excreting High Amounts of Indoleacetic Acid', *Canadian Journal of Microbiology*, 29(8), pp. 916–923.

Hedges, L. J. and Lister, C. E. (2005) *Nutritional Attributes of Salad Vegetables*. Christchurch: New Zealand.

Husen, E. (2003) 'Screening of Soil Bacteria for Plant Growth Promotion Activities In Vitro', *Indonesian Journal of Agricultural Science*, 4(1), pp. 27–31.

Hynes, R. K. *et al.* (2008) 'Isolation, Selection, and Characterization of Beneficial Rhizobacteria from Pea, Lentil, and Chickpea Grown in Western Canada.', *Canadian Journal of Microbiology*, 54(4), pp. 248–258.

Ibanez, F. *et al.* (2017) 'Bacterial Endophytes of Plants: Diversity, Invasion Mechanisms and Effects on the Host', in Maheshwari, D. K. (ed.) *Endophytes: Biology and Biotechnology*. Springer International Publishing, pp. 25–40.

Imanirampa, L. and Alele, P. E. (2016) 'Antifungal Activity of *Cleome gynandra* L. Aerial Parts for Topical Treatment of *Tinea capitis*: An In Vitro Evaluation', *BMC Complementary and Alternative Medicine*, 16(1), p. 194.

Ingle, K. P. and Padole, D. A. (2017) 'Phosphate Solubilizing Microbes: An Overview', *International Journal of Current Microbiology and Applied Sciences*, 6(1), pp. 844–852.

Islam, M. *et al.* (2007) 'Isolation and Identification of Potential Phosphate Solubilizing Bacteria from the Rhizoplane of *Oryza sativa* L. cv. BR29 of Bangladesh', *Zeitschrift für Naturforschung*, 62(1–2), pp. 103–110.

Kai, M., Effmert, U. and Piechulla, B. (2016) 'Bacterial-Plant-Interactions: Approaches to Unravel the Biological Function of Bacterial Volatiles in the Rhizosphere', *Frontiers in Microbiology*, 7(108).

Kakujaha-Matundu, O. (1996) 'Subsistence Farmers Perception of Environmental Problems and Monetary Estimates of Agricultural and Non-Agricultural Resources in the Okakarara Area', *Desert Research Foundation of Namibia Occassional Paper 5*.

Kalayu, G. (2019) 'Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers', *International Journal of Agronomy*, 2019.

Kamatenesi-Mugisha, M. and Oryem-Origa, H. (2007) 'Medicinal Plants Used to Induce Labour During Childbirth in Western Uganda', *Journal of Ethnopharmacology*, 109(2007), pp. 1–9.

Kamei, Adam, Dolai, A. K. and Kamei, Apou (2014) 'Role of Hydrogen Cyanide Secondary Metabolite of Plant Growth Promoting Rhizobacteria as Biopesticides of Weeds', *Global Journal of Science Frontier Research (D)*, 14(6), pp. 109–112.

Kavitha, V. and Ramadas, V. S. (2013) 'Nutritional Composition of Raw Fresh and Shade Dried Form of Spinach Leaf (*Spinach oleracea*)', *JPR:BioMedRx: An International Journal*, 1(8), pp. 767–770.

Khakipour, N. *et al.* (2008) 'Production of Auxin Hormone by Fluorescent *Pseudomonads*', *American-Eurasian Journal of Agricultural & Environmental Science*, 4(6), pp. 687–692.

Khan, A. A. *et al.* (2009) 'Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and their Role in Crop Production', *Journal of Agricultural and Biological Science*, 1(1), pp. 48–58.

Khan, M. S., Zaidi, A. and Wani, P. A. (2007) 'Role of Phosphate-Solubilizing Microorganims in Sustainable Agriculture - A Review', *Agronomy for Sustainable Development*, 27(1), pp. 29–43.

Kobayashi, T. and Nishizawa, N. K. (2012) 'Iron Uptake, Translocation, and Regulation in Higher Plants.', *Annual Review of Plant Biology*, 63, pp. 131–152.

Kolberg, H. (2001) 'Indigenous Namibian Leafy Vegetables: A Literature Survey and Project Proposal', *Agricola*, 12, pp. 54–60.

Krasilinikov, N. A. (1957) 'On the Role of Soil Micro-Organism in Plant Nutrition', *Microbiologiya*, 26, pp. 659–672.

Krewulak, K. D. and Vogel, H. J. (2008) 'Structural Biology of Bacterial Iron Uptake', *Biochimica et Biophysica Acta - Biomembranes*, 1778(9), pp. 1781–1804.

Kujeke, G. T., Edziwa, X. and Icishahayo, D. (2017) 'Field Performance of Spider Plant (*Cleome gynandra* L.) Under Different Agronomic Practices', *African Journal of Food, Agriculture, Nutrition and Development*, 17(3), pp. 12179–12197.

Kumar, V., Behl, R. K. and Narula, N. (2001) 'Establishment of Phosphate Solubilizing Strains of *Azotobacter chroococcum* in Rhizosphere and their Effect on Wheat Under Green House Condition', *Microbiological Research*, 156(1), pp. 87–93.

Liu, L., Kloepper, J. W. and Tuzun, S. (1995) 'Induction of Systemic Resistance in Cucumber Against Bacterial Angular Leaf Spot by Plant Growth-Promoting Rhizobacteria', *Phytopathology*, 85(8), pp. 843–847.

Ljung, K. *et al.* (2002) 'Biosynthesis, Conjugation, Catabolism and Homeostasis of Indole-3-Acetic Acid in *Arabidopsis thaliana*', *Plant Molecular Biology*, 50(2), pp. 309–332.

Lokesha, R. (2018) 'The African Spider Plant (*Gynandropsis pentaphylla* – DC. Syn. *Cleome gynandra* Linn.) (Capparaceae): Phytochemistry, Pharmacological and Biotechnological Properties - A Review', *International Journal of Pharmacognosy and Chinese Medicine*, 2(4), pp. 1–9.

Maheshwari, D. K., Dheeman, S. and Agarwal, M. (2015) 'Phytohormone-Producing PGPR for Sustainable Agriculture', in Maheshwari, D. K. (ed.) *Bacterial Metabolites in Sustainable Agroecosystem*. Springer International Publishing, pp. 159–182.

Mahmud, K. *et al.* (2020) 'Current Progress in Nitrogen Fixing Plants and Microbiome Research', *Plants*, 9(1), p. 97.

Manandhar, N. P. (2002) *Plants and People of Nepal*. Portland, Oregon: Timber Press.

Mano, H. and Morisaki, H. (2008) 'Endophytic Bacteria in the Rice Plant', *Microbes and Environments*, 23(2), pp. 109–117.

Marianela, S. *et al.* (2018) 'Microbial Endophytes that Live within the Seeds of Two Tomato Hybrids Cultivated in Argentina', *Agronomy*, 8(8), p. 136.

Marshall, D. M. *et al.* (2007) '*Cleome*, a Genus Closely Related to *Arabidopsis*, Contains Species Spanning a Developmental Progression from C_3 to C_4 Photosynthesis', *The Plant Journal*, 51(5), pp. 886–896.

Masuka, A., Goss, M. and Mazarura, U. (2012) 'Morphological Characterization of Four Selected Spider Plant (*Cleome gynandra* L.) Morphs from Zimbabwe and Kenya', *Asian Journal of Agriculture and Rural Development*, 2(4), pp. 646–657.

McGlynn, S. E. *et al.* (2013) 'Classifying the Metal Dependence of Uncharacterized Nitrogenases', *Frontiers in Microbiology*, 3, p. 419.

Mendelsohn, J. (2006) *Farming Systems in Namibia*. Cape Town: RAISON.

Miethke, M. and Marahiel, M. A. (2007) 'Siderophore-Based Iron Acquisition and Pathogen Control', *Microbiology and Molecular Biology Reviews*, 71(3), pp. 413–451.

Miransari, M. and Smith, D. L. (2014) 'Plant Hormones and Seed Germination', *Environmental and Experimental Botany*, 99, pp. 110–121.

Mishra, S. S., Moharana, S. K. and Dash, M. R. (2011) 'Review on *Cleome gynandra*', *International Journal of Research in Pharmacy and Chemistry*, 3.

Mitter, B. *et al.* (2017) 'A New Approach to Modify Plant Microbiomes and Traits by Introducing Beneficial Bacteria at Flowering into Progeny Seeds', *Frontiers in Microbiology*, 8(11).

Mnzava, N. A. (1990) 'Studies on Tropical Vegetables. Part: Amino and Patty Acid Composition in seed of *Cleome* (*Gynandropsis gynandra* L. Briq) Selections of Zambia', *Food Chemistry*, 35(4), pp. 287–293.

Mohammadi, K. (2012) 'Phosphorus Solubilizing Bacteria: Occurrence, Mechanisms and Their Role in Crop Production', *Resources and Environment*, 2(1), pp. 80–85.

Muhanji, G. *et al.* (2011) 'African Indigenous Vegetable Enterprises and Market Access for Small-Scale Farmers in East Africa', *International Journal of Agricultural Sustainability*, 9(1), pp. 194–202.

Murphy, B. R., Doohan, F. M. and Hodkinson, T. R. (2018) 'From Concept to Commerce: Developing a Successful Fungal Endophyte Inoculant for Agricultural Crops', *Journal of Fungi*, 4(1), p. 24.

Neilands, J. B. and Leong, S. A. (1986) 'Siderophores in Relation to Plant Growth and Disease', *Annual Review of Plant Physiology*, 37, pp. 187–208.

Nelson, E. B. (2004) 'Microbial Dynamics and Interactions in the Spermosphere', *Annual Review of Phytopathology*, 42(1), pp. 271–309.

Ng'etich, O. K., Aguyoh, J. N. and Ogweno, J. O. (2012) 'Growth, Yield and Physiological Responses of Spider Plant (*Cleome gynandra* L.) to Calcium Ammonium Nitrate Rates', *International Journal of Agronomy and Plant Production*, 3(9), pp. 346–355.

O'Callaghan, M. (2016) 'Microbial Inoculation of Seed for Improved Crop Performance: Issues and Opportunities', *Applied Microbiology and Biotechnology*, 100(13), pp. 5729–5746.

Oberhansli, T., Defago, G. and Haas, D. (1991) 'Indole-3-Acetic Acid (IAA) Synthesis in the Biocontrol Strain CHA0 of *Pseudomonas fluorescens*: Role of Tryptophan Side Chain Oxidase', *Journal of General Microbiology*, 137(10), pp. 2273–2279.

Olanrewaju, O. S., Glick, B. R. and Babalola, O. O. (2017) 'Mechanisms of Action of Plant Growth Promoting Bacteria', *World Journal of Microbiology and Biotechnology*, 33(11), pp. 2364-2369.

Omondi, E. O. *et al.* (2016) 'Molecular Markers for Genetic Diversity Studies in African Leafy Vegetables', *Advances in Bioscience and Biotechnology*, 7(3), pp. 188–197.

Omondi, E. O. *et al.* (2017) 'Nutritional Compound Analysis and Morphological Characterization of Spider Plant (*Cleome gynandra*) - an African Indigenous Leafy Vegetable', *Food Research International*, 100(1), pp. 284–295.

Onyango, C. M., Onwonga, R. N. and Kimenju, J. W. (2016) 'Assessment of Spider Plant (*Cleome gynandra* L.) Germplasm for Agronomic Traits in Vegetable and Seed Production: A Green House Study', *American Journal of Experimental Agriculture*, 10(1), pp. 1–10.

Opiro, R. *et al.* (2013) 'Tick-Repellent Properties of Four Plant Species Against *Rhipicephalus appendiculatus* Neumann (*Acarina: Ixodidae*) Tick Species', *Journal of Agricultural Research and Development*, 3(2), pp. 17–21.

Parsa, S. *et al.* (2016) 'Fungal Endophytes in Germinated Seeds of the Common Bean, *Phaseolus vulgaris*', *Fungal Biology*, 120(5), pp. 783–790.

Patten, C. L. and Glick, B. R. (1996) 'Bacterial Biosynthesis on Indole-3-Acetic Acid', *Canadian Journal of microbiology*, 42(3), pp. 201–220.

Pérez-Montaño, F. *et al.* (2014) 'Plant Growth Promotion in Cereal and Leguminous Agricultural Important Plants: From Microorganism Capacities to Crop Production', *Microbiological Research*, 169(5–6), pp. 325–336.

Pradhan, N. and Sukla, L. B. (2005) 'Solubilization of Inorganic Phosphate by Fungi Isolated from Agricultural Soil', *African Journal of Biotechnology*, 5(10), pp. 850–854.

Premachandra, D., Hudek, L. and Brau, L. (2016) 'Bacterial Modes of Action for Enhancing of Plant Growth', *Journal of Biotechnology & Biomaterials*, 6(3), p. 236.

Prinsen, E. *et al.* (1993) '*Azospirillum brasilense* Indole-3-Acetic Acid Biosynthesis: Evidence for a Non-Tryptophan Dependent Pathway.', *Molecular Plant-Microbe Interactions*, 6(5), pp. 609-615.

Rajkumar, M. *et al.* (2010) 'Potential of Siderophore-Producing Bacteria for Improving Heavy Metal Phytoextraction', *Trends in Biotechnology*, 28(3), pp. 142–149.

Ramamoorthy, V. *et al.* (2001) 'Induction of Systemic Resistance by Plant Growth Promoting Rhizobacteria in Crop Plants Against Pest and Diseases', *Crop Protection*, 20(1), pp. 1–11.

van Rensburg, W. S. J. *et al.* (2007) 'African Leafy Vegetables in South Africa', *Water SA*, 33(3), pp. 317–326.

Rijavec, T. and Lapanje, A. (2016) 'Hydrogen Cyanide in the Rhizosphere: Not Suppressing Plant Pathogens, but Rather Regulating Availability of Phosphate', *Frontiers in Microbiology*, 18(7), p. 1785.

Rodin, R. J. (1985) 'Plants in the Kwanyama Culture', in *The Ethnobotany of the Kwanyama Ovambos*. Kansas: Allen Press Inc, pp. 23–34.

Rodríguez, H. and Fraga, R. (1999) 'Phosphate Solubilizing Bacteria and their Role in Plant Growth Promotion.', *Biotechnology advances*, 17(4–5), pp. 319–339.

Ronn, R., Vestergard, M. and Ekelund, F. (2012) 'Interactions Between Bacteria, Protozoa and Nematodes in Soil', *Acta Protozool*, 51(3), pp. 223–235.

Rudgers, J. A. *et al.* (2009) 'A Fungus Among Us: Broad Patterns of Endophyte Distribution in the Grasses', *Ecology*, 90(6), pp. 1531–1539.

Ryan, R. P. *et al.* (2008) 'Bacterial Endophytes: Recent Developments and Applications', *FEMS Microbiology Letters*, 278(1), pp. 1–9.

Sagar, A. *et al.* (2018) 'Comparative Analysis of Production of Hydrogen Cyanide with Production of Siderophore and Phosphate Solubilization activity in Plant Growth Promoting Bacteria', *Vegetos*, 31(2), pp. 130–135.

Saleem, M. *et al.* (2007) 'Perspective of Plant Growth Promoting Rhizobacteria (PGPR) containing ACC Deaminase in Stress Agriculture', *Journal of Industrial Microbiology and Biotechnology*, 34(10), pp. 635–648.

Santi, C., Bogusz, D. and Franche, C. (2013) 'Biological Nitrogen Fixation in Non-Legume Plants', *Annals of Botany*, 111(5), pp. 743–767.

Dos Santos, P. C. *et al.* (2012) 'Distribution of Nitrogen Fixation and Nitrogenase-Like Sequences Amongst Microbial Genomes', *BMC Genomics*, 13(1), p. 162.

Santoyo, G. *et al.* (2016) 'Plant Growth-Promoting Bacterial Endophytes', *Microbiological Research*, 183, pp. 92–99.

Schardl, C. L., Leuchtmann, A. and Spiering, M. J. (2004) 'Symbioses of Grasses with Seedborne Fungal Endophytes', *Annual Review of Plant Biology*, 55(1), pp. 315–340.

Shackleton, S. E. *et al.* (1998) 'Use and Trading of Wild Edible Herbs in the Central Lowveld Savanna Region, South Africa', *Economic Botany*, 52(3), pp. 251–259.

Shade, A., Jacques, M. and Barret, M. (2017) 'Ecological Patterns of Seed Microbiome Diversity, Transmission, and Assembly', *Current Opinion in Microbiology*, 37, pp. 15–22.

Shahzad, R. *et al.* (2018) 'What Is There in Seeds? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth', *Frontiers in Plant Science*, 9(24).

Shearin, Z. R. C. *et al.* (2017) 'Fungal Endophytes from Seeds of Invasive, Non-Native *Phragmites australis* and Their Potential Role in Germination and Seedling Growth', *Plant and Soil*, 422(2), pp. 183–194.

Shilla, O. *et al.* (2019) '*Cleome gynandra* L. Origin, Taxonomy and Morphology: A Review', *African Journal of Agricultural Research*, 14(32), pp. 1568–1583.

Shridhar, B. S. (2012) 'Review: Nitrogen Fixing Microorganisms', *International Journal of Microbiological Research*, 3(1), pp. 46–52.

Silué, D. (2009) *Spider Plant: An Indigenous Species with Many Uses*. Duluti, Arusha: Tanzania.

Singh, S. *et al.* (2014) 'Phosphate Solubilizing Rhizobia Promote the Growth of Chickpea under Buffering Conditions', *International Journal of Pure & Applied Bioscience*, 2(5), pp. 97–106.

Sivasakthi, S., Usharani, G. and Saranraj, P. (2014) 'Biocontrol Potentiality of Plant Growth Promoting Bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis*: A Review', *African Journal of Agricultural Research*, 9(16), pp. 1265–1277.

de Souza, R., Ambrosini, A. and Passaglia, L. M. P. (2015) 'Plant Growth-Promoting Bacteria as Inoculants in Agricultural Soils', *Genetics and Molecular Biology*, 38(4), pp. 401–419.

Spaepen, S., Vanderleyden, J. and Remans, R. (2007) 'Indole-3-Acetic Acid in Microbial and Microorganism-Plant Signaling', *FEMS Microbiology Reviews*, 31(4), pp. 425–448.

Starkey, P. *et al.* (2017) 'Promoting Rural Access and Mobility in Northern Namibia: An Integrated Approach', in *8th Africa Transportation Technology Transfer Conference*. Livingstone, pp. 1–15.

Stone, M. and Williams, D. (1992) 'On the Evolution of Functional Secondary Metabolites', *Molecular Microbiology*, 6(1), pp. 29–34.

Tian, F. *et al.* (2009) 'Genetic Diversity of Siderophore-Producing Bacteria of Tobacco Rhizosphere', *Brazilian Journal of Microbiology*, 40(61), pp. 276–284.

Tilak, K. V. *et al.* (2005) 'Diversity of Plant Growth and Soil Health Supporting Bacteria', *Current Science*, 89(1), pp. 136–150.

Timmusk, S. *et al.* (2017) 'Perspectives and Challenges of Microbial Application for Crop Improvement', *Frontiers in Plant Science*, 8(49), pp. 1–10.

Truyens, S. *et al.* (2014) 'Bacterial Seed Endophytes: Genera, Vertical Transmission and Interaction with Plants (Minireview)', *Environmental Microbiology Reports*, 7(1), pp. 40–50.

Tyc, O. *et al.* (2014) 'Impact of Interspecific Interactions on Antimicrobial Activity Among Soil Bacteria', *Frontiers in Microbiology*, 5(567), pp. 1–10.

Tyc, O. *et al.* (2020) 'The Ecological Role of Bacterial Seed Endophytes Associated with Wild Cabbage in the United Kingdom', *MicrobiologyOpen*, 9(1).

Vejan, P. *et al.* (2016) 'Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability-A Review', *Molecules*, 21(5), pp. 1–17.

Verma, S. K., Kharwar, R. N. and White, J. F. (2019) 'The Role of Seed-Vectored Endophytes in Seedling Development and Establishment', *Symbiosis*, 78(2), pp. 107–113.

Vessey, J. K. (2003) 'Plant Growth Promoting Rhizobacteria as Biofertilizers', *Plant and Soil*, 255(2), pp. 571–586.

Walia, A. *et al.* (2017) 'Endophytic Bacteria: Role in Phosphate Solubilization', in Maheshwari, D. K. and Annapurna, K. (eds) *Endophytes: Crop Productivity and Protection*. Springer International Publishing, pp. 61–93.

Walker, T. S. *et al.* (2003) 'Root Exudation and Rhizosphere Biology', *Plant Physiology*, 132(1), pp. 44–51.

Walpola, B. C. and Yoon, M. (2012) 'Prospectus of Phosphate Solubilizing Microorganisms and Phosphorus Availability in Agricultural Soils: A Review', *African Journal of Microbiology Research*, 6(37), pp. 6600–6605.

Walsh, U. F., Morrissey, J. P. and O'Gara, F. (2001) '*Pseudomona*s for Biocontrol of Phytopathogens from Functional Genomics to Commercial Exploitation', *Current Opinion in Biotechnology*, 12(3), pp. 289–95.

Wangolo, E. *et al.* (2015) 'Effects of Shoot Tip and Flower Removal on Growth and Yield of Spider Plant (*Cleome gynandra* L.) in Kenya', *American Journal of Experimental Agriculture*, 8(6), pp. 367–376.

Weller, D. M. *et al.* (2002) 'Microbial Populations Responsible for Specific Soil Suppressiveness to Plant Pathogens', *Annual Review of Phytopathology*, 40, pp. 309–348.

Whipps, J. M. (2001) ‘Microbial Interactions and Biocontrol in the Rhizosphere’, *Journal of Experimental Botany*, 52(1), pp. 487–511.

Wu, T. *et al.* (2017) ‘Morphological Patterns in a World Collection of *Cleome gynandra*’, *Genetic Resources and Crop Evolution*. Springer Netherlands, 65(1), pp. 271–283.

Yadegari, M. *et al.* (2010) ‘Plant Growth Promoting Rhizobacteria Increase Growth, Yield and Nitrogen Fixation in *Phaseolus Vulgaris*’, *Journal of Plant Nutrition*, 33(12), pp. 1733–1743.

Yousefi, A. A. *et al.* (2011) ‘Phosphate Solubilizing Bacteria and Arbuscular Mycorrhizal Fungi Impacts on Inorganic Phosphorus Fractions and Wheat Growth’, *World Applied Sciences Journal*, 15(9), pp. 1310–1318.

Zhao, Y. *et al.* (2001) ‘A Role for Flavin Monooxygenase-Like Enzymes in Auxin Biosynthesis’, *Science*, 291(5502), pp. 306–309.

CHAPTER 3

CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIAL SEED ENDOPHYTES OF *CLEOME GYNANDRA* L. FROM NORTHERN REGIONS OF NAMIBIA

Abstract

Endophytic bacteria have the ability to enhance plant health, growth and yield. In addition, they have the potential to act as bioinoculants. *Cleome gynandra* L. (*C. gynandra*) is a wild drought-tolerant and underutilized leafy vegetable found in the rural semi-arid northern regions of Namibia. It has shown exceptional potential in traditional medicine, agronomy, economy and climate adaptation. The objectives of this study were to morphologically characterize isolated bacterial strains and assess their plant growth promoting (PGP) capabilities. Twenty morphologically distinct isolates were obtained and assessed for plant growth promoting traits. Isolated endophytes exhibited capabilities to produce growth regulators as follows: nitrogen fixation (40%), indole acetic acid production (IAA) (100%), siderophore production (80%), ammonia production (85%), fluorescence production (10%), hydrogen cyanide production (30%) and phosphate solubilization (15%). Phylogenetic analysis of these isolates revealed that all the isolates belonged to the *Proteobacteria* phylum (excluding *Phytobacter ursingii*). It was concluded that *C. gynandra* seeds harbour endophytic bacteria that could improve plant growth in hostile environments, enhance crop productivity and be used for biotechnological applications in agriculture.

Keywords: agriculture, bacterial seed endophytes, *C. gynandra*, plant growth promotion, seed inoculants.

Introduction

Rapid population growth in the 20th century, coupled with environmental damage and climate change, has brought about challenges to the agricultural yield output of the traditionally grown field crops to feed the world population (Glick, 2012; Majeed, Muhammad and Ahmad, 2018). To address the expected food needs, conventional agricultural production was intensified to ensure food security (Timmusk *et al.*, 2017; Majeed, Muhammad and Ahmad, 2018). This was achieved through the Green Revolution that enabled the sharp increase of input of synthetic chemical fertilizers and pesticides, irrigation and the development of high yielding crop hybrids (Chauhan *et al.*, 2012; Abraham *et al.*, 2014; Gange and Gadhave, 2018). These fertilizers have become essential components of modern agriculture because they provide essential plant nutrients such as nitrogen, phosphorus and potassium (Chauhan *et al.*, 2012).

The excessive use of industrial chemicals fertilizers, herbicides and pesticides has led to environmental hazards such as leaching and nutrient-rich run-off that cause eutrophication of surface water bodies; greenhouse gas emissions; groundwater contamination, food poisoning and soil quality degradation (Pingali, 2012; Abraham *et al.*, 2014). Therefore, an alternative and more sustainable approach which can reduce the use of chemical application and improve agriculture to ensure food security must be put in place (Jha *et al.*, 2013; Chandini *et al.*, 2019).

A plant growing under field conditions harbours a well-structured and regulated complex microbial community that inhabit the rhizosphere, as well as the epiphytic and endophytic phyllosphere (Backer *et al.*, 2018; Numan *et al.*, 2018). Many of the bacteria found in and around plants have the ability to stimulate and facilitate plant growth and

consequently are called plant growth promoting bacteria (PGPB) (Akila *et al.*, 2011; Glick, 2012; Jha *et al.*, 2013; Gupta *et al.*, 2014). These bacteria stimulate the plant by invading internal plant tissue with their main point of entry being the rhizosphere (root zone) (Gaiero *et al.*, 2013; Ibanez *et al.*, 2017). Endophytes are microorganisms that dwell inside plants without causing any harm (Schulz and Boyle, 2006; Mercado-Blanco and Lugtenberg, 2014). A study conducted on maize seeds indicated that its endophytic bacterial community can be conserved during evolution and natural selection (Johnston-monje and Raizada, 2011). Seed endophytes are of particular interest for they are vertically transmitted from generation to generation (Rosenblueth and Martínez-romero, 2006; Truyens *et al.*, 2014). They are endemic to nature and are thought to influence the plant's physiology through regulating the plants nutrient and hormone levels (Hardoim *et al.*, 2012). In the rhizosphere, roots are invaded through root hair colonisation, infection threads between root hairs and disrupted epidermal cell layers (Compant, Clement and Sessitsch, 2010; Reinhold-Hurek and Hurek, 2011). Another form of colonisation that plant growth promoting bacteria use is the infection of seeds to ensure their existence in new plants (Ibanez *et al.*, 2017).

All plants studies to date have shown to symbiotically harbour endophytic microorganisms that help them in several ways such as acting as biofertilizers and biopesticides (Rosenblueth and Martínez-romero, 2006; Araujo *et al.*, 2008; Cocq *et al.*, 2017). These studies have centred on crops of economic importance leaving out indigenous plants such as *C. gynandra* (Chweya and Mnzava, 1997; Maheswari, Anbukkarasi and Hemalatha, 2014; Omondi *et al.*, 2017; López *et al.*, 2018). Plant growth promoting bacteria have been used as soil inoculants to improve the yields of agricultural crops (Khalid, Arshad and Zahir, 2004; Golding, 2012; de Souza, Ambrosini and

Passaglia, 2015; Chaturvedi, Singh and Gupta, 2016). Their efficiency transpires through a variety of mechanisms that develop during the early stages of seedling development as seed endophytes become a part of the host plant rhizosphere (Yang, Kloepper and Ryu, 2009; White *et al.*, 2017; Verma, Kharwar and White, 2019). This contributes to plant growth promoting traits such as nitrogen fixation, siderophore production, indole-3-acetic acid (IAA) production, phosphate solubilization, ammonia production, hydrogen cyanide production and fluorescence production (Adesemoye and Egamberdieva, 2013; Abbamondi *et al.*, 2016). These mechanisms either directly facilitate resource acquisition or indirectly inhibit pathogen activity on plant growth (Ahemad and Kibret, 2014; Gupta *et al.*, 2015; Verma and White, 2017). Bacterial species belonging to the genera *Pseudomonas Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus,* and *Serratia* have been reported to have plant growth promoting capabilities (Matiru and Dakora, 2004; Truyens *et al.*, 2014; Agbodjato *et al.*, 2015).

Cleome gynandra L., is a leafy vegetable that is indigenous to rural areas in Namibia on agricultural land and near human settlements (Guarino, 1997). (Guarino, 1997; van Rensburg *et al.*, 2007). It is probably a native of Africa and is now widely distributed in tropical and subtropical regions throughout the world (Chweya and Mnzava, 1997). Mostly collected from the wild, *C. gynandra* is known to be very nutritious and consumed in most African countries (Guarino, 1997; Silué, 2009; Kujeke, Edziwa and Icishahayo, 2017). It is an annual herb that is identified by its white flowers, long seed pods and compound leaves that are divided into folioles. *C. gynandra* is a plant with a C4 photosynthetic pathway that enables it to withstand high temperatures, intense sunlight and drought (Silué, 2009; Shahzad *et al.*, 2018). With its natural habitat consisting of

wastelands and many other soil types, make this plant indubitably suitable for various agricultural prospects (Chweya and Mnzava, 1997). This study aimed to isolate and assess *C. gynandra* seed endophytes diversity, characterize their plant growth promoting traits and test their agricultural potential as plant growth promoting (PGP) factors.

3.1 Materials and Methods

3.1.1 Study area and sample collection

A 100 whole healthy *C. gynandra* seed pods were collected. These seeds were used as the source material for endophytic bacteria isolation. All seeds used in this study were collected in May 2018 from the Omusati, Oshana, Ohangwena and Oshikoto regions in the Northern part of Namibia (**Figure 3.1**; **Figure 3.2**). Individual samples were placed in sterile plastic bags that contained silica gel to remove excess moisture, and stored at 4˚C until further processing in the laboratory of the Department of Biological Sciences of the University of Namibia. There was a total of five sampling points from each region (**Table 3.1**). The soil in these regions is mostly composed of cambisols, calcisols and arenosols (Mendelsohn, 2007). The soil texture is sandy, fine and soft with good water-retention capacity, however, it has moderate fertility as it is not rich in essential nutrients (Mendelsohn *et al.*, 2002). The rainy season is from October to April with these regions receiving an average rainfall from 250-300mm, 350-400mm and occasionally 500-550mm per annum (Mendelsohn *et al.*, 2002).

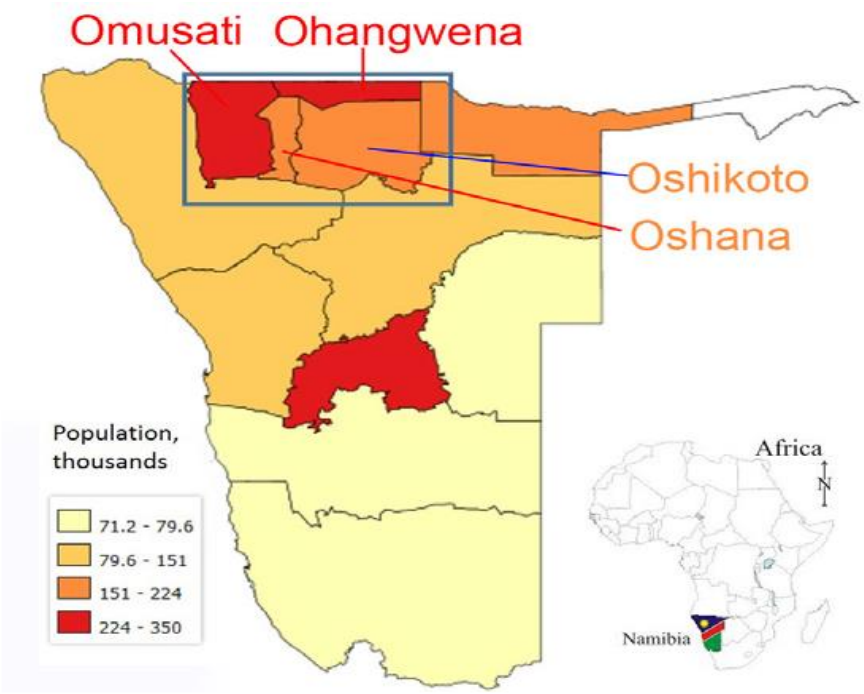


Figure 3.1: Map depicting the central northern regions of Namibia (Starkey *et al.*, 2017).

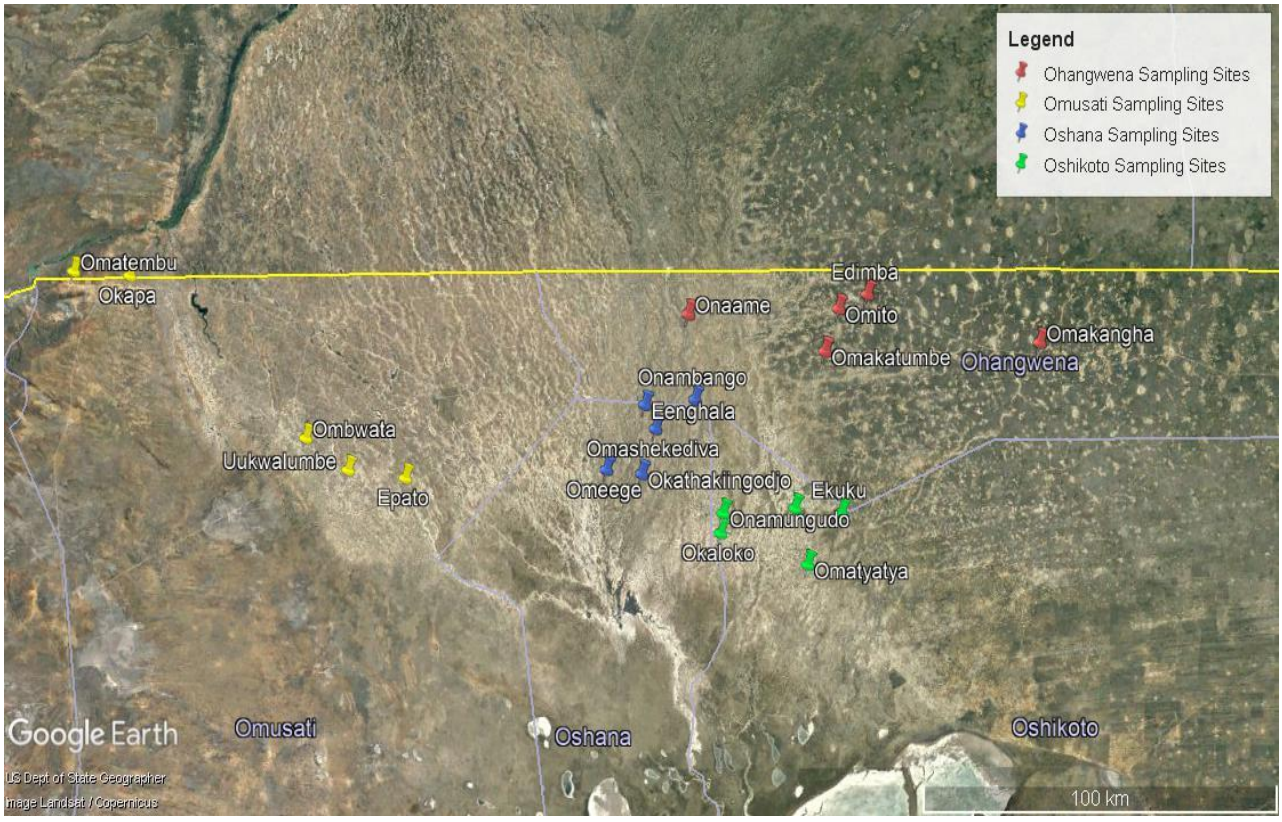


Figure 3.2: Map depicting the sampling sites. Google Earth Pro (Version 7.3.2.5776) [software]. Mountain View, CA: Google Inc. (2018).

Table 3.1: Sampling sites GPS coordinates

Site name	Latitude coordinate	Longitude coordinate
Omusati Region:		
Uukwalumbe	S17˚51.437’	E015˚02.209’
Ombwata	S17˚47.100’	E014˚55.525’
Epato	S17˚52.596’	E015˚11.287’
Okapa	S17˚24.929’	E014˚27.628’
Omatembu	S17˚24.182’	E014˚18.585’
Oshana Region:		
Omeege	S17˚51.723’	E015˚43.123’
Omashekediva	S17˚46.310’	E015˚50.886’
Eenghala	S17˚43.069’	E015˚49.236’
Okathakiingodjo	S17˚52.311’	E015˚48.655’
Onambango	S17˚42.401’	E015˚57.140’
Ohangwena Region:		
Edimba	S17˚28.434’	E016˚24.433’
Onaame	S17˚30.975’	E015˚56.196’
Omakangha	S17˚34.919’	E016˚51.698’
Omito	S17˚30.336’	E016˚19.894’
Omakatumbe	S17˚36.045’	E016˚17.706’
Oshikoto region:		
Omatyatya	S18˚04.557’	E016˚14.875’
Okaloko	S18˚00.275’	E016˚01.097’
Ompugulu	S17˚57.007’	E016˚12.890’
Onamungudo	S17˚57.613’	E016˚01.516’
Ekuku	S17˚57.670’	E016˚20.237’

3.1.2 Endophytic bacteria isolation

The collected plant material (seed pods) were first disinfected with 70% ethanol for 1 minute and then treated with 10% sodium hypochlorite solution for 10 minutes. The samples were then rinsed with distilled water three times to remove any surface microorganisms that might interfere with further testing. To verify successful surface sterilization, 300 µl of the final rinse water was plated onto trypticase soy agar (TSA) plates (15 g Agar, 15 g Casein Peptone, 5 g Sodium Chloride, 5 g Soya Peptone, 1000 ml

in distilled water) and served as a control. Plates were incubated at 28°C for 15 days with periodic examination for microbial growth (Coêlho *et al.*, 2011).

Endophytic bacteria were isolated from seed pods collected in the 4 Northern regions of Namibia. Entire seed pods were ground using a mortar and pestle. The plant extract was then mixed with 6ml of aqueous 0.9% NaCl solution and incubated at 28°C for three hours to allow endophytes to be released. The extract was then serially diluted up to the dilution factor 10^{-4} after which 0.1 ml aliquots were then plated on TSA. All plates, the control included, were incubated for 2 weeks at 28°C (Araujo *et al.*, 2002). Colonies were selected every 5 days based on morphological differences. Pure cultures were obtained by sub culturing single morphologically distinct colonies onto fresh TSA plates (Araujo *et al.*, 2002). Bacterial cultures for each isolate were prepared by growing them in nutrient broth at 28˚C for 24 hours. After incubation, 500 µl of each culture and 500 µl of prepared 50% glycerol were mixed in a 1.5 ml Eppendorf tube, to form a 1:1 volume ratio. The isolates were labelled accordingly and stored in a -80˚C freezer.

3.1.3 Genomic DNA extraction and 16S rRNA gene amplification

Based on cell and colony morphology, twenty different morphotypes were identified from C. gynandra seed endophytes. Total genomic DNA of overnight pure cultures was extracted using a ZR Fungal/Bacterial DNA MiniPrepTM Kit from Zymo Research (USA), in accordance with the manufacturer's instructions. The extracted DNA was stored at 4˚C until use. The DNA was subjected to PCR amplification using the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG3-') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), targeting the 1.5 kb region of the 16S rRNA genes (Hynes et al., 2008) using an ESCO Swift™ MaxPro Thermal Cycler (Esco Group,

Singapore). The final PCR reaction mixture contained 25 µl DreamTaq Green PCR Master Mix 2× (ThermoScientific: Thermofisher, USA), 16 µl Nuclease-free water, 2 µl of each primer (1µM concentration) and 5 µl isolated Genomic DNA as a template which amounted to a total volume of 50 µl (Coêlho et al., 2011). The PCR conditions were as follows: pre-denaturation at 95˚C for 4 min, 35 cycles of denaturation at 95ºC for 1 min, annealing at 50ºC for 30 sec, extension at 72ºC for 1 min and a final extension at 7 ºC for 10 min, with the holding temperature being 4˚C (Kang, Lee & Cho, 2013). Amplicons were quantified by running a 1% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized using a Clear View UV Transilluminator (Cleaver Scientific Ltd, United Kingdom). PCR product sequencing and purification were conducted at Inqaba Biotec (Pretoria, South Africa). The obtained sequences were edited using Chromas (Version 2.6.4). and aligned using BioEdit Sequence Alignment Editor (Version 7.0.5.3). The consensus sequences were subjected to the BLASTn search program for nucleotide sequence homology (Altschul et al., 1997). The obtained sequences were compared with the sequences in the National Center for Biotechnology Information (NCBI) database using the Standard Nucleotide BLAST. The search was made specific to the 16S ribosomal RNA sequences (Bacteria and Archaea) database for bacterial identification.

3.1.4 Phylogenetic analysis

A phylogenetic tree was constructed from the obtained consensus sequences. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis X (MEGA X) (Kumar *et al.*, 2018). The identified sequences were aligned using the Clustal X program (Larkin *et al.*, 2007). The evolutionary history was inferred using the Neighbour-Joining method (Satou and Nei, 1987). The optimal tree with the sum of

branch length (0.138924950) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. This analysis involved 57 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 464 positions in the final dataset.

3.1.5 Bioassays for plant growth promoting traits:

3.1.5.1 Siderophore production

The isolates were evaluated for the qualitative production of siderophores on blue agar chrome azurol s (CAS) plates (60.5 mg CAS ($C_{23}H_{13}Cl_2Na_3O_9S$) in 50 ml water mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl), 72.9 mg HDTMA (hexadecyltrimethylammonium bromide) in 40 ml water, 42.23g Kings B medium, 900 ml distilled water) (Schwyn and Neilands, 1987). Wells were formed onto each CAS agar plate. Three hundred microlitres of 24-hour old broth cultures of the isolates were placed into each well. CAS plates were then incubated at 28°C for 3-6 days. The development of yellow to orange halo was indicative of siderophore production (Chaitanya *et al.*, 2014). Distilled water was used as a negative control and *Klebsiella pneumonia* was used as a positive control (Louden, Haarmann and Lynne, 2011). All assays were carried out in triplicate.

3.1.5.2 Phosphate solubilization

The phosphate solubilization abilities of the isolated strains were tested on Pikovskaya medium (0.5 g Yeast extract, 0.5 g $Ca_3(PO_4)_2$, 0.1 g $MgSo_4$, 10 g $C_6H_{12}O_6$, 0.0001 g $Fe(II)SO_4$, 0.2 g KCl, 0.0001 g $MnSO_4$ & 20 g Agar in 1000 ml of distilled water), where growth is associated with the capacity to use inorganic phosphate in the form of $Ca_3(PO_4)_2$ as the sole source of insoluble phosphate (Pikovskaya, 1948). Isolates were spot inoculated onto the plates and incubated at 28˚C for 7 days. The appearance of a transparent zone around the bacterial colonies indicated the phosphate solubilization ability of the isolate. Distilled water was used as a negative control and *Klebsiella pneumonia* was used as a positive control (Minaxi *et al.*, 2012). Assays were carried out in triplicate.

3.1.5.3 Determination of indole production

Indole production was tested in bacterial suspension grown in nutrient broth in the dark supplemented with tryptophan (2 mg ml-1) as the precursor of IAA and compared to the ones grown without the addition of the tryptophan for day 5 and 7 respectively. Isolates were incubated for two weeks at 28˚C (Lwin *et al.*, 2012). IAA presence was checked in a culture supernatant (50 µl) mixed with 100 µl of Salkowski reagent (4.5 g of $FeCl_3$ per litre in 10.8 M H_2SO_4) in a 96 well plate, in triplicates, forming a 1:2 ratio (Gordon and Weber, 1950). The plate was subsequently incubated at room temperature, under dark condition for 30 – 40 minutes. The development of a pink colour indicated IAA production (Lwin *et al.*, 2012). Absorbency was measured using a SpectraMax® M2 Microplate Reader and SoftMax® Pro 6.4 Software (Molecular Devices Inc, USA) at 530 nm to determine the concentration of IAA which was then compared to a standard graph (Minaxi

et al., 2012). Absorbency was subjected to One Way ANOVA. Pure IAA was used to prepare the standard concentrations for the graph (Appendix 1), which was used to compare the concentrations measured from the spectroscopy. IAA solution (5%) was used as a positive control whereas nutrient broth was used as a negative control (Minaxi *et al.*, 2012).

3.1.5.4 Ammonia production

To screen for ammonia production, 1 ml of 24-hour old bacterial cultures were inoculated separately in peptone water (10 ml) and incubated at 36±˚2C for 48-72 hours. Nessler's reagent (0.5 ml) was added to the bacterial suspension. Ammonia production was indicated by the development of a brown to yellow colour (Cappuccino and Sherman, 1992). Distilled water was used as a negative control and *Klebsiella pneumonia* was used as a positive control (Agbodjato *et al.*, 2015).

3.1.5.5 Amplification of Nitrogenase *nif*H gene

To test for putative nitrogen fixing bacteria, the approximately 400 bp *nif*H gene was amplified by nested PCR using the primer sets FGPH19 (5'-TACGGCAARGGTGGNATHG-3') and PoIR (5'-ATSGCCATCATYTCRCCGGA-3') for the first PCR run and PoIF (5'-TGCGAYCCSAARGCBGACTC-3') and AQER (5'-GACGATGTAGATYTCCTG-3') primer sets for the second PCR run (Zhan and Sun, 2011). The reactions were carried out in an ESCO Swift™ MaxPro Thermal Cycler (Esco Group, Singapore). The reaction mixture for the first stage of nested PCR contained 12.5 µl DreamTaq Green Master Mix 2× (ThermoScientific: Thermofisher, USA), 10.5 µl Nuclease-free Water, 0.5 µl of each primer at a concentration of 0.5 µM and 1 µl of

Genomic DNA, totalling a volume of 25 µl (Dias *et al.*, 2012). The reaction conditions were as follows: pre-denaturation at 94°C for 4 min, 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. The reaction mixture for the second stage of the nested PCR contained 12.5 µl DreamTaq Green Master Mix 2× (ThermoScientific: Thermofisher, USA), 10.5 µl Nuclease-free Water, 0.5 µl of each primer at a concentration of 0.5 µM and 1 µl of template from the first nested PCR stage, totaling a volume of 25 µl (Dias *et al.*, 2012). The reaction conditions were as follows: pre-denaturation at 94°C for 4 min, 30 cycles of elongation at 94°C for 1 min, annealing at 56˚C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Products were held at 4°C. The positive control strain used was *Enterobacter* sp and the negative control was nuclease free water. The products were visualized using a 1.2% agarose gel and 100 bp ladder (Dias *et al.*, 2012).

3.1.6 Screening for Secondary metabolite production:

3.1.6.1 Hydrogen cyanide (HCN) production

Endophytic bacteria isolates were screened for HCN production on TSA plates supplemented with glycine (4.4g/L). Using 24-hour old broth culture of the isolates, a loop-full of each isolate was streaked onto the plates. Whatman number 1 filter paper was soaked in 0.5% picric acid solution and then place on the underside of each Petri dish lid. Parafilm was used to seal the plates, which were then incubated for 5-7 days at 30˚C (Freeman *et al.*, 1975). The cyanogenic potential was indicated by the change of colour in the filter paper from yellow to brown or reddish-brown (Allu, Kumar and Audipudi, 2014). Assays were carried out in triplicate.

3.1.6.2 Fluorescence production

Fluorescence production was determined using King's B agar (20 g Peptone, 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 20 g Agar, 1000 ml distilled water), (King, Ward and Raney, 1954). Endophytic bacteria were streaked on the plates. The plates were incubated for 48 hours at 28±2˚C. After the incubation period, the plates were observed for fluorescence production under UV light (Allu, Kumar and Audipudi, 2014). Assays were carried out in triplicate.

3.2 Results and Discussion

Bacterial isolates were selected based on morphological features such as colour (red, yellow and white), colony size (small), and texture (smooth). A total of 20 endophytic bacterial isolates, that have plant growth promoting capabilities, were isolated from seeds of *Cleome gynandra* L. located in the four central northern regions of Namibia. It should however be noted the number of isolates is not a true reflection of the diverse microbial community as some microbes could be unculturable while others required specific enrichment media to grow (Kandjimi, Uzabakiriho and Chimwamurombe, 2015). Isolates were categorized by their traits. They elicited traits as follows (**Table 3.2**); 16 siderophore producers, 3 phosphate solubilizers, 20 IAA producers (**Appendix 2**), 17 ammonia producers, 6 hydrogen producers, 2 fluorescence producers and 8 nitrogen fixers.

3.2.1 *C. gynandra* bacterial seed endophyte isolation and identification

Seed endophytes have been isolated from various sources previously (Verma and White, 2017; Tyc *et al.*, 2020), however, no reports are available on the seed endophytes isolated

from *Cleome gynandra*. Through 16S rRNA sequence identification and the BLASTn search tool, this study's *C. gynandra* seed endophytic bacteria were identified and classified into five genera, namely *Klebsiella*, *Enterobacter*, *Phytobacter*, *Kluyvera*, *Kosakonia* (**Table 3.3**) Additional classification indicated that all isolates belonged to the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacterales* and family *Enterobacteriaceae*. A total of 80 isolates were initially isolated from *C. gynandra* seeds and only 20 isolates (five from each region) were selected based on their distinctive morphological traits and were further screened *in vitro* for a wide range of PGP traits. Tested PGP traits included nitrogen fixation, mineral phosphate solubilization, IAA production, siderophore production, ammonia production, hydrogen cyanide production and fluorescence production. The isolate that occurred most frequently in all the regions was *Enterobacter cloacae*. The isolates that were unique to each region were *Klebsiella aerogenes* (OSC-2) for Oshana region, *Enterobacter mori* (OHC-3) and *Phytobacter ursingii* (OHC-4) for Ohangwena region, *Kluyvera cryocrescens* (OKC-9) for Oshikoto region and *Kosakonia sacchari* (OMC-6) for Omusati region.

3.2.2 Phylogenetic analysis of 16S rRNA isolates

The most similar reference sequences with the highest homology, query coverage and the lowest E values were selected and aligned by the Clustal X program. An outgroup was not used because outgroups that do not have polymorphic sites matching up to the sequences increase the likelihood of conflict in the tree, which leads to incorrect root assignment (Wheeler, 1990; Huelsenbeck, Bollback and Levine, 2002) Phylogenetic analyses revealed that the majority (90%) of the endophytic isolates shared high sequence similarities (greater than 98%) with their identified relative species (**Table 3.3**). The

constructed tree (**Figure 3.4)** contains the closest related strains for each isolate. Isolates are shown to belong to the *Klebsiella*, *Enterobacter*, *Phytobacter*, *Kluyvera* and *Kosakonia* genera, which all fall under the Enterobacteriaceae family. A study conducted on soil bacterial communities in Mexico produced similar results as endophytic bacteria belonging to the *Proteobacteria* phyla were found to be one of the dominant phyla at 38% (Lüneberg *et al.*, 2018). However, studies conducted in arid environments have shown the presence of *Bacillus* (Marasco *et al.*, 2013; Soussi *et al.*, 2016). Its absence in this study could be due to soil properties not being conducive for its growth resulting in it being less abundant. Although the isolates displayed high identity similarities, a significant number of them had low bootstrap values. It can be concluded that the low values could be caused by the rate of evolution and mutation of their genes, unrelated sequences being aligned together or sequences being too similar.

3.2.3 Plant Growth Promoting Traits and Screening for Secondary Metabolites

The endophytic isolates were subjected to six tests to determine their plant growth promoting capabilities as presented in **Table 3.2**. Siderophore producing bacteria have evolved to develop specialized mechanisms to solubilize iron from organic substances, promote plant growth directly and indirectly by supplying plants with this solubilized iron and protect the host plant against pathogens (Arora, Tewari and Singh, 2013; Gupta *et al.*, 2015; Ferreira *et al.*, 2019). Findings from this study have shown that the production of iron-chelating was the most common plant growth promoting trait observed among 80% of the isolates, namely *K. aerogenes, K. grimontii, K. pneumoniae, E. cloacae, E. mori* and *K. sacchari*. Similar results were reported for bacterial genera *Pseudomonas, Enterobacter, Rhizobium* and *Bacillus* in the tobacco rhizosphere, where 85% of the

isolates demonstrated siderophore producing abilities (Tian *et al.*, 2009; Sujatha and Ammani, 2013). Siderophores reduce the population of deleterious microorganisms which creates less competition for iron in the rhizosphere (Sharma *et al.*, 2003), making them more prevalent and giving them higher potential in the rhizosphere (Leong, 1986; Neilands and Leong, 1986). Plants from the Omusati region had the highest proportion (5/20) of siderophore producers whereas those from the Oshana region had the lowest at three. Plant samples from the Ohangwena and Oshikoto regions had four siderophore producers respectively.

Phosphate solubilizing plant growth promoting bacteria have garnered the attention of agricultural microbiologists as soil inoculants used to improve plant growth and yield because they enhance the availability of phosphorus to the plants (Bhattacharyya and Jha, 2012). From the four regions only three isolates (15% of all isolates), namely *Enterobacter cloacae* (OSC-1) from Oshana region, *Klebsiella pneumonia* (OKC-7) from Oshikoto region and *Enterobacter cloacae* (OMC-1) from Omusati region, formed solubilization halos on Pikovskaya medium. Isolates from Ohangwena region were unable to solubilize phosphate. Such a low percentage of isolates that show phosphate solubilization ability is not unique to our study as other studies also show limited numbers of 37% of microbes being able to solubilize phosphate (Sagar *et al.*, 2018). The ability of plant growth promoting rhizobacteria strains to solubilize insoluble phosphorus (P) and convert it to plant-available form is an important characteristic under conditions where P is a limiting factor for crop production (Pradhan and Sukla, 2005; Khan, Zaidi and Wani, 2007; Ramesh *et al.*, 2014). Studies have demonstrated that phosphate solubilizing endophytes have the potential to plant growth under drought conditions (Mapelli *et al.*, 2013; Soussi *et al.*, 2016). This may be considered a positive indicator of utilizing the

microbes as biofertilizers for crop production and beneficial for sustainable agriculture (Walpola and Yoon, 2012).

IAA is the most studied auxin and there is strong evidence that IAA producing endophytes can increase and improve plant yield (Glick, 2012). Most microorganisms (80%) can detect IAA produced by plants (Patten and Glick, 1996) which can account for it being discovered in high percentages. In an attempt to better select endophytic isolates with high plant growth promotion potential traits, IAA production was tested. The results revealed that all 20 isolates had IAA producing capabilities. The 20 isolates produced IAA at varying concentrations, ranging from 0.1523 – 0.2921 µg IAA/ml^{-1} (**Appendix 2**). Among endophytic strains, *Enterobacter cloacae* (OSC-5) and *Phytobacter ursingii* (OHC-4) outstood for producing significantly higher amounts of IAA compared to the other strains, with the highest amount (0.2921 µg IAA/ml^{-1}) coming from the Oshana region. The lowest amount was produced from *Enterobacter cloacae* (OKC-8) at 0.1523 µg IAA/ml^{-1}, isolated from the Oshikoto region (**Figure 3.3**). A study conducted on peppers grown under drought stress showed that 100% of endophytes were able to produce IAA (Marasco *et al.*, 2012). Other studies showed that 89% of strains produced IAA, with the highest producing strains *Agrobacterium* and *Rhizobium* producing IAA above 7.8 µg IAA/ml^{-1} (Khalid, Arshad and Zahir, 2004; El-deeb *et al.*, 2012; Abbamondi *et al.*, 2016), which is significantly higher than the amounts obtained in this study. Statistical analysis (Appendix 3) revealed a p-value of 0.000. Because the p-value is below the significant level of 0.05, it has been determined that there is a significant difference between the means. This could be a result of the rate at which each microbe can produce IAA.

Ammonia producing endophytes provide a nutrient source for the host plant. Ammonia production is a trait that yielded the second number of positive producers with 17 out of

20 (85%) isolates testing positive. These isolates include *E. cloacae, K. pneumoniae, P. ursingii, K. cryoscrescens, K. grimontii* and *K. sacchari*. *Bacillus* strains isolated from maize produced similar rates of ammonia with 80% of them being producers (Agbodjato *et al.*, 2015). The highest number came from Oshikoto and Omusati region with five ammonia producers each while the lowest came from the Oshana region with only three producers.

The amplified nitrogenase *nif*H gene fragments were successfully amplified from the genomic DNA of 8 out of the 20 isolates with the expected length of ± 320 pairs. Four bacterial species were identified to contain the *nif*H gene using nested PCR and they were *Klebsiella aerogenes* (OSC-2), *Enterobacter cloacae* (OSC-5, OSC-3, OSC-1, OSC-7, OHC-2), *Klebsiella pneumoniae* (OHC-1) and *Kluyvera cryocrescens* (OKC-9). These bacteria are metabolically versatile and are found in a diverse range of environments (Pérez-Montaño *et al.*, 2014). They have been suggested as sugarcane endophytic plant promoters and nitrogen-fixing bacteria (Zhu *et al.*, 2013; Chen *et al.*, 2014). In previous studies and in the absence of chemical nitrogen fertilizers, *Klebsiella* showed nitrogen-fixing capabilities when it was inoculated into wheat in greenhouse and field experiments (Iniguez, Dong and Triplett, 2004). The use of nitrogen-fixing bacteria as an inoculant increased the average yield of sweet cherries by 21.7% (Esitken *et al.*, 2006) and increased dry weight and nitrogen content of roots, shoots and seedlings in sugarcane at a statistically significant 95% confidence level (Lin *et al.*, 2012).

Hydrogen cyanide (HCN) is a volatile secondary metabolite produced by deleterious rhizobacteria where it is used as a biocontrol against weeds and other plant pathogens (Kamei, Dolai and Kamei, 2014). HCN producing rhizobacteria have recently shown contributions to geochemical processes such as chelation of iron, which in turn increase

nutrient availability and thus indirectly improved plant growth (Rijavec and Lapanje, 2016; Sagar *et al.*, 2018). The Ohangwena, Oshikoto and Omusati regions yielded two hydrogen cyanide producers respectively, with Oshana region producing none. This is a low yield considering that a vast number of organisms can naturally produce hydrogen cyanide (Kamei, Dolai and Kamei, 2014). HCN producing endophytes in this study made up 30% of the isolates, and they belong to the *Enterobacter* and *Klebsiella* genera. A similar percentage (40%) was recorded for bacteria (*Bacillus, Pseudomonas, Enterobacter, Acinetobacter* and *Micrococcus*) isolated from beans (Kumar *et al.*, 2012). Fluorescence production brought in the lowest yield of results with only two isolates from the Ohangwena region, namely *Klebsiella pneumonia* (OHC-1) and *Phytobacter ursingii* (OHC-4), yielding positive results.

Fluorescence producing endophytes and their potential as inoculants have been realized as they were able to yield increased growth in chickpea, potato, sugar beet and radishes (Sivasakthi, Usharani and Saranraj, 2014). A similar report on a red chilli plant indicated that all tested bacteria (belonging to *Pseudomonas* genera) were able to produce fluorescence (Allu, Kumar and Audipudi, 2014).

The present study observed all isolates being able to elicit more than one plant growth promoting trait. *Enterobacter cloacae* (OMC-1) was able to display five plant growth promoting characteristics that correspond with reports that have shown *Enterobacter* spp. possess multiple plant growth promoting activities (Tsuda *et al.*, 2001; Hynes *et al.*, 2008; Khalifa *et al.*, 2016). Several strains of *Enterobacter cloacae* have been considered to be plant growth promoting rhizobacteria (Liu *et al.*, 2013). *E. cloacae* has been widely distributed in nature primarily being isolated from the rhizosphere and has shown nitrogen fixation abilities, with one specific strain displaying biocontrol potential against pathogens

found in corn and cucumbers (Hinton and Bacon, 1995). A study in India showed that *E. cloacae* has been recovered from a soybean rhizosphere and enhanced the soybean growth through the mobilization of phosphate (Ramesh *et al.*, 2014). The *Enterobacter cloacae* strain in this study displayed siderophore production, phosphate solubilization, ammonia production, hydrogen cyanide production and indole-3-acetic acid production. Arid ecosystems have diverse microbial communities that dwell within them (Soussi *et al.*, 2016). Endophytic bacteria found in this ecosystems are suggested to support and improve plant health and growth as well as make them resistant to abiotic stresses such as drought (Marasco *et al.*, 2012; Soussi *et al.*, 2016). Further clarification on factors that lead plant growth promoting bacteria to regulate drought resistance will allow for these bacteria to have better potential in biotechnology and agriculture (Marasco *et al.*, 2013; Soussi *et al.*, 2016).

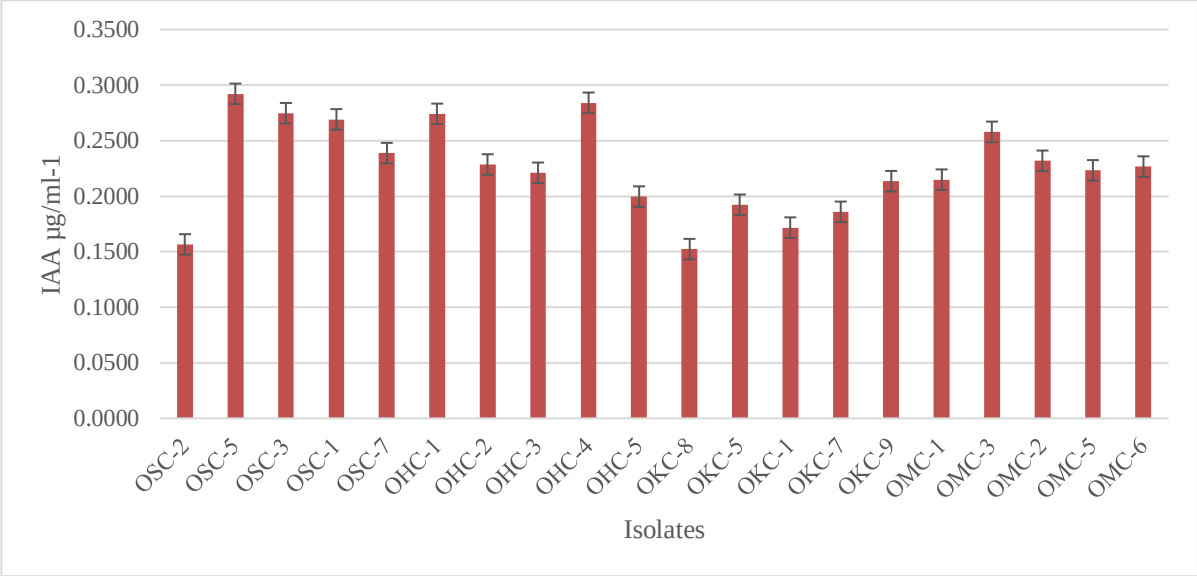


Figure 3.3: Average IAA production (µg/ml^{-1}) by isolated endophytes. Vertical bars indicate mean ± SE.

Table 3.2: Screening for PGP and secondary metabolite traits

Isolate	*nif*H	Siderophore Production	Phosphate Solubilization	NH3 Production	HCN Production	Fluorescence Production	IAA Production
Klebsiella aerogenes (OSC-2)	+	+	-	-	-	-	+
Enterobacter cloacae (OSC-5)	+	-	-	+	-	-	+
Enterobacter cloacae (OSC-3)	+	+	-	+	-	-	+
Enterobacter cloacae (OSC-1)	+	-	+	-	-	-	+
Enterobacter cloacae (OSC-7)	+	+	-	+	-	-	+
Klebsiella pneumoniae (OHC-1)	+	+	-	+	-	+	+
Enterobacter cloacae (OHC-2)	+	+	-	-	+	-	+
Enterobacter mori (OHC-3)	-	+	-	+	+	-	+
Phytobacter ursingii (OHC-4)	-	-	-	+	-	+	+
Klebsiella pneumoniae (OHC-5)	-	+	-	+	-	-	+
Enterobacter cloacae (OKC-8)	-	+	-	+	-	-	+
Enterobacter cloacae (OKC-5)	-	+	-	+	-	-	+
Enterobacter cloacae (OKC-1)	-	-	-	+	+	-	+
Klebsiella pneumoniae (OKC-7)	-	+	+	+	+	-	+
Kluyvera cryocrescens (OKC-9)	+	+	-	+	-	-	+
Enterobacter cloacae (OMC-1)	-	+	+	+	+	-	+
Enterobacter cloacae (OMC-3)	-	+	-	+	-	-	+
Klebsiella grimontii (OMC-2)	-	+	-	+	+	-	+
Enterobacter cloacae (OMC-5)	-	+	-	+	-	-	+
Kosakonia sacchari (OMC-6)	-	+	-	+	-	-	+

Table 3.3: Source of bacterial isolates and BLAST results of 16S rRNA

Isolate Designation	Most Related Species	Percentage Identity	Accession Number	Site Name	Region
OSC-2	*Klebsiella aerogenes* KCTC 2190	98%	NR102493.2	Omashekediva	Oshana
OSC-5	*Enterobacter cloacae* subsp. dissolvens strain LMG 2683	100%	NR044978.1	Eenghala	Oshana
OSC-3	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Eenghala	Oshana
OSC-1	*Enterobacter cloacae* subsp. dissolvens strain ATCC 23373	99%	NR118011.1	Okathakiingodjo	Oshana
OSC-7	*Enterobacter cloacae* subsp. dissolvens strain LMG 2683	100%	NR044978.1	Onambango	Oshana
OHC-1	*Klebsiella pneumoniae* strain DSM 30104	98%	NR117686.1	Edimba	Ohangwena
OHC-2	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Edimba	Ohangwena
OHC-3	*Enterobacter mori* LMG 25706 strain R18-2	100%	NR116430.1	Omakangha	Ohangwena
OHC-4	*Phytobacter ursingii* strain ATCC 27989	100%	NR159305.1	Omito	Ohangwena
OHC-5	*Klebsiella pneumoniae* strain DSM 30104	97%	NR117686.1	Omakatumbe	Ohangwena
OKC-8	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Omatyatya	Oshikoto
OKC-5	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Ompugulu	Oshikoto
OKC-1	*Enterobacter cloacae* subsp. dissolvens strain ATCC 23373	99%	NR118011.1	Onamungudo	Oshikoto
OKC-7	*Klebsiella pneumoniae* strain DSM 30104	97%	NR117686.1	Ekuku	Oshikoto
OKC-9	*Kluyvera cryocrescens* strain NBRC 102467	99%	NR114108.1	Ekuku	Oshikoto
OMC-1	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Uukwalumbe	Omusati
OMC-3	*Enterobacter cloacae* strain DSM 30054	99%	NR117679.1	Epato	Omusati
OMC-2	*Enterobacter cloacae* strain ATCC 13047	99%	NR102794.2	Okapa	Omusati
OMC-5	*Enterobacter cloacae* subsp. dissolvens strain ATCC 23373	99%	NR118011.1	Omatembu	Omusati
OMC-6	*Kosakonia sacchari* strain SP1	98%	NR118333.1	Omatembu	Omusati

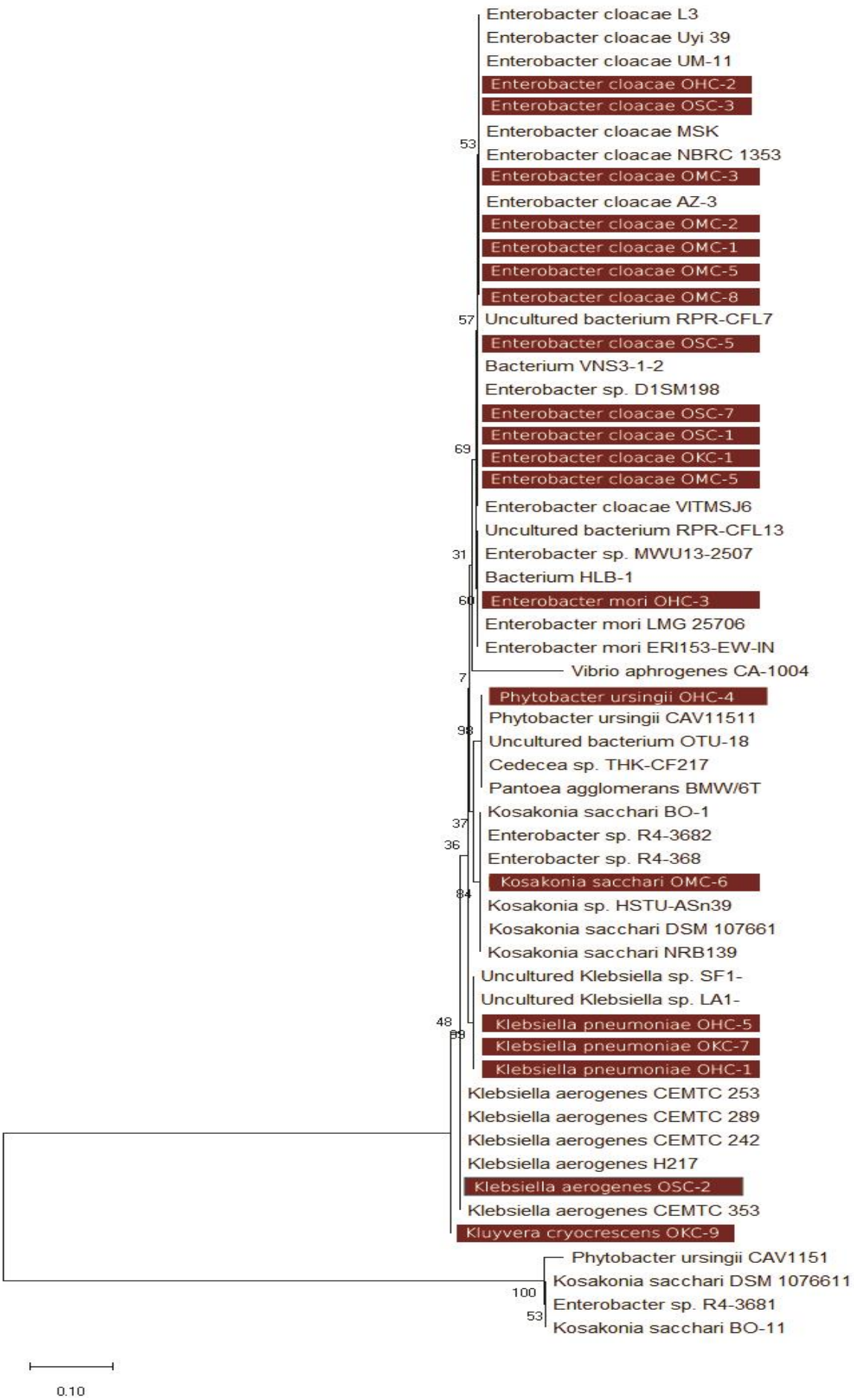

Enterobacter cloacae L3
Enterobacter cloacae Uyi 39
Enterobacter cloacae UM-11
Enterobacter cloacae OHC-2
Enterobacter cloacae OSC-3
53
Enterobacter cloacae MSK
Enterobacter cloacae NBRC 1353
Enterobacter cloacae OMC-3
Enterobacter cloacae AZ-3
Enterobacter cloacae OMC-2
Enterobacter cloacae OMC-1
Enterobacter cloacae OMC-5
Enterobacter cloacae OMC-8
57
Uncultured bacterium RPR-CFL7
Enterobacter cloacae OSC-5
Bacterium VNS3-1-2
Enterobacter sp. D1SM198
Enterobacter cloacae OSC-7
Enterobacter cloacae OSC-1
69
Enterobacter cloacae OKC-1
Enterobacter cloacae OMC-5
Enterobacter cloacae VITMSJ6
Uncultured bacterium RPR-CFL13
31
Enterobacter sp. MWU13-2507
Bacterium HLB-1
60
Enterobacter mori OHC-3
Enterobacter mori LMG 25706
Enterobacter mori ERI153-EW-IN
Vibrio aphrogenes CA-1004
7
Phytobacter ursingii OHC-4
Phytobacter ursingii CAV11511
98
Uncultured bacterium OTU-18
Cedecea sp. THK-CF217
Pantoea agglomerans BMW/6T
Kosakonia sacchari BO-1
37
Enterobacter sp. R4-3682
36
Enterobacter sp. R4-368
Kosakonia sacchari OMC-6
84
Kosakonia sp. HSTU-ASn39
Kosakonia sacchari DSM 107661
Kosakonia sacchari NRB139
Uncultured Klebsiella sp. SF1-
Uncultured Klebsiella sp. LA1-
48
Klebsiella pneumoniae OHC-5
Klebsiella pneumoniae OKC-7
Klebsiella pneumoniae OHC-1
Klebsiella aerogenes CEMTC 253
Klebsiella aerogenes CEMTC 289
Klebsiella aerogenes CEMTC 242
Klebsiella aerogenes H217
Klebsiella aerogenes OSC-2
Klebsiella aerogenes CEMTC 353
Kluyvera cryocrescens OKC-9
Phytobacter ursingii CAV1151
Kosakonia sacchari DSM 1076611
100
Enterobacter sp. R4-3681
53
Kosakonia sacchari BO-11
0.10

Figure 3.4: A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of endophytic seed bacteria (isolates are labelled according to their designation from Table 3.4 and are highlighted in red). Bootstrap confidence values were obtained with 1000 replicates and bootstrap values of more than 50% are shown at branch points. The scale bar represents the observed number of substitutions per nucleotide site (0.10).

Namibia is an arid country that is characterized by extreme ecological environments to which plants have to adapt (Mendelsohn *et al.*, 2002). Organisms such as plants and bacteria that thrive in such extreme conditions adopt mechanisms and survival strategies to alleviate abiotic stresses (Soussi *et al.*, 2016). These conditions will therefore select endophytes that would help them curb or mitigate these types of stresses from generation to generation (Marasco *et al.*, 2012, 2013). Plant growth promoting bacteria are important components of sustainable agriculture as they are cost-effective, safe and can enhance plant growth and yield (Glick, 2012; Blaszczyk *et al.*, 2014). Direct and indirect benefits of plant growth promotion have been reported to be successful due to the presence of endophytic seed bacteria (Magnani *et al.*, 2010; Mercado-Blanco and Lugtenberg, 2014). Research findings in this study are in agreement with other research conducted on isolated strains and their plant growth promoting potential. Strains that can display multiple traits are more desirable for agricultural use as bioinoculants, biocontrol agents and biofertilizers. The correct selection of isolates being used as inoculants is crucial. Strains should be tested for effectiveness through plant growth promotion activity. To ensure the selected endophytes are safe to use and will not invade the environment or other plants, tests can be done in early product development to exclude pathogenic microorganisms (Berg, 2009). *Enterobacter cloacae* (OMC-1), *Klebsiella pneumonia* (OHC-1) and *Kluyvera cryocrescens* (OKC-9) were the three isolates that displayed the most diverse and highest amount of plant growth promoting traits. Evidence of plant growth promoting

activity indicates that these isolated strains can successfully improve overall plant health and improve crop yield (Ryan *et al.*, 2008). Cost-effective production systems using inexpensive materials (Visnovsky *et al.*, 2008) and food industry by-products (Costa *et al.*, 2001), make microbial inoculants an economically feasible option to chemical fertilizers (O'Callaghan, 2016). Endophytic seed bacteria isolated in this study need to be further studied for prospective agricultural applications.

3.3 Conclusion

This study revealed that *Cleome gynandra* L. seeds harbour endophytes. Twenty bacterial isolates were isolated from *C. gynandra* seeds obtained from the Oshana, Omusati, Ohangwena and Oshikoto regions respectively. The isolated endophytes (100%) displayed plant growth promoting traits including siderophore production, phosphate solubilization, indole-3-acetic acid production, ammonia production, hydrogen cyanide production, fluorescence production and nitrogen fixation. All isolates belong to the *Proteobacteria* phylum. The lack of genetic diversity could be attributed to abiotic stresses that prevented other microbes from thriving and being more abundant. It can be concluded that findings from this study have highlighted that endophytic seed bacteria have potential application in sustainable agriculture methods. The understanding of the plant growth potential of the endophytes in this study will provide the information required for future commercialization. Further research and development on all plant growth promoting seed endophytes and their interactions are required.

REFERENCES

Abbamondi, G. R. *et al.* (2016) 'Plant Growth-Promoting Effects of Rhizospheric and Endophytic Bacteria Associated with Different Tomato Cultivars and New Tomato Hybrids', *Chemical and Biological Technologies in Agriculture*. Springer International Publishing, 3(1).

Abraham, B. *et al.* (2014) 'The System of Crop Intensification : Reports from the Field on Improving Agricultural Production, Food Security, and Resilience to Climate Change for Multiple Crops', *Agriculture & Food Security*, 3(4), pp. 1–12.

Adesemoye, A. O. and Egamberdieva, D. (2013) 'Beneficial Effects of Plant Growth-Promoting Rhizobacteria on Improved Crop Production: Prospects for Developing Economies', in D.K Maheshwari et al (ed.) *Bacteria in Agrobiology: Crop Productivity*. Berlin, Heidelberg: Springer-Verlag, pp. 45–63.

Agbodjato, N. A. . *et al.* (2015) 'Characterization of Potential Plant Growth Promoting Rhizobacteria Isolated from Maize (*Zea mays* L.) in Central and Northern Benin (West Africa)', *Applied and Environmental Soil Science*, 2015.

Ahemad, M. and Kibret, M. (2014) 'Mechanisms and Applications of Plant Growth Promoting Rhizobacteria: Current Perspective', *Journal of King Saud University - Science*. King Saud University, 26(1), pp. 1–20.

Akila, R. *et al.* (2011) 'Combined Application of Botanical Formulations and Biocontrol Agents for the Management of *Fusarium oxysporum* f .sp. *cubense* (Foc) causing *Fusarium* Wilt in Banana', *Biological Control*. Elsevier Inc., 57(3), pp. 175–183.

Allu, S., Kumar, N. P. and Audipudi, A. V (2014) 'Isolation, Biochemical and PGP

Characterization of Endophytic *Pseudomonas aeruginosa* Isolated from Chilli Red Fruit Antagonistic Against Chilli Anthracnose Disease', *International Journal of Microbial Application Science*, 3(2), pp. 318–329.

Altschul, S. F. *et al.* (1997) 'Gapped BLAST and PSI-BLAST : A New Generation of Protein Database Search Programs', *Nucleic Acids Research*, 25(17), pp. 3389–3402.

Araujo, W. L. *et al.* (2002) 'Diversity of Endophytic Bacterial Populations and Their Interaction with *Xylella fastidiosa* in Citrus Plants', *Applied and Environmental Microbiology*, 68(10), pp. 4906–4914.

Araujo, W. L. *et al.* (2008) 'Interaction Between Endophytes and Plant Host: Biotechnological Aspects', in Barka, E. A. and Clement, C. (eds) *Plant-Microbe Interactions*. Research Signpost, pp. 95–115.

Arora, Naveen Kumar, Tewari, S. and Singh, R. (2013) 'Multifaceted Plant-Associated Microbes and Their Mechanisms Diminish the Concept of Direct and Indirect PGPRs', in Arora, N.K. (ed.) *Plant Microbe Symbiosis: Fundamentals and Advances*. India: Springer, pp. 411–446.

Backer, R. *et al.* (2018) 'Plant Growth-Promoting Rhizobacteria : Context, Mechanisms of Action, and Roadmap to Commercialization of Biostimulants for Sustainable Agriculture', *Frontiers in Plant Science*, 9(1473), pp. 1–17.

Bhattacharyya, P. N. and Jha, D. K. (2012) 'Plant Growth-Promoting Rhizobacteria (PGPR): Emergence in Agriculture', *World Journal of Microbiology and Biotechnology*, 28(4), pp. 1327–1350.

Blaszczyk, L. *et al.* (2014) '*Trichoderma* spp. - Application and Prospects For Use in Organic

Farming and Industry', *Journal of Plant Protection Research*, 54(4), pp. 309–317.

Cappuccino, J. C. and Sherman, N. (1992) *Microbiology: A Laboratory Manual*. New York: Benjamin/Cummings.

Chaitanya, K. *et al.* (2014) 'Polymer Producing Bacteria Showing Siderophore Activity with Chrome Azurol S (CAS) Agar Plate Assay', *International Journal of Scientific and Research Publications*, 4(12).

Chandini *et al.* (2019) 'The Impact of Chemical Fertilizers on our Environment and Ecosystem', in Sharma, P. (ed.) *Research Trends in Environmental Sciences*. 2nd edn. New Dehli: AkiNik Publications, pp. 69–86.

Chaturvedi, H., Singh, V. and Gupta, G. (2016) 'Potential of Bacterial Endophytes as Plant Growth Promoting Factors', *Journal of Plant Pathology & Microbiology*, 7(9), p. 376.

Chauhan, P. S. *et al.* (2012) 'Environmental Impacts of Organic Fertilizers Usage in Agriculture', in Singh, Rajeev Pratap (ed.) *Organic Fertilizers: Types, Production and Environmental Impact*. New York: Nova Science Publishers, Inc., pp. 62–84.

Chen, M. *et al.* (2014) 'Complete Genome Sequence of *Kosakonia sacchari* Type Strain SP1 T', *Standards in Genomic Sciences*, 9(3), pp. 1311–1318.

Chweya, J. A. and Mnzava, N. A. (1997) *Cat's Whiskers: Cleome gynandra L., Biodiversity International*. Rome: Italy: International Plant Genetic Resources Institute.

Cocq, K. L. E. *et al.* (2017) 'Exploitation of Endophytes for Sustainable Agricultural Intensification (Microreview)', *Molecular Plant Pathology*, 18(3), pp. 469–473.

Coêlho, M. M. *et al.* (2011) 'Isolation of Endophytic Bacteria from *Arboreal* Species of the

Amazon and Identification by Sequencing of the 16S rRNA Encoding Gene', *Genetics and Molecular Biology*, 34(4), pp. 676–680.

Compant, S., Clement, C. and Sessitsch, A. (2010) 'Plant Growth-Promoting Bacteria in the Rhizo- and Endosphere of Plants: Their Role, Colonization, Mechanisms Involved and Prospects for Utilization', *Soil Biology & Biochemistry*, 42(5), pp. 669–678.

Costa, E. *et al.* (2001) 'Production of the Biocontrol Agent *Pantoea agglomerans* Strain CPA-2 Using Commercial Products and By-Products.', *Applied Microbiology and Biotechnology*, 56, pp. 367–371.

Dias, A. C. F. *et al.* (2012) 'Abundance and Genetic Diversity of nifH Gene Sequences in Anthropogenically Affected Brazilian Mangrove Sediments', *Applied and Environmental Microbiology*, 78(22), pp. 7960–7967.

El-deeb, B. *et al.* (2012) 'Characterization of Endophytic Bacteria Associated with Rose Plant (*Rosa damascena trigintipeta*) During Flowering Stage and Their Plant Growth Promoting Traits', *Journal of Plant Interactions*, 7(3), pp. 248–253.

Esitken, A. *et al.* (2006) 'Effects of Floral and Foliar Application of Plant Growth Promoting Rhizobacteria (PGPR) on Yield, Growth and Nutrition of Sweet Cherry', *Scientia Horticulturae*, 110(4), pp. 324–327.

Felsenstein, J. (1985) 'Confidence Limits on Phylogenies: An Approach Using the Bootstrap', *Evolution*, 39(4), pp. 783–791.

Ferreira, C. M. H. *et al.* (2019) 'Comparison of Five Bacterial Strains Producing Siderophores with Ability to Chelate Iron under Alkaline Conditions', *AMB Express*. Springer Berlin Heidelberg, 9(78).

Freeman, L. R. *et al.* (1975) 'Production of Hydrogen Cyanide by *Pseudomonas fluorescens*', *Applied Microbiology*, 29(4), pp. 560–561.

Gaiero, J. *et al.* (2013) 'Inside The Root: Bacterial Root Endophytes and Plant Growth Promotion', *American Journal of Botany*, 100(9), pp. 1738–1750.

Gange, A. C. and Gadhave, K. R. (2018) 'Plant Growth-Promoting Rhizobacteria Promote Plant Size Inequality', *Scientific Reports*. Springer US, 8(13828), pp. 1–10.

Glick, B. R. (2012) 'Plant Growth-Promoting Bacteria: Mechanisms and Applications', *Scientifica*, 2012(5).

Golding, A.-L. (2012) 'Plant Growth Promoting H_2-Oxidizing Bacteria as Seed Inoculants for Cereal Crops', *Agricultural Sciences*, 3(4), pp. 510–516.

Gordon, S. A. and Weber, R. P. (1950) 'Colorimetric Estimation of Indoleacetic Acid', in *Laboratory Manual*. Chicago: Argonne National Laboratory, pp. 192–195.

Guarino, L. (1997) 'Local Leafy Vegetables', in *Traditional African Vegetables*. Rome (Italy): International Plant Genetic Resources Institute, pp. 75–81.

Gupta, A. *et al.* (2014) 'Whole Genome Sequencing and Analysis of Plant Growth Promoting Bacteria Isolated from the Rhizosphere of Plantation Crops Coconut, Cocoa and Arecanut', *PLoS ONE*, 9(8).

Gupta, G. *et al.* (2015) 'Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture', *Journal of Microbial & Biochemical Technology*, 7(2), pp. 96–102.

Hardoim, P. R. *et al.* (2012) 'Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth

Stages', *PLoS ONE,* 7(2).

Hinton, D. M. and Bacon, C. W. (1995) '*Enterobacter cloacae* is an Endophytic Symbiont of Corn', *Mycopathologia*, 129(2), pp. 117–125.

Huelsenbeck, J. P., Bollback, J. P. and Levine, A. M. (2002) 'Inferring the Root of a Phylogenetic Tree', *Systemic Biology*, 51(1), pp. 32–43.

Hynes, R. K. *et al.* (2008) 'Isolation, Selection, and Characterization of Beneficial Rhizobacteria from Pea, Lentil, and Chickpea Grown in Western Canada.', *Canadian Journal of Microbiology*, 54(4), pp. 248–258.

Ibanez, F. *et al.* (2017) 'Bacterial Endophytes of Plants : Diversity, Invasion Mechanisms and Effects on the Host', in Maheshwari, D. K. (ed.) *Endophytes: Biology and Biotechnology*. Springer International Publishing, pp. 25–40.

Iniguez, A. L., Dong, Y. and Triplett, E. W. (2004) 'Nitrogen Fixation in Wheat Provided by *Klebsiella pneumoniae* 342', *Molecular Plant-Microbe Interactions*, 17(10), pp. 1078–1085.

Jha, Prabhat *et al.* (2013) 'Association of Rhizospheric / Endophytic Bacteria with Plants : A Potential Gateway to Sustainable Agriculture', *Greener Journal of Agricultural Sciences*, 3(2), pp. 73–84.

Johnston-monje, D. and Raizada, M. N. (2011) 'Conservation and Diversity of Seed Associated Endophytes in Zea across Boundaries of Evolution, Ethnography and Ecology', *PLoS ONE*, 6(6).

Kamei, Adam, Dolai, A. K. and Kamei, Apou (2014) 'Role of Hydrogen Cyanide Secondary

Metabolite of Plant Growth Promoting Rhizobacteria as Biopesticides of Weeds', *Global Journal of Science Frontier Research (D)*, 14(6), pp. 109–112.

Kandjimi, O. S., Uzabakiriho, J.-D. and Chimwamurombe, P. M. (2015) 'Isolation and Characterization of Culturable Bacteria from Bulk Soil Samples and the Rhizosphere of Arid-Adapted *Tylosema esculentum* (Burchell). A. Schreiber (Marama bean) in Namibia', *African Journal of Biotechnology*, 14(11), pp. 944–952.

Kang, Y. M., Lee, C. K. and Cho, K. M. (2013) 'Diversity and Antimicrobial Activity of Isolated Endophytic Bacteria from Deodeok (*Codonopsis lanceolata*) of Different Locations and Ages', *African Journal of Microbiology Research*, 7(12), pp. 1015–1028.

Khalid, A., Arshad, M. and Zahir, Z. A. (2004) 'Screening Plant Growth-Promoting Rhizobacteria for Improving Growth and Yield of Wheat', *Journal of Applied Microbiology*, 96(3), pp. 473–480.

Khalifa, A. Y. Z. *et al.* (2016) 'Characterization of the Plant Growth Promoting Bacterium, *Enterobacter cloacae* MSR1, Isolated from Roots of Non-Nodulating *Medicago sativa*', *Saudi Journal of Biological Sciences*. King Saud University, 23(1), pp. 79–86.

Khan, M. S., Zaidi, A. and Wani, P. A. (2007) 'Role of Phosphate-Solubilizing Microorganisms in Sustainable Agriculture - A Review', *Agronomy for Sustainable Development*, 27(1), pp. 29–43.

Kimura, M. (1980) 'A Simple Method for Estimating Evolutionary Rate of Base Substitutions Through Comparative Studies of Nucleotide Sequences', *Journal of Molecular Evolution*, 16(2), pp. 111–120.

King, E. O., Ward, M. K. and Raney, D. E. (1954) 'Two Simple Media for the Demonstration of

Pyocyanin and *Fluorescin*', *Journal of Laboratory and Clinical Medicine*, 44(2), pp. 301–307.

Kujeke, G. T., Edziwa, X. and Icishahayo, D. (2017) 'Field Performance of Spider Plant (*Cleome gynandra* L.) Under Different Agronomic Practices', *African Journal of Food, Agriculture, Nutrition and Development*, 17(3), pp. 12179–12197.

Kumar, A. *et al.* (2012) 'Isolation, Screening and Characterization of Bacteria from Rhizospheric Soils for Different Plant Growth Promotion (PGP) Activities: An In Vitro Study', *Recent Research in Science and Technology*, 4(1), pp. 1–5.

Kumar, S. *et al.* (2018) 'MEGA X: Molecular Evolutionary Genetics Analysis Across Computing Platforms', *Molecular Biology and Evolution*, 35(6), pp. 1547–1549.

Larkin, M. A. *et al.* (2007) 'Clustal W and Clustal X version 2.0', *Bioinformatics*, 23(21), pp. 2947–2948.

Leong, J. (1986) 'Siderophores: Their Biochemistry and Possible Role in the Biocontrol of Plant Pathogens', *Annual Review of Phytopathology*, 24, pp. 187–209.

Lin, L. *et al.* (2012) 'Plant Growth-Promoting Nitrogen-Fixing Enterobacteria Are in Association with Sugarcane Plants Growing in Guangxi, China', *Microbes and Environments*, 27(4), pp. 391–398.

Liu, W. *et al.* (2013) 'Comparative Genome Analysis of *Enterobacter cloacae*', *PLoS ONE*, 8(9).

López, S. *et al.* (2018) 'Microbial Endophytes That Live Within Seeds of Two Tomato Hybrid Cultivated in Argentina', *Agronomy*, 8(8), p. 136.

Louden, B. C., Haarmann, D. and Lynne, A. (2011) 'Use of Blue Agar CAS Assay for Siderophore Detection', *Journal of Microbiology & Biology Education*, 12(1), pp. 51–53.

Lüneberg, K. *et al.* (2018) 'Drylands Soil Bacterial Community is Affected by Land Use Change and Different Irrigation Practices in the Mezquital Valley, Mexico', *Scientific Reports*, 8(1), p. 1413.

Lwin, K. M. *et al.* (2012) 'Isolation of Plant Hormone (Indole-3-Acetic Acid - IAA) Producing Rhizobacteria and Study on their Effects on Maize Seedling', *Engineering Journal*, 16(5), pp. 137–144.

Magnani, G. . *et al.* (2010) 'Diversity of Endophytic Bacteria in Brazilian Sugarcane', *Genetics and Molecular Research*, 9(1), pp. 250–258.

Maheswari, T. U., Anbukkarasi, K. and Hemalatha, T. (2014) 'Bacterial Endophytes as Biofertilizer for Pulse Crops', *Popular Kheti*, 2(3), pp. 89–93.

Majeed, A., Muhammad, Z. and Ahmad, H. (2018) 'Plant Growth Promoting Bacteria: Role in Soil Improvement, Abiotic and Biotic Stress Management of Crops', *Plant Cell Reports*. Springer Berlin Heidelberg, 37(12), pp. 1599–1609.

Mapelli, F. *et al.* (2013) 'Potential for Plant Growth Promotion of Rhizobacteria Associated with S*alicornia* growing in Tunisian hypersaline soils', *BioMed Research International*, 2013(6).

Marasco, R. *et al.* (2012) 'A Drought Resistance-Promoting Microbiome Is Selected by Root System under Desert Farming', *PLoS ONE*, 7(10).

Marasco, R. *et al.* (2013) 'Are Drought-Resistance Promoting Bacteria Cross-Compatible with Different Plant Models?', *Plant Signaling & Behavior*, 8(10).

Matiru, V. N. and Dakora, F. D. (2004) 'Minireview. Potential Use of Rhizobial Bacteria as Promoters of Plant Growth for Increased Yield in Landraces of African Cereal Crops', *African Journal of Biotechnology*, 3(1), pp. 1–7.

Mendelsohn, J. *et al.* (2002) *Atlas of Namibia*. Cape Town: David Philip Publishers.

Mendelsohn, J. (2007) *A Digest of Information on Key Aspects of Oshikoto, Ohangwena, Omusati and Oshana ' s Geography*. Windhoek.

Mercado-Blanco, J. and Lugtenberg, B. J. J. (2014) 'Biotechnological Applications of Bacterial Endophytes', *Current Biotechnology*, 3(1), pp. 60–75.

Minaxi *et al.* (2012) 'Characterization of Multifaceted *Bacillus* sp. RM-2 for its Use as Plant Growth Promoting Bioinoculant for Crops Grown in Semi Arid Deserts', *Applied Soil Ecology*. Elsevier B.V., 59, pp. 124–135.

Neilands, J. B. and Leong, S. A. (1986) 'Siderophores in Relation to Plant Growth and Disease', *Annual Review of Plant Physiology*, 37, pp. 187–208.

Numan, M. *et al.* (2018) 'Plant Growth Promoting Bacteria As An Alternative Strategy for Salt Tolerance in Plants : A Review', *Microbiological Research*. Elsevier, 209(2018), pp. 21–32.

O'Callaghan, M. (2016) 'Microbial Inoculation of Seed for Improved Crop Performance: Issues and Opportunities', *Applied Microbiology and Biotechnology*, 100(13), pp. 5729–5746.

Omondi, E. O. *et al.* (2017) 'Nutritional Compound Analysis and Morphological

Characterization of Spider Plant (*Cleome gynandra*) - an African Indigenous Leafy Vegetable', *Food Research International*. Elsevier, 100(1), pp. 284–295.

Patten, C. L. and Glick, B. R. (1996) 'Bacterial Biosynthesis on Indole-3-Acetic Acid', *Canadian Journal of Microbiology*, 42(3), pp. 201–220.

Pikovskaya, R. I. (1948) 'Mobilization of Phosphorus in Soil Connection with the Vital Activity of Some Microbial Species', *Microbiology*, 17, pp. 362–370.

Pingali, P. L. (2012) 'Green Revolution: Impacts, Limits, and the Path Ahead', *Pnas*, 109(31), pp. 12302–12308.

Pradhan, N. and Sukla, L. B. (2005) 'Solubilization of Inorganic Phosphate by Fungi Isolated from Agricultural Soil', *African Journal of Biotechnology*, 5(10), pp. 850–854.

Ramesh, A. *et al.* (2014) 'Phosphorus Mobilization from Native Soil P-Pool upon Inoculation with Phytate-Mineralizing and Phosphate- Solubilizing *Bacillus aryabhattai* Isolates for Improved P-Acquisition and Growth of Soybean and Wheat Crops in Microcosm Conditions', *Agricultural Research*, 3(2), pp. 118–127.

Reinhold-Hurek, B. and Hurek, T. (2011) 'Living Inside Plants: Bacterial Endophytes', *Current Opinion in Plant Biology*. Elsevier Ltd, 14(4), pp. 435–443.

van Rensburg, W. S. J. *et al.* (2007) 'African Leafy Vegetables in South Africa', *Water SA*, 33(3), pp. 317–326.

Rijavec, T. and Lapanje, A. (2016) 'Hydrogen Cyanide in the Rhizosphere : Not Suppressing Plant Pathogens, but Rather Regulating Availability of Phosphate', *Frontiers in Microbiology*, 7, p. 1785.

Rosenblueth, M. and Martínez-romero, E. (2006) ‘Bacterial Endophytes and Their Interactions with Hosts’, *Molecular Plant-Microbe Interactions*, 19(8), pp. 827–837.

Ryan, R. P. *et al.* (2008) ‘Bacterial Endophytes : Recent Developments and Applications’, *FEMS Microbiology Letters*, 278(1), pp. 1–9.

Sagar, A. *et al.* (2018) ‘Comparative Analysis of Production of Hydrogen Cyanide with Production of Siderophore and Phosphate Solubilization activity in Plant Growth Promoting Bacteria’, *Vegetos*, 31(2), pp. 130–135.

Satou, N. and Nei, M. (1987) ‘The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees’, *Molecular Biology and Evolution*, 4(4), pp. 406–425.

Schulz, B. and Boyle, C. (2006) *Microbial Root Endophytes*. 5th edn. Edited by T. Sieber. Berlin, Germany: Springer-Verlag GmbH.

Schwyn, B. and Neilands, J. . (1987) ‘Universal CAS assay for the Detection and Determination of Siderophores’, *Analytical Biochemistry*, 160(1), pp. 47–56.

Shahzad, R. *et al.* (2018) ‘What Is There in Seeds ? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth’, *Frontiers in Plant Science*, 9(24).

Sharma, A. *et al.* (2003) ‘Plant Growth-Promoting Bacterium *Pseudomonas* sp . Strain GRP 3 Influences Iron Acquisition in Mung Bean (*Vigna radiat*a L. Wilzeck)’, *Soil Biology & Biochemistry*, 35(7), pp. 887–894.

Silué, D. (2009) *Spider Plant : An Indigenous Species with Many Uses*. Duluti, Arusha:

Tanzania.

Sivasakthi, S., Usharani, G. and Saranraj, P. (2014) 'Biocontrol Potentiality of Plant Growth Promoting Bacteria (PGPR) - *Pseudomonas fluorescens* and *Bacillus subtilis* : A Review', *African Journal of Agricultural Research*, 9(16), pp. 1265–1277.

Soussi, A. *et al.* (2016) 'Plant-Associated Microbiomes in Arid Lands : Diversity, Ecology and Biotechnological Potential', *Plant and Soil*, 405(1), pp. 357–370.

de Souza, R., Ambrosini, A. and Passaglia, L. M. P. (2015) 'Plant Growth-Promoting Bacteria as Inoculants in Agricultural Soils', *Genetics and Molecular Biology*, 38(4), pp. 401–419.

Starkey, P. *et al.* (2017) 'Promoting Rural Access and Mobility in Northern Namibia : An Integrated Approach', in *8th Africa Transportation Technology Transfer Conference*. Livingstone, pp. 1–15.

Sujatha, N. and Ammani, K. (2013) 'Siderophore Production by the Isolates of *Fluorescent Pseudomonads*', *International Journal of Current Research and Review*, 5(20), pp. 1–7.

Tian, F. *et al.* (2009) 'Genetic Diversity Of Siderophore-Producing Bacteria Of Tobacco Rhizosphere', *Brazilian Journal of Microbiology*, 40(61), pp. 276–284.

Timmusk, S. *et al.* (2017) 'Perspectives and Challenges of Microbial Application for Crop Improvement', *Frontiers in Plant Science*, 8(49), pp. 1–10.

Truyens, S. *et al.* (2014) 'Bacterial Seed Endophytes : Genera, Vertical Transmission and Interaction with Plants (Minireview)', *Environmental Microbiology Reports*, 7(1), pp. 40–50.

Tsuda, K. *et al.* (2001) 'Evaluation of the Endophyte *Enterobacter cloacae* SM10 Isolated from Spinach Roots for Biological Control against *Fusarium* Wilt of Spinach', *Journal of General Plant Pathology*, 67(1), pp. 78–84.

Tyc, O. *et al.* (2020) 'The Ecological Role of Bacterial Seed Endophytes Associated with Wild Cabbage in the United Kingdom', *MicrobiologyOpen*, 9(1).

Verma, S. K., Kharwar, R. N. and White, J. F. (2019) 'The Role of Seed-Vectored Endophytes in Seedling Development and Establishment', *Symbiosis*, 78(2), pp. 107–113.

Verma, S. K. and White, J. F. (2017) 'Indigenous Endophytic Seed Bacteria Promote Seedling Development and Defend Against Fungal Disease in Browntop Millet (*Urochloa ramosa* L.)', *Journal of Applied Microbiology*, 124(3), pp. 764–778.

Visnovsky, G. A. *et al.* (2008) 'Influence of Culture Medium Composition, Dissolved Oxygen Concentration and Harvesting Time on the Production of *Serratia entomophila*, a Microbial Control Agent of the New Zealand Grass Grub.', *Biocontrol Science and Technology*, 18, pp. 87–100.

Walpola, B. C. and Yoon, M. (2012) 'Prospectus of Phosphate Solubilizing Microorganisms and Phosphorus Availability in Agricultural Soils : A Review', *African Journal of Microbiology Research*, 6(37), pp. 6600–6605.

Wheeler, W. C. (1990) 'Nucleic Acid Sequence Phylogeny and Random Outgroups', *Cladistics*, 6(4), pp. 363–367.

White, J. F. *et al.* (2017) 'Disease Protection and Allelopathic Interactions of Seed-Transmitted Endophytic *Pseudomonads* of Invasive Reed Grass (*Phragmites australis*)', *Plant and Soil*, 422, pp. 195–208.

Yang, J., Kloepper, J. W. and Ryu, C.-M. (2009) 'Rhizosphere Bacteria Help Plants Tolerate Abiotic Stress', *Trends in Plant Science*, 14(1), pp. 1–4.

Zhan, J. and Sun, Q. (2011) 'Diversity of Free-Living Nitrogen-Fixing Microorganisms in Wastelands of Copper Mine Tailings during the Process of Natural Ecological Restoration', *Journal of Environmental Sciences*, 23(3), pp. 476–487.

Zhu, B. *et al.* (2013) '*Enterobacter sacchari* sp. nov., A Nitrogen-Fixing Bacterium Associated with Sugar Cane (*Saccharum officinarum* L.)', *International Journal of Systematic and Evolutionary Microbiology*, 63(7), pp. 2577–2582.

CHAPTER 4

CLEOME GYNANDRA L. SEED ENDOPHYTES PROMOTE RAPE *(BRASSICA NAPUS* L.) SEEDLING GERMINATION RATE, PLANT GROWTH AND LEAF YIELD

Abstract

Endophytic seed bacteria have been considered for agricultural applications as they are known to elicit plant growth promoting traits such as nitrogen fixation, siderophore production, phosphate solubilization, fluorescence production, hydrogen cyanide and indole-3-acetic acid (IAA) production. They improve plant health, growth and potentially increase the plant yield. Endophytic bacteria have recently garnered attention as potential bioinoculants. Few studies have tested them in this application and developed them for commercial use. The objective of this study was to evaluate the effects of microorganism-based bio-fertilizers in single, double and triple strain combinations isolated from *Cleome gynandra* L. on the domesticated crop *Brassica napus* L. (Rape English Giant). Endophyte strains used in this study were, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Kluyvera cryocrescens*. Plant growth parameters observed were wet mass, dry mass, chlorophyll readings, root length, plant height and leaf yield. There was a statistically significant difference for plant height ($p=0.037$), root length ($p=0.024$), and wet ($p=0.000$) and dry ($p=0.000$) mass between the treatments. The post hoc LSD test determined that the single strain (*K. pneumoniae*) and triple strain combination (*E. cloacae*, *K. pneumoniae* and *K. cryocrescens*) performed the best against the other treatments, controls and in all growth parameters. This study concluded that *C. gynandra* seeds contain endophytic bacteria with the potential to improve plant growth for the production of rape seed and could be used as inoculants to establish a sustainable crop production system.

Keywords: commercialization, endophytic seed bacteria, plant growth promotion, seed inoculant, yield enhancing

Introduction

Endophytes are organisms that occur and interact within intracellular or intercellular plant tissue (Schulz and Boyle, 2006; Reinhold-Hurek and Hurek, 2011; Cocq *et al.*, 2017). Those plant tissues include roots, stems, leaves, fruits and seeds (Rosenblueth and Martínez-romero, 2006; Hardoim *et al.*, 2012; White *et al.*, 2017). Endophytes have been investigated for their potential plant growth promoting properties and their fitness to biotic and abiotic stress (Araujo *et al.*, 2008; Mercado-Blanco and Lugtenberg, 2014). There is an increasing body of evidence that suggests that seed endophytes are of interest as they have shown plant growth, enhanced plant tolerance to biotic and abiotic stresses and enhanced plant defence mechanisms (Reinhold-Hurek and Hurek, 2011; Truyens *et al.*, 2014; Shahzad *et al.*, 2018).

Plant growth promoting endophytes have been utilized to stimulate plant growth through the production of auxins, secondary metabolites and through direct and indirect mechanisms of action (Bhattacharyya and Jha, 2012; Vejan *et al.*, 2016). Mechanisms of action in this study include (i) nitrogen fixation and ammonia production to increase nitrogen availability for plant uptake (Gabriela *et al.*, 2015); (ii) production of volatile secondary metabolites in particular hydrogen cyanide, to improve overall plant growth (Ryu *et al.*, 2003); (iii) production of auxin indole-3-acetic acid to regulate plant growth (de Souza, Ambrosini and Passaglia, 2015); (iv) production of siderophores to make iron available in the soil for plant uptake (Flores-Felix, Silva and Rivera, 2015); (v) production of fluorescence to inhibit plant pathogens (Arora, Khare and Oh, 2008); (vi) phosphate solubilization to increase the availability of phosphate for plant uptake (Wani, Khan and Zaida, 2007). Plant growth promoting bacteria have shown potentiality to confer resistance to water stress in tomatoes (Mayak, Tirosh and Glick, 2004). A study

conducted on peppers showed that endophytes have plant growth promoting traits that perform in synergy, which allowed the plant to be drought-resistant under water stress (Marasco *et al.*, 2013).

Farming practices require the use of costly and harmful chemical fertilizers to sustain the high yield needed to keep up with the demand for agricultural produce (Sansanwal *et al.*, 2017; Trivedi *et al.*, 2018). Researchers have shown an interest in organic agricultural approaches in farming (Orhan *et al.*, 2006; Glick, 2012; de Souza, Ambrosini and Passaglia, 2015). Endophytes have been used successfully as biofertilizers, to improve plant growth and sustain plant and soil health (Orhan *et al.*, 2006; Kalayu, 2019). The best method for endophytes being applied to agricultural systems has not been established, however, reports have shown that using endophytes as soil or seed inoculants has been successful (Cocq *et al.*, 2017; Murphy, Doohan and Hodkinson, 2018). Endophytic inoculants have been noted to increase nitrogen uptake, nodulation, growth and yield of crops (Bhattacharyya and Jha, 2012). In comparison to well-studied rhizosphere, seed endophytes are poorly understood (Cankar *et al.*, 2005).

Cleome gynandra L., also commonly known as the Cat's Whisker, is an indigenous, wild and underexploited leafy green vegetable (Guarino, 1997; Wasonga *et al.*, 2015; Kujeke, Edziwa and Icishahayo, 2017). It grows on a wide range of soils but thrives on well-manured, fertile soil (van Rensburg *et al.*, 2007; Shilla *et al.*, 2019). Its status of being a weed causes the plant to be neglected and prevents the crop from being improved for breeding (higher leaf yield, longer vegetation growth period) (Chweya and Mnzava, 1997; Chataika *et al.*, 2020). *C. gynandra* has been reported to be highly nutritious and possessing insecticidal properties (Chweya and Mnzava, 1997; Abukutsa-Onyango, 2007). Despite its limitations such as producing a low yield (Chweya and Mnzava, 1997),

Cleome's seeds have shown the potential to harbour plant growth promoting endophytes. This makes *C. gynandra* seed endophytes suitable candidates to serve as seed inoculants. Rape English Giant (*Brassica napus* L.) is an annual leafy vegetable crop that is considered a profitable agricultural enterprise worldwide (Loehr, Seif and Nyambo, 1998; Kuntashula *et al.*, 2004). Its leaves are rich in ascorbic acid, thiamine and vitamin A (Ganya, Svotwa and Katsaruware, 2018). It grows in a wide variety of soils, has a short growing season and has a high water demand due to its large leaf surface area (Walton *et al.*, 1999; Ganya, Svotwa and Katsaruware, 2018).

The *Brassica* genus belongs to the *Brassicaceae* family (Rieger, Preston and Powles, 1999). This family is a near relative to the *Capparaceae* family to which *C. gynandra* belongs (Chweya and Mnzava, 1997). Because they are such close relatives, Rape English Giant was deemed a suitable candidate for the greenhouse trials in the present study. This study aimed to test the effectiveness of using plant growth promoting bacteria (PGPB) isolated from *Cleome gynandra* L. seeds as a seed inoculant with the potential to enhance in vivo growth of the domesticated crop *Brassica napus* L.

4.1 Materials and Methods

4.1.1 Research Site Location

The experiment was carried out from May 2019 to July 2019 at the University of Namibia's Greenhouse Facility (22.6122˚S, 17.0584˚E). The altitude of the location is 1655 m above sea level with average greenhouse temperatures ranging from 15 to 32 ˚C. No rainfall was recorded during the experiment.

4.1.2 Bacterial Isolation and Screening for Plant Growth Promoting Traits

Protocol for isolation and screening of plant growth promoting traits has been outlined in chapter 3.

4.1.3 Bacterial Inoculum and Treatment Preparation:

4.1.3.1 Bacterial Strains and Inoculant Preparation

Three bacterial strains isolated from *Cleome gynandra* L. seeds exhibiting several plant growth-promoting traits (**Table 4.1**) were used in this study. The bacterial cultures were subjected to the McFarland's method for turbidity. Prepared McFarland solution ($BaCl_2$ (0.048M, 0.5 ml) was added to 99.5 ml of 0.18M H_2SO_4) was read in a spectrophotometer at the wavelength of 625nm. Three selected bacterial strains were inoculated in sterile nutrient broth and incubated at 28˚C for 24 hours. The turbidity of the bacterial cultures were read in a spectrophotometer at 625 nm and adjusted to the 0.5 McFarland scale (Lwin *et al.*, 2012; Eduardo *et al.*, 2018).

Table 4.1: Bacterial isolates and the plant growth promoting traits they display.

Isolate	PGP Trait
Enterobacter cloacae	Siderophore production, Phosphate solubilization, NH_3 production, HCN production, IAA production
Klebsiella pneumoniae	*nif*H, Siderophore production, NH_3 production, Fluorescence production, IAA production
Kluyvera cryocrescens	*nif*H, Siderophore production, NH_3 production, IAA production

NH_3 = Ammonia **HCN** = Hydrogen cyanide **IAA** = Indole-3-acetic acid ***nif*H** = Nitrogen fixation

4.1.3.2 Treatments

Isolates were selected based on several plant growth promoting traits (**Table 4.1**). Three bacterial strains, namely, *Enterobacter cloacae* (OMC-1), *Klebsiella pneumoniae* (OHC-1) and *Kluyvera cryocrescens* (OKC-9), were chosen as treatments for the pot experiment based on their performance amongst multiple (more than three) plant growth promoting

properties. Strains were used in a singular and consortia form (**Table 4.2**). Treatments were arranged in a randomized complete block design with nine blocks and three replicates.

Table 4.2: Treatments used for experiment.

Treatment Label	Treatment
T_1	A (*Enterobacter cloacae*)
T_2	B (*Klebsiella pneumoniae*)
T_3	C (*Kluyvera cryocrescens*)
T_4	AB
T_5	AC
T_6	BC
T_7	ABC
T_8	Water
T_9	Fertilizer (Nuleaf Organic Fertilizer)

4.1.3.3 Effect of Endophytic Bacterial Inoculation on Rape Seed Germination and Mean Germination Time

Sterile Whatman no. 1 filter paper was placed in sterile petri dishes and labelled according to each treatment. Endophytes were grown in 10 ml of nutrient broth for 24 hours at 28˚C. Each bacterial treatment (0.5 ml) was added to 1.5 ml of distilled water to moisten the filter paper. Starke Ayres® Nutrifeed (0.5 ml) in combination with 1.5 ml distilled water was used as a positive control (T_9) and 2 ml of distilled water was used as a negative control (T_8). Using sterile forceps, seeds (five), were gently placed and spread into petri dishes. The time for this step was recorded (08H00). The petri dishes containing the seeds were placed in a dark cupboard at room temperature. Aseptic measures such as sterilization of all equipment were used to avoid contamination. The filter paper was moistened with 1 ml of distilled water every 12 hours. Germination percentage

(Germination Percentage = seeds germinated/total seeds × 100) was recorded every 24 hours for 6 days as described by Manmathan and Lapitan (2013).

4.1.3.4 Effect of Endophytic Bacterial Inoculation on Growth

Starke Ayres ® Rape English Giant seeds were inoculated with 50 ml of each bacterial treatment. The seeds were left to soak in the treatment for 24 hours after which the seeds were drained. Five seeds were sown into equally measured, soil filled plant pots at 1 cm depth (Lwin *et al.*, 2012).

4.1.3.5 Pot Trial Experiment of *C. gynandra* Seed Endophyte as Seed Inoculants

In this experiment, plants pots (12 cm x 9 cm) were sterilized using a bleach solution and filled with approximately 222 g of dry soil. The pots were arranged in a randomized complete block design with three replicates for each treatment, totalling 310 pots in the greenhouse. Each treatment was applied directly to the seeds. Plants were watered daily with equal amounts of water (approximately 250 ml). The temperature was measured daily for the duration of the experiment. Chlorophyll concentration readings were measured every three days, on the fourth youngest leaf on the main stem after the leaves emerged (± 20 days after germination) using the Konica Minolta Chlorophyll Meter SPAD-502Plus (Konica Minolta Inc, Japan). After 45 days, fresh mass, root length, and leaf yield were determined and compared. The plants were then dried at 70˚C for six hours and weighed to determine the dry mass (Lwin *et al.*, 2012).

4.1.4 Data Analysis

Growth parameter measurements were recorded on the same day to avoid any differences based on the developmental stages of the plants' growth. The collected data were subjected to multivariate analysis of variance (MANOVA) using IBM® SPSS® Statistics software (Corp, 2019). The means were separated using post hoc LSD at a 0.05 significance level.

4.2 Results and Discussion

4.2.1 Germination Assay

A positive germination percent is considered to be 99% and above (Lwin *et al.*, 2012). In this study, T_1 (*E. cloacae*), T_4 (*E. cloacae*; *K. pneumoniae*), T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) and T_8 (Negative control) displayed the highest positive effect on germination with all of them having a germination percent of 100%. Treatment 5 (*E. cloacae*; *K. cryocrescens*), 6 (*K. pneumoniae*; *K. cryocrescens*), and 9 (Positive control) yielded the lowest results with a germination percent of 87% indicating that these treatments neither promoted nor inhibited the germination rate.

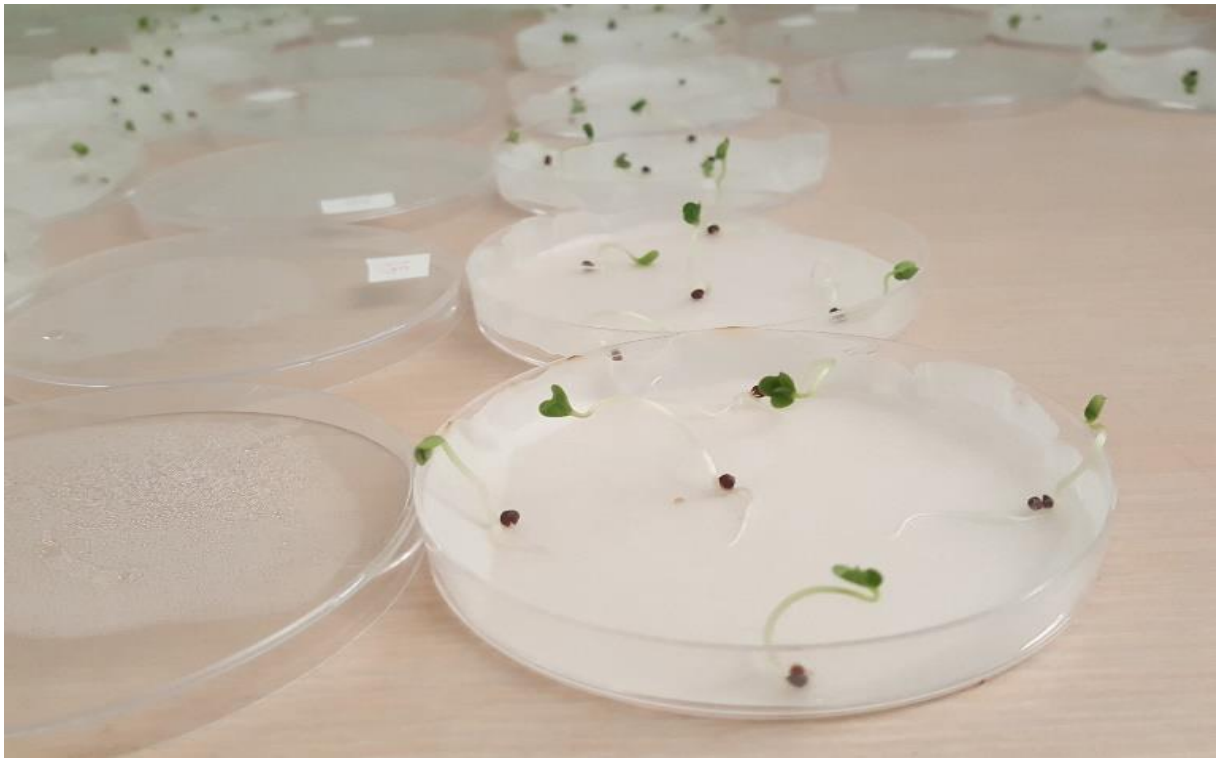


Figure 4.1: Germination assay.

Table 4.3: Germination Percent of Each Treatment.

Treatments	Germination Percent
T_1 (*E. cloacae*)	100%
T_2 (*K. pneumoniae*)	93%
T_3 (*K. cryocrescens*)	93%
T_4 (*E. cloacae*; *K. pneumoniae*)	100%
T_5 (*E. cloacae*; *K. cryocrescens*)	87%
T_6 (*K. pneumoniae*; *K. cryocrescens*)	87%
T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*)	100%
T_8 (Water) Control	100%
T_9 (Fertilizer) Control	87%

4.2.2 Pot Trial Experiment and Plant Growth Parameters

Final harvest of plants was done after 45 days where root length, plant height, leaf yield and fresh mass were recorded. The expected time frame for germination was four days. Treatments T_1 (*E. cloacae*) to T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) were able

to germinate within two days. This is an indication that the selected microbes were able to increase the germination rate by 50%. This could be due to the rate of colonisation that different endophytes possess. Treatments in this study have shown the ability to have a positive effect on the germination rate on a domesticated crop. All the isolated strains resulted in significantly higher germination rates with a 50% improvement under the following single and combination treatments: T_1 (*E. cloacae*), T_4 (*E. cloacae*; *K. pneumoniae*), T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) and T_8 (Negative control). The leaves grew in a pattern of three folioles per stalk with T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) producing the largest leaf size. All inoculated treatments recorded higher leaf number in comparison to uninoculated controls except for T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*). There was an increase in the root length from the treatments in comparison to the controls. T_2 (*K. pneumoniae*) had the highest value for root length at 21.21 cm, recording an increase of 3% more than the controls, while the lowest value went to T_8 at 13.36 cm (**Table 4.4**).

There was an increase in the root length from the treatments in comparison to the controls. However, the bacterial treatments were not able to increase the plant height when compared to the positive and negative controls (**Table 4.4**). The lowest value for plant height was recorded as 19.47 cm for T_8, whereas the highest value for plant height was 25.59 cm for T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*). The results for plant height are in agreement with a study conducted Zahir *et al.*, (2010). The bacterial treatments were not able to increase the chlorophyll levels when compared to the positive and negative controls (**Figure 4.3**), however, T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) produced the highest values at 6% on day one, four and ten and 7% on day seven, while T_1 (*E. cloacae*) yielded the lowest values overall. This is supported by findings from

previous research done by Yildirim *et al.*, (2011) on broccoli. The negative control had higher levels of chlorophyll in comparison to the positive control and microbial treatments. This could be attributed to the negative control synthesizing more chlorophyll in order to absorb more light. Results show that the positive control produced the highest amounts for wet and dry mass, in comparison to the microbial treatments. Although most microbial treatments scored significantly lower than the control, treatment T_4 (*E. cloacae*; *K. pneumoniae*) was able to produce amounts 30% lower for wet mass and 10% lower for dry mass respectively. The negative control had a higher wet mass in comparison to some microbial treatments. This could be due to its nitrogen levels, which have been linked to increased wet mass. Research on *Cleome gynandra* L., has seen an increase in wet and dry mass with an application of a nitrogen rich fertilizer (Ng'etich, Aguyoh and Ogweno, 2012). The results presented from the previous study are similar to the present study as wet and dry mass amounts were highest in T_9 (Positive control) which was a nitrogen containing fertilizer. Single strain *Klebsiella pneumoniae* (T_2), as well as consortia of and combination strains *Enterobacter cloacae* and *Kluyvera cryocrescens* (T_5) were able to increase the relative yield of multiple growth parameters in comparison to the positive and negative control (**Table 4.4**). The maximum effect on all growth parameters was observed on the combination treatment T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*). This could be due to their synergistic effects amongst each other, which could in turn indirectly improve the plant growth. In a report conducted by Iniguez, Dong and Triplett (2004), *Klebsiella*, a known nitrogen-fixing bacteria (Wei *et al.*, 2014), was reported to increase chlorophyll levels, plant height and root and shoot weight in wheat plants under greenhouse conditions, which thus supports the results in this study. *Enterobacter cloacae, Klebsiella pneumoniae* and *Kluyvera cryocrescens* are endophytic bacteria that

have been reported to possess multiple plant growth promoting activities (Chelius and Triplett, 2000; Khan, Zaidi and Wani, 2007; Tian *et al.*, 2009; Singh, 2013; Khalifa *et al.*, 2016). Some inoculants that had negative effects or no effect on growth parameters could be attributed to the production of phytotoxins that inhibited plant growth (Brown and Rovira, 1999).

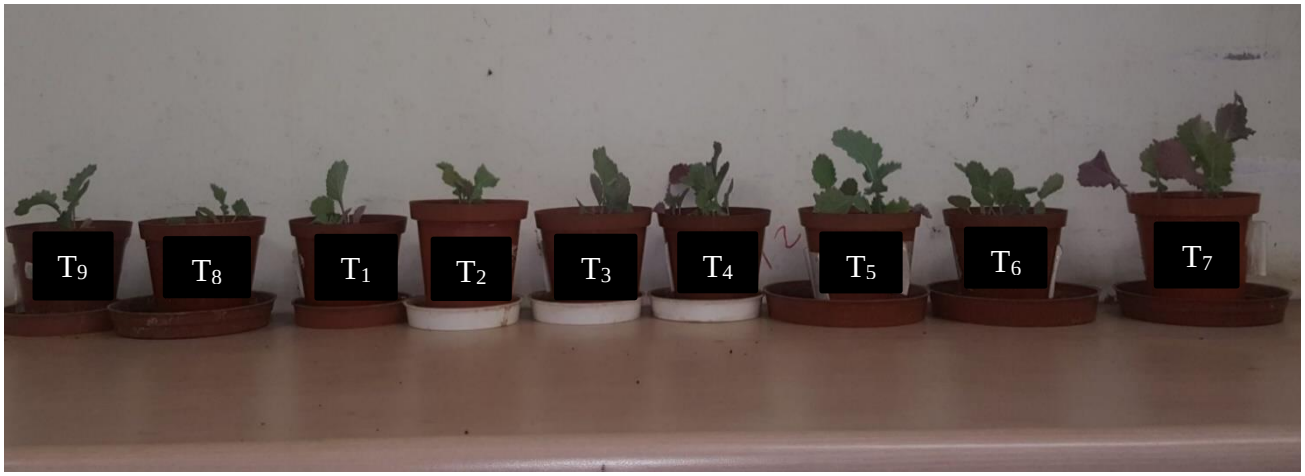
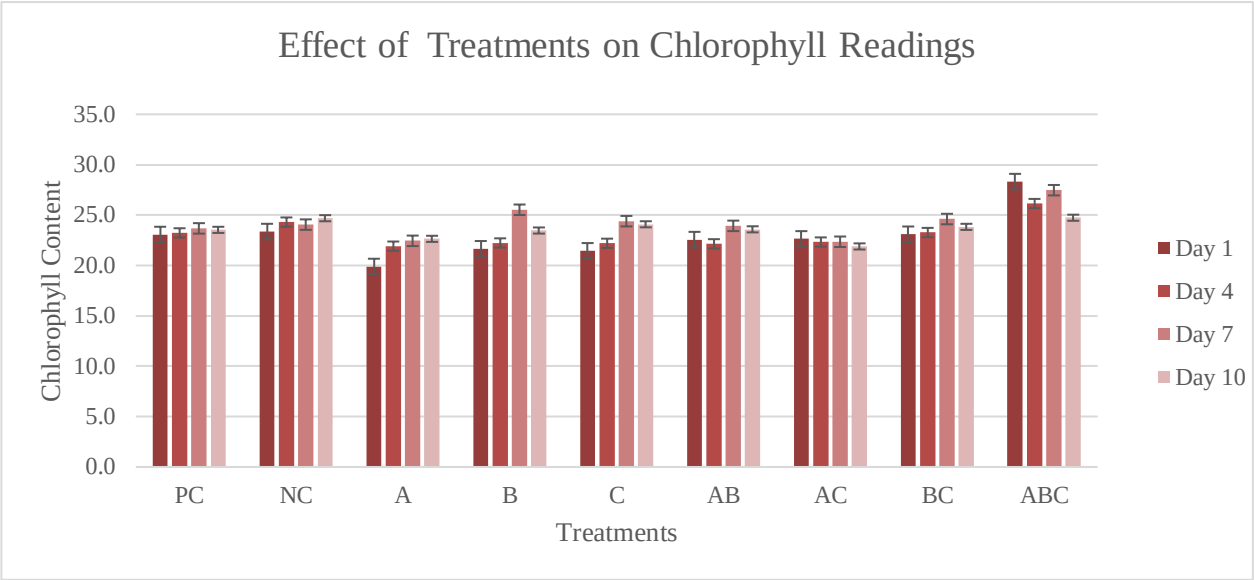


Figure 4.2: Displaying the effects of each treatment on plant growth.



PC = Positive Control (Fertilizer) **NC** = Negative Control (Water)

A = *Enterobacter cloacae*

B = *Klebsiella pneumoniae* **C** = *Kluyvera cryocrescens*

Figure 4.3: The effects of each treatment on chlorophyll content. Vertical bars indicate the mean ± SE.

Table 4.4: Comparison of treatment effects on leaf number, root length, plant height, wet mass and dry mass.

Treatment	Leaf Number	Root Length (cm)	Plant Height (cm)	Wet Mass (g)	Dry Mass (g)
T_1	15.10±3.62	15.73±3.36	21.83±3.64	1.59±0.99	0.71±0.59
T_2	12.77±3.80	21.21±32.97	21.21±3.76	1.09±0.53	0.48±0.27
T_3	15.03±4.16	15.89±4.24	22.69±5.31	1.28±0.66	0.45±0.28
T_4	16.30±3.33	16.60±4.02	22.76±4.27	3.18±1.42	1.10±0.63
T_5	15.23±2.99	13.76±3.83	22.15±4.12	2.98±1.34	0.86±0.47
T_6	14.60±4.21	16.46±2.86	23.85±2.74	2.68±1.10	0.94±0.46
T_7	6.40±2.46	17.44±2.93	25.59±3.26	2.67±1.90	0.89±0.67
T_8	8.85±4.04	13.36±4.01	19.47±4.96	2.15±1.50	0.34±0.28
T_9	8.21±3.41	17.09±4.18	23.80±4.66	5.18±2.89	1.22±0.70

Data are presented as mean±standard deviation

T_1 = A (*Enterobacter cloacae*) **T_6** = BC

T_2 = B (*Klebsiella pneumoniae*) **T_7** = ABC

T_3 = C (*Kluyvera cryocrescens*) **T_8** = Water (Negative Control)

T_4 = AB
T_5 = AC
T_9 = Fertilizer (Positive Control)

A test of normality determined that plant height was the only collection of data that were normally distributed based on the examination of its normal Q-Q plots (**Appendix 3**). All the data collected were subjected to a multivariate analysis of variance (MANOVA) (**Appendix 3**). Multivariate analysis has been used to identify and analyze multiple important traits of different bacterial species in previous studies (Al Khanjari *et al.*, 2008; Yada *et al.*, 2010), which has also been done in the present study. It was determined that there was a significant difference for root length ($p = 0.024$), plant height ($p = 0.037$), wet mass ($p = 0.000$) and dry mass ($p = 0.000$). In the case of the leaf number, T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) was the only one to indicate that it had no significant ($p = 0.092$) effect on the number of leaves yielded in comparison to the positive control. T_5 (*E. cloacae*; *K. cryocrescens*) was the only inoculant that significantly promoted root length in comparison to the other inoculants and controls. Inoculant T_6 showed significance with regards to plant height. Treatment T_2 (*K. pneumoniae*) and T_3 (*K. cryocrescens*) had the highest means for wet mass compared to the other treatments and controls. Treatment T_3 (*K. cryocrescens*) showed that there was no significant difference ($p = 0.059$) compared to the negative control for dry mass while T_4 (*E. cloacae*; *K. pneumoniae*), T_6 (*K. pneumoniae*; *K. cryocrescens*) and T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) displayed no difference against the positive control. All treatments showed that there was no significant difference in chlorophyll readings with regards to the positive and negative control. Treatments with the best overall performance were T_2 (*K. pneumoniae*) and T_7 (*E. cloacae*; *K. pneumoniae*; *K. cryocrescens*) which was determined by the post hoc LSD test (Hilton and Armstrong, 2006) (**Appendix 3**).

The primary goal of agriculture is to produce high quality, safe and affordable food for the increasing world population (Avis *et al.*, 2008; Backer *et al.*, 2018). An emerging trend in agriculture has seen chemical fertilizers being replaced with microbial counterparts to reduce health concerns (Conn and Franco, 2004; Timmusk *et al.*, 2014; Kalayu, 2019). The best strategy for endophyte application in agriculture systems is not known (Cocq *et al.*, 2017). The use of microbes as seed inoculant under field trials and greenhouse conditions has been documented in many studies, reporting these bacteria to produce plant growth regulators (Ozturk, Caglar and Sahin, 2003; Zahir, Arshad and Frankenberger, 2004) and have promising agronomic potential as seed inoculants (Zaida *et al.*, 2009; Minaxi *et al.*, 2012; de Souza, Ambrosini and Passaglia, 2015). The use of microbial inoculation has been successful in sugar cane (Silva *et al.*, 2012), however, field efficacy has not been thoroughly studied. Interactions between plant growth promoting bacteria and plants are not well understood, with regards to field applications and different environments (Niranjan, Shetty and Reddy, 2005). In some cases, it has been unsuccessful due to problems with the establishment of biological agent and other uncontrollable variables (O'Callaghan, 2016; Cocq *et al.*, 2017). Availability of more information will enable the development and acceptance of new agricultural technologies which can improve plant development (Akanbi *et al.*, 2007). Determining the behaviour of endophytes under different conditions is critical to understanding their life cycle in order to assess their risk of becoming pathogenic, through shifts in abiotic conditions or adaptation to the new host. (Redman, Dunigan and Rodriguez, 2001; Robinson *et al.*, 2016). Based on previous research (Murphy, Doohan and Hodkinson, 2018; Shahzad *et al.*, 2018), the improved growth parameters evaluated in this study could be attributed to the plant growth promoting capabilities of the microorganisms selected. The information

provided in this study looks to contribute to the understanding of endophytes and how they can be applied to efficient agricultural systems.

4.3 Conclusion

This study provided evidence that *Cleome gynandra* L. seeds from Oshana, Omusati, Ohangwena and Oshikoto regions respectively, harbour bacterial endophytes with several plant growth promoting traits and can serve as seed inoculants on domesticated crops. Findings from this study have confirmed that *C. gynandra* endophytic seed bacteria have promising potential as seed inoculants. They have displayed capabilities to improve the leaf number, plant height, root length and increase the germination rate of *Brassica napus* L. seeds. It is recommended that more greenhouse experiments and field trials are required to understand the endophytes capabilities and contribute to the commercialization of the bacterial strains.

REFERENCES

Akanbi, W. B. *et al.* (2007) 'The Use of Compost Extract as Foliar Spray Nutrient Source and Botanical Insecticide in *Telfairia occidentalis*.', *World Journal of Agricultural Sciences*, 3, pp. 642–652.

Araujo, W. L. *et al.* (2008) 'Interaction Between Endophytes and Plant Host: Biotechnological Aspects', in Barka, E. A. and Clement, C. (eds) *Plant-Microbe Interactions*. Research Signpost, pp. 95–115.

Arora, N. K., Khare, E. and Oh, J. H. (2008) 'Diverse Mechanisms Adopted by *Pseudomonas* Fluorescent PGC2 During the Inhibition of R*hizoctonia solani* and *Phytophthora*

capsici', *World Journal of Microbiology and Biotechnology*, 24, pp. 581–585.

Avis, T. J. *et al.* (2008) 'Multifaceted Beneficial Effects of Rhizosphere Microorganisms on Plant Health and Productivity', *Soil Biology & Biochemistry*, 40(7), pp. 1733–1740.

Backer, R. *et al.* (2018) 'Plant Growth-Promoting Rhizobacteria : Context, Mechanisms of Action, and Roadmap to Commercialization of Biostimulants for Sustainable Agriculture', *Frontiers in Plant Science*, 9(1473), pp. 1–17.

Bhattacharyya, P. N. and Jha, D. K. (2012) 'Plant Growth-Promoting Rhizobacteria (PGPR): Emergence in Agriculture', *World Journal of Microbiology and Biotechnology*, 28(4), pp. 1327–1350.

Brown, G. D. and Rovira, A. D. (1999) 'The Rhizosphere and Its Management to Improve Plant Growth', *Advances in Agronomy*, 66, pp. 1–102.

Cankar, K. *et al.* (2005) 'Bacterial Endophytes from Seeds of Norway Spruce (*Picea abies* L . Karst)', *FEMS Microbiology Letters*, 244(2), pp. 341–345.

Chelius, M. K. and Triplett, E. W. (2000) 'Immunolocalization of Dinitrogenase Reductase Produced by *Klebsiella pneumoniae* in Association with *Zea mays* L .', *Applied and Environmental Microbiology*, 66(2), pp. 783–787.

Chweya, J. A. and Mnzava, N. A. (1997) *Cat's Whiskers: Cleome gynandra L.*, *Biodiversity International*. Rome: Italy: International Plant Genetic Resources Institute.

Cocq, K. L. E. *et al.* (2017) 'Exploitation of Endophytes for Sustainable Agricultural Intensification (Microreview)', *Molecular Plant Pathology*, 18(3), pp. 469–473.

Conn, V. M. and Franco, C. M. M. (2004) 'Effect of Microbial Inoculants on the Indigenous

Actinobacterial Endophyte Population in the Roots of Wheat as Determined by Terminal Restriction Fragment Length Polymorphism', *Applied and Environmental Microbiology*, 70(11), pp. 6407–6413.

Corp, I. (2019) 'IBM SPSS Statistics for Windows'. Armonk, NY: IBM Corp.

Eduardo, L. G. *et al.* (2018) 'Low Accuracy of the McFarland Method for Estimation of Bacterial Populations', *African Journal of Microbiology Research*, 12(31), pp. 736–740.

Flores-Felix, J. D., Silva, L. R. and Rivera, L. P. (2015) 'Plant Probiotics as a Tool to Produce Highly Functional Fruits: The Case of Phyllobacterium and Vitamin C in Strawberries', *PLoS ONE*, 10(4), pp. 1–13.

Gabriela, F. *et al.* (2015) 'Azospirillum Inoculation Effects on Growth, Product Quality and Storage Life of Lettuce Plants Grown Under Salt Stress', *Scientia Horticulturae*, 195, pp. 154–162.

Ganya, S., Svotwa, E. and Katsaruware, R. D. (2018) 'Performance of Two Rape (*Brassica napus*) Cultivars under Different Fertilizer Management Levels in the Smallholder Sector of Zimbabwe', *International Journal of Agronomy*, 2018.

Glick, B. R. (2012) 'Plant Growth-Promoting Bacteria: Mechanisms and Applications', *Scientifica*, 2012(5).

Guarino, L. (1997) 'Local Leafy Vegetables', in *Traditional African Vegetables*. Rome (Italy): International Plant Genetic Resources Institute, pp. 75–81.

Hardoim, P. R. *et al.* (2012) 'Dynamics of Seed-Borne Rice Endophytes on Early Plant Growth Stages', *PLoS ONE*, 7(2).

Hilton, A. C. and Armstrong, R. (2006) 'Post Hoc ANOVA Tests', *Microbiologist*, 6, pp. 34–36.

Iniguez, A. L., Dong, Y. and Triplett, E. W. (2004) 'Nitrogen Fixation in Wheat Provided by *Klebsiella pneumoniae* 342', *Molecular Plant-Microbe Interactions*, 17(10), pp. 1078–1085.

Kalayu, G. (2019) 'Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers', *International Journal of Agronomy*, 2019.

Khalifa, A. Y. Z. *et al.* (2016) 'Characterization of the Plant Growth Promoting Bacterium, *Enterobacter cloacae* MSR1, Isolated from Roots of Non-Nodulating Medicago sativa', *Saudi Journal of Biological Sciences*. King Saud University, 23(1), pp. 79–86.

Khan, M. S., Zaidi, A. and Wani, P. A. (2007) 'Role of Phosphate-Solubilizing Microorganisms in Sustainable Agriculture - A Review', *Agronomy for Sustainable Development*, 27(1), pp. 29–43.

Al Khanjari, S. *et al.* (2008) 'Morphological Diversity of Omani Wheat', *Genetic Resources and Crop Evolution*, 55(8), pp. 1185–1195.

Kujeke, G. T., Edziwa, X. and Icishahayo, D. (2017) 'Field Performance of Spider Plant (*Cleome gynandra* L.) under Different Agronomic Practices', *African Journal of Food, Agriculture, Nutrition and Development*, 17(3), pp. 12179–12197.

Kuntashula, E. *et al.* (2004) 'Potential of Biomass Transfer Technologies in Sustainable Vegetable Production in the Wetlands (DAMBOS) of Eastern Zambia', *Experimental Agriculture*, 40(1), pp. 37–51.

Loehr, B., Seif, A. and Nyambo, B. (1998) 'Vegetable IPM in Africa: Current Status and Future Prospects with Emphasis on Eastern and Southern Africa', *Acta Horticulturae*, 513(10), pp. 99–104.

Lwin, K. M. *et al.* (2012) 'Isolation of Plant Hormone (Indole-3-Acetic Acid - IAA) Producing Rhizobacteria and Study on their Effects on Maize Seedling', *Engineering Journal*, 16(5), pp. 137–144.

Manmathan, H. and Lapitan, N. L. . (2013) 'Measuring Germination Percentage in Wheat', *Bio-protocol*, 3(16).

Marasco, R. *et al.* (2013) 'Are Drought-Resistance Promoting Bacteria Cross-Compatible with Different Plant Models?', *Plant Signaling & Behavior*, 8(10).

Mayak, S., Tirosh, T. and Glick, B. R. (2004) 'Plant Growth-Promoting Bacteria Confer Resistance in Tomato Plants to Salt Stress', *Plant Physiology and Biochemistry*, 42(6), pp. 565–572.

Mercado-Blanco, J. and Lugtenberg, B. J. J. (2014) 'Biotechnological Applications of Bacterial Endophytes', *Current Biotechnology*, 3(1), pp. 60–75.

Minaxi *et al.* (2012) 'Characterization of Multifaceted *Bacillus* sp. RM-2 for its Use as Plant Growth Promoting Bioinoculant for Crops Grown in Semi Arid Deserts', *Applied Soil Ecology*. Elsevier B.V., 59, pp. 124–135.

Murphy, B. R., Doohan, F. M. and Hodkinson, T. R. (2018) 'From Concept to Commerce: Developing a Successful Fungal Endophyte Inoculant for Agricultural Crops', *Journal of Fungi*, 4(1), p. 24.

Ng'etich, O. K., Aguyoh, J. N. and Ogweno, J. O. (2012) 'Growth, Yield and Physiological Responses of Spider Plant (*Cleome gynandra* L.) to Calcium Ammonium Nitrate Rates', *International Journal of Agronomy and Plant Production*, 3(9), pp. 346–355.

Niranjan, R., Shetty, H. S. and Reddy, M. S. (2005) 'Plant Growth-Promoting Rhizobacteria: Potential Green Alternative for Plant Productivity', in Siddiqui, Z. A. (ed.) *PGPR: Biocontrol and Biofertilization*. Dordrecht: Springer, pp. 197–216.

O'Callaghan, M. (2016) 'Microbial Inoculation of Seed for Improved Crop Performance: Issues and Opportunities', *Applied Microbiology and Biotechnology*, 100(13), pp. 5729–5746.

Orhan, E. *et al.* (2006) 'Effects of Plant Growth Promoting Rhizobacteria (PGPR) on Yield, Growth and Nutrient Contents in Organically Growing Raspberry', *Scientia Horticulturae*, 111(1), pp. 38–43.

Ozturk, A., Caglar, O. and Sahin, F. (2003) 'Yield Response of Wheat and Barley to Inoculation of Plant Growth Promoting Rhizobacteria at Various Levels of Nitrogen Fertilization', *Journal of Plant Nutrition and Soil Science*, 166(2), pp. 1–5.

Redman, R. S., Dunigan, D. D. and Rodriguez, R. J. (2001) 'Fungal Symbiosis from Mutualism to Parasitism: Who Controls the Outcome, Host or Invader?', *New Phytologist*, 151, pp. 705–716.

Reinhold-Hurek, B. and Hurek, T. (2011) 'Living Inside Plants: Bacterial Endophytes', *Current Opinion in Plant Biology*. Elsevier Ltd, 14(4), pp. 435–443.

van Rensburg, W. S. J. *et al.* (2007) 'African Leafy Vegetables in South Africa', *Water SA*, 33(3), pp. 317–326.

Rieger, M. A., Preston, C. and Powles, S. B. (1999) 'Risks of gene flow from transgenic-herbicide resistant canola (*Brassica napus*) to weedy relatives in southern Australian cropping systems', *Australian Journal of Agricultural Research*, 50(2), pp. 115–128.

Robinson, R. J. *et al.* (2016) 'Wheat Seed Embryo Excision Enables the Creation of Axenic Seedlings and Koch's Postulates Testing of Putative Bacterial Endophytes', *Scientific Reports*, 6(25), p. 581.

Rosenblueth, M. and Martínez-romero, E. (2006) 'Bacterial Endophytes and Their Interactions with Hosts', *Molecular Plant-Microbe Interactions*, 19(8), pp. 827–837.

Ryu, C. M. *et al.* (2003) 'Bacterial Volatiles Promote Growth in Arabidopsis', *Proceedings of the National Academy of Sciences of the United States of America*, 100, pp. 4927–4932.

Sansanwal, R. *et al.* (2017) 'Role of Endophytes in Agriculture', *Chemical Science Review and Letters*, 6(24), pp. 2397–2407.

Schulz, B. and Boyle, C. (2006) *Microbial Root Endophytes*. 5th edn. Edited by T. Sieber. Berlin, Germany: Springer-Verlag GmbH.

Shahzad, R. *et al.* (2018) 'What Is There in Seeds ? Vertically Transmitted Endophytic Resources for Sustainable Improvement in Plant Growth', *Frontiers in Plant Science*, 9(24).

Shilla, O. *et al.* (2019) '*Cleome gynandra* L. origin, taxonomy and morphology: A review', *African Journal of Agricultural Research*, 14(32), pp. 1568–1583.

Silva, M. F. *et al.* (2012) 'Survival of Endophytic Bacteria in Polymer-Based Inoculants and Efficiency of Their Application to Sugarcane', *Plant and Soil*, 356, pp. 231–243.

Singh, J. S. (2013) 'Potential Microbes for Sustainable Agriculture', *Resonance: Journal of Science Education*, (March), pp. 275–281.

de Souza, R., Ambrosini, A. and Passaglia, L. M. P. (2015) 'Plant Growth-Promoting Bacteria as Inoculants in Agricultural Soils', *Genetics and Molecular Biology*, 38(4), pp. 401–419.

Tian, F. *et al.* (2009) 'Genetic Diversity Of Siderophore-Producing Bacteria Of Tobacco Rhizosphere', *Brazilian Journal of Microbiology*, 40(61), pp. 276–284.

Timmusk, S. *et al.* (2014) 'Drought-Tolerance of Wheat Improved by Rhizosphere Bacteria from Harsh Environments: Enhanced Biomass Production and Reduced Emissions of Stress Volatiles', *PLoS ONE*, 9(5).

Trivedi, G. *et al.* (2018) 'Role of Endophytes in Agricultural Crops Under Drought Stress : Current and Future Prospects', *Journal of Applied Microbiology*, 3(4), pp. 174–188.

Truyens, S. *et al.* (2014) 'Bacterial Seed Endophytes: Genera, Vertical Transmission and Interaction with Plants (Minireview)', *Environmental Microbiology Reports*, 7(1), pp. 40–50.

Vejan, P. *et al.* (2016) 'Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability-A Review', *Molecules*, 21(5), pp. 1–17.

Walton, G. *et al.* (1999) 'Phenology, Physiology and Agronomy', in Salisbury, P. et al. (eds) *Canola in Australia: the first thirty years*, pp. 9–14.

Wani, P. A., Khan, M. S. and Zaida, A. (2007) 'Synergistic Effect of the Inoculation with Nitrogen-Fixing and Phosphate-Solubilizing Rhizobacteria on Performance of Field-

Grown Chickpea', *Journal of Plant Nutrition and Soil Science*, 170(2), pp. 283–287.

Wasonga, D. O. *et al.* (2015) 'Morphological Characterization and Selection of Spider Plant (*Cleome gynandra*) Accessions from Kenya and South Africa', *Asian Journal of Agricultural Sciences*, 7(4), pp. 36–44.

Wei, C. *et al.* (2014) 'Endophytic Nitrogen-Fixing *Klebsiella variicola* Strain DX120E Promotes Sugarcane Growth', *Biology and Fertility of Soils*, 50(4), pp. 657–666.

White, J. F. *et al.* (2017) 'Disease Protection and Allelopathic Interactions of Seed-Transmitted Endophytic Pseudomonads of Invasive Reed Grass (*Phragmites australis*)', *Plant and Soil*, 422, pp. 195–208.

Yada, B. *et al.* (2010) 'Morphological Characterization of Ugandan Sweet Potato Germplasm', *Crop Science*, 50(6), pp. 2364–2371.

Yildirim, E. *et al.* (2011) 'Growth, Nutrient Uptake, and Yield Promotion of Broccoli by Plant Growth Promoting Rhizobacteria with Manure', *HortScience*, 46(6), pp. 932–936.

Zahir, A., Arshad, Z. M. and Frankenberger, W. F. (2004) 'Plant Growth Promoting Rhizobacteria', *Advances in Agronomy*, 81, pp. 97–168.

Zahir, Z. A. *et al.* (2010) 'Substrate-Dependent Auxin Production by *Rhizobium phaseoli* Improves the Growth and Yield of *Vigna radiata* L. under Salt Stress Conditions', *Journal of Microbiology and Biotechnology*, 20(9), pp. 1288–1294.

Zaida, A. *et al.* (2009) 'Plant Growth Promotion by Phosphate Solubilizing Bacteria', *Acta Microbiologica et Immunologica Hungarica*, 56(3), pp. 263–284.

CONCLUSION

Many endophytes associated with plants are known to confer plant growth promoting traits. This thesis revealed that *Cleome gynandra* L. seeds collected from the Oshana, Omusati, Ohangwena and Oshikoto regions respectively, harbour endophytes. Twenty bacterial isolates possessing plant growth promoting and secondary metabolite producing capabilities were isolated from *C. gynandra* seeds. The endophytes (excluding *Phytobacter ursingii*) belong to the *Proteobacteria* phyla and their importance as plant growth promoters can be deduced from all the research published to date. Although

research suggests beneficial microorganisms are essential to agriculture, only a few microorganisms have been used as seed treatments/inoculants. As bio-inoculants, seed endophytes can improve the overall health and growth of plants. Bacterial strains in this study have shown the ability to serve as seed inoculants on the domesticated crop *Brassica napus* L. (Rape English Giant), which is a phylogenetic relative of *C. gynandra*. They were able to increase the germination rate by 50% as well as have an effect on plant height, root length and leaf number. Further investigations and greenhouse/field trials are required to ensure that endophytic bacteria from this study can be commercialized and applied as bioinoculants. The use of endophytes that are native to plant and soil types in indigenous crops in Namibia should be encouraged in order to move towards protecting current natural resources and provide environmental sustainability. They could contribute to sustainable, cost effective and more environment-friendly methods of agriculture. Although endophytes can be mass produced and are suited for biotechnological application, studies only focus on culturable endophytes (Mercado-Blanco and Lugtenberg, 2014). There are still knowledge gaps on seed endophytes and their mechanisms. With a more in-depth understanding of bacterial seed endophytes, current and future scientists will be able to unlock the biotechnological potential of plant-microbe interactions and open them to a host of new applications. The process of commercialization requires the isolation of strains, screening, mass production, industry linkage, quality control and field efficacy testing (Nandakumar *et al.*, 2001). Successful commercialization would depend on the linkage between scientific academic bodies, industries (Nakkeeran, Fernando and Siddiqui, 2005) and government bodies. This thesis serves as a reference for future research and development on all plant-growth promoting seed endophytes and their interactions pertaining to *Cleome gynandra* L.

REFERENCES

Mercado-Blanco, J. and Lugtenberg, B. J. J. (2014) 'Biotechnological Applications of Bacterial Endophytes', *Current Biotechnology*, 3(1), pp. 60–75.

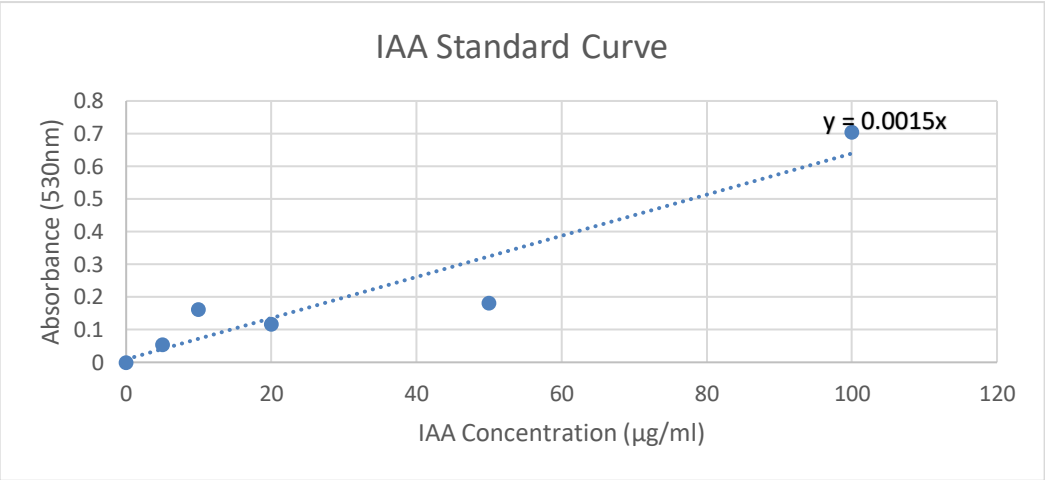
Nakkeeran, S., Fernando, D. and Siddiqui, Zaki A (2005) 'Plant Growth Promoting Rhizobacteria Formulations and its Scope in Commercialization for the Management of Pests and Diseases', in Siddiqui, Z.A. (ed.) *PGPR: Biocontrol and Biofertilization*. Dordrecht, The Netherlands: Springer, pp. 257–296.

Nandakumar, R. *et al.* (2001) 'A New Bio-Formulation Containing Plant Growth Promoting Rhizobacterial Mixture for the Management of Sheath Blight and Enhanced Grain Yield in Rice', *Biocontrol*, 46(4), pp. 493–510.

Appendices

Appendix 1

IAA standard curve graph.



Appendix 2

Table 1: Screening for IAA production

Isolate	Replicates (Absorbance=530) 1	2	3	Average	Standard Deviation	IAA Producer
OSC-2	0.2153	0.1168	0.1376	0.1572	±0.0512	+
OSC-5	0.2659	0.3086	0.3018	0.2921	±0.0229	+
OSC-3	0.2747	0.2819	0.2674	0.2747	±0.0073	+
OSC-1	0.2125	0.3349	0.2599	0.2691	±0.0617	+
OSC-7	0.2329	0.248	0.2353	0.2387	±0.0081	+
OHC-1	0.1963	0.2985	0.3274	0.2741	±0.0689	+
OHC-2	0.2092	0.2335	0.2428	0.2285	±0.0173	+
OHC-3	0.2077	0.2157	0.2397	0.2210	±0.0167	+
OHC-4	0.2295	0.307	0.3156	0.2840	±0.0474	+
OHC-5	0.234	0.18	0.1848	0.1996	±0.0299	+
OKC-8	0.165	0.1529	0.139	0.1523	±0.0130	+
OKC-5	0.1965	0.2122	0.168	0.1922	±0.0224	+
OKC-1	0.1948	0.144	0.1762	0.1717	±0.0257	+
OKC-7	0.2013	0.1653	0.1911	0.1859	±0.0186	+
OKC-8	0.2566	0.1916	0.1922	0.2135	±0.0374	+
OMC-1	0.2296	0.2083	0.2066	0.2148	±0.0128	+
OMC-3	0.2575	0.2473	0.2689	0.2579	±0.0108	+
OMC-2	0.2317	0.2249	0.2389	0.2318	±0.0070	+
OMC-5	0.3069	0.1891	0.1737	0.2232	±0.0729	+
OMC-6	0.256	0.2017	0.2222	0.2266	±0.0274	+

Appendix 3

Statistical analysis data generated from SPSS software

Overall Normality Test

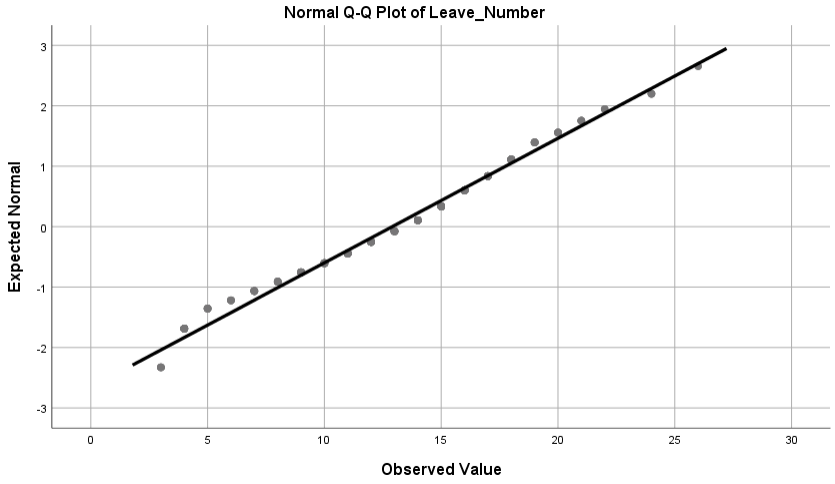
Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Leave_Number	.087	250	.000	.979	250	.001
Root_Length	.255	250	.000	.244	250	.000
Plant_Height	.040	250	.200*	.995	250	.603
Wet_Mass	.168	250	.000	.844	250	.000
Dry_Mass	.156	250	.000	.872	250	.000

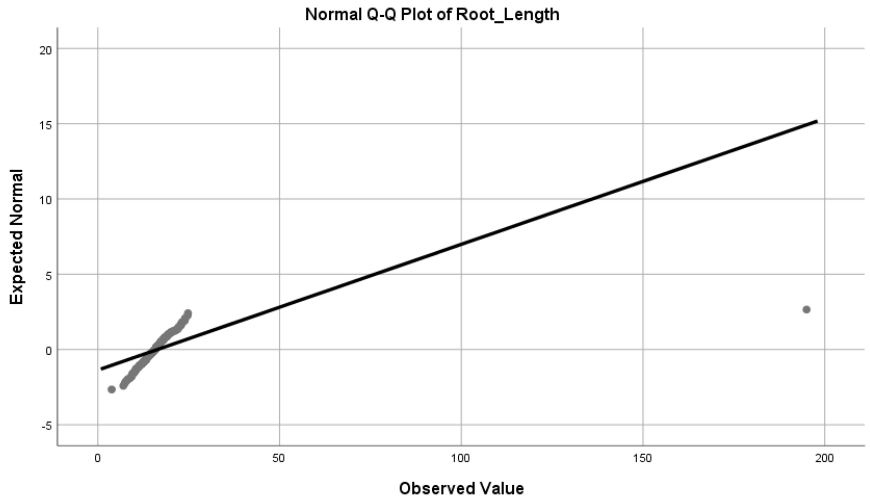
*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

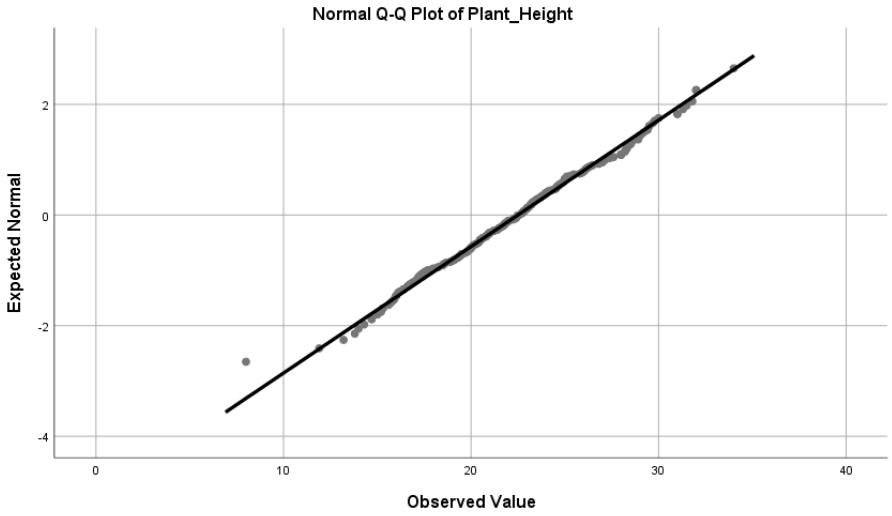
Leaf Number Q-Q Plot



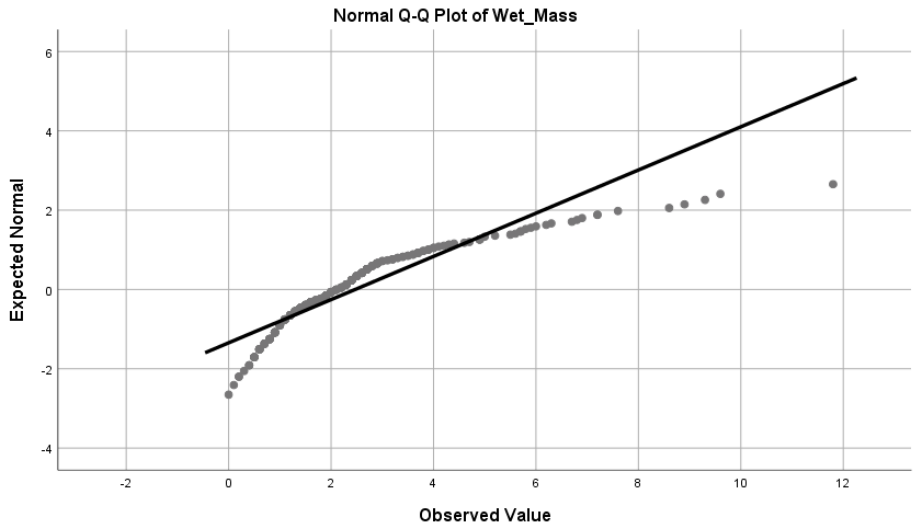
Root Length Q-Q Plot



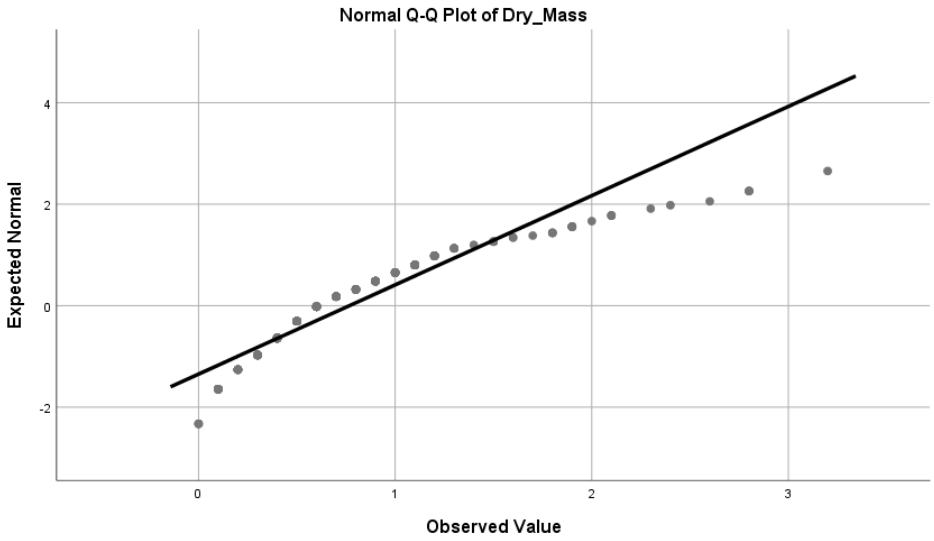
Plant Height Q-Q Plot



Wet Mass Q-Q Plot



Dry Mass Q-Q Plot



Chlorophyll Readings One Way ANOVA

ANOVA

Chlorophyll Averages

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1317.884	203	6.492	1.742	.151

Within Groups	41.000	11	3.727		
Total	1358.884	214			

Between-Subjects Factors

		Value Label	N
Growth Parameters	1	A (Enterobacter cloacae)	30
	2	B (Klebsiella pneumoniae)	30
	3	C (Kluyvera cryocrescens)	30
	4	AB	30
	5	AC	30
	6	BC	30
	7	ABC	20
	8	Positive Control	24
	9	Negative Control	26

Descriptive Statistics

	Growth Parameters	Mean	Std. Deviation	N
Leaf_Number	A (Enterobacter cloacae)	15.100	3.6232	30
	B (Klebsiella pneumoniae)	12.767	3.8028	30
	C (Kluyvera cryocrescens)	15.033	4.1563	30
	AB	16.300	3.3337	30
	AC	15.233	2.9906	30
	BC	14.600	4.2067	30
	ABC	6.400	2.4581	20

	Positive Control	8.208	3.4133	24
	Negative Control	8.846	4.0368	26
	Total	12.904	4.8536	250
Root_Length	A (Enterobacter cloacae)	15.727	3.3594	30
	B (Klebsiella pneumoniae)	21.207	32.9656	30
	C (Kluyvera cryocrescens)	15.890	4.2363	30
	AB	16.597	4.0227	30
	AC	13.757	3.8324	30
	BC	16.457	2.8551	30
	ABC	17.440	2.9305	20
	Positive Control	17.092	4.1815	24
	Negative Control	13.362	4.0092	26
	Total	16.382	11.9672	250
Plant_Height	A (Enterobacter cloacae)	21.830	3.6419	30
	B (Klebsiella pneumoniae)	21.217	3.7578	30
	C (Kluyvera cryocrescens)	22.687	5.3146	30
	AB	22.757	4.2698	30
	AC	22.147	4.1248	30
	BC	23.847	2.7356	30
	ABC	25.590	3.2643	20
	Positive Control	23.796	4.6646	24
	Negative Control	19.473	4.9580	26
	Total	22.495	4.3738	250
Wet_Mass	A (Enterobacter cloacae)	1.593	.9903	30
	B (Klebsiella pneumoniae)	1.090	.5333	30
	C (Kluyvera cryocrescens)	1.277	.6611	30
	AB	3.183	1.4208	30
	AC	2.977	1.3392	30
	BC	2.683	1.1014	30

	ABC	2.670	1.8991	20
	Positive Control	5.183	2.8874	24
	Negative Control	2.146	1.4957	26
	Total	2.471	1.8353	250
Dry_Mass	A (Enterobacter cloacae)	.710	.5909	30
	B (Klebsiella pneumoniae)	.477	.2700	30
	C (Kluyvera cryocrescens)	.447	.2751	30
	AB	1.097	.6338	30
	AC	.857	.4681	30
	BC	.943	.4599	30
	ABC	.890	.6711	20
	Positive Control	1.221	.7009	24
	Negative Control	.338	.2801	26
	Total	.767	.5688	250

Post Hoc Tests

Growth Parameters

Multiple Comparisons

LSD

Dependent Variable	(I) Growth Parameters	(J) Growth Parameters	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Leaf_Number	A (Enterobacter cloacae)	B (Klebsiella pneumoniae)	2.333*	.9388	.014	.484	4.183
		C (Kluyvera cryocrescens)	.067	.9388	.943	-1.783	1.916
		AB	-1.200	.9388	.202	-3.049	.649
		AC	-.133	.9388	.887	-1.983	1.716
		BC	.500	.9388	.595	-1.349	2.349
		ABC	8.700*	1.0496	.000	6.632	10.768
		Positive Control	6.892*	.9957	.000	4.930	8.853
		Negative Control	6.254*	.9742	.000	4.335	8.173
	B (Klebsiella pneumoniae)	A (Enterobacter cloacae)	-2.333*	.9388	.014	-4.183	-.484
		C (Kluyvera cryocrescens)	-2.267*	.9388	.017	-4.116	-.417
		AB	-3.533*	.9388	.000	-5.383	-1.684
		AC	-2.467*	.9388	.009	-4.316	-.617
		BC	-1.833	.9388	.052	-3.683	.016
		ABC	6.367*	1.0496	.000	4.299	8.434
		Positive Control	4.558*	.9957	.000	2.597	6.520
		Negative Control	3.921*	.9742	.000	2.001	5.840

	C (Kluyvera cryocrescens)	A (Enterobacter cloacae)	-.067	.9388	.943	-1.916	1.783
		B (Klebsiella pneumoniae)	2.267*	.9388	.017	.417	4.116
		AB	-1.267	.9388	.179	-3.116	.583
		AC	-.200	.9388	.831	-2.049	1.649
		BC	.433	.9388	.645	-1.416	2.283
		ABC	8.633*	1.0496	.000	6.566	10.701
		Positive Control	6.825*	.9957	.000	4.864	8.786
		Negative Control	6.187*	.9742	.000	4.268	8.106
	AB	A (Enterobacter cloacae)	1.200	.9388	.202	-.649	3.049
		B (Klebsiella pneumoniae)	3.533*	.9388	.000	1.684	5.383
		C (Kluyvera cryocrescens)	1.267	.9388	.179	-.583	3.116
		AC	1.067	.9388	.257	-.783	2.916
		BC	1.700	.9388	.071	-.149	3.549
		ABC	9.900*	1.0496	.000	7.832	11.968
		Positive Control	8.092*	.9957	.000	6.130	10.053
		Negative Control	7.454*	.9742	.000	5.535	9.373
	AC	A (Enterobacter cloacae)	.133	.9388	.887	-1.716	1.983
		B (Klebsiella pneumoniae)	2.467*	.9388	.009	.617	4.316
		C (Kluyvera cryocrescens)	.200	.9388	.831	-1.649	2.049

		AB	-1.067	.9388	.257	-2.916	.783
		BC	.633	.9388	.501	-1.216	2.483
		ABC	8.833*	1.0496	.000	6.766	10.901
		Positive Control	7.025*	.9957	.000	5.064	8.986
		Negative Control	6.387*	.9742	.000	4.468	8.306
	BC	A (Enterobacter cloacae)	-.500	.9388	.595	-2.349	1.349
		B (Klebsiella pneumoniae)	1.833	.9388	.052	-.016	3.683
		C (Kluyvera cryocrescens)	-.433	.9388	.645	-2.283	1.416
		AB	-1.700	.9388	.071	-3.549	.149
		AC	-.633	.9388	.501	-2.483	1.216
		ABC	8.200*	1.0496	.000	6.132	10.268
		Positive Control	6.392*	.9957	.000	4.430	8.353
		Negative Control	5.754*	.9742	.000	3.835	7.673
	ABC	A (Enterobacter cloacae)	-8.700*	1.0496	.000	-10.768	-6.632
		B (Klebsiella pneumoniae)	-6.367*	1.0496	.000	-8.434	-4.299
		C (Kluyvera cryocrescens)	-8.633*	1.0496	.000	-10.701	-6.566
		AB	-9.900*	1.0496	.000	-11.968	-7.832
		AC	-8.833*	1.0496	.000	-10.901	-6.766
		BC	-8.200*	1.0496	.000	-10.268	-6.132
		Positive Control	-1.808	1.1008	.102	-3.977	.360
		Negative Control	-2.446*	1.0814	.025	-4.576	-.316

	Positive Control	A (Enterobacter cloacae)	-6.892*	.9957	.000	-8.853	-4.930
		B (Klebsiella pneumoniae)	-4.558*	.9957	.000	-6.520	-2.597
		C (Kluyvera cryocrescens)	-6.825*	.9957	.000	-8.786	-4.864
		AB	-8.092*	.9957	.000	-10.053	-6.130
		AC	-7.025*	.9957	.000	-8.986	-5.064
		BC	-6.392*	.9957	.000	-8.353	-4.430
		ABC	1.808	1.1008	.102	-.360	3.977
		Negative Control	-.638	1.0292	.536	-2.665	1.390
	Negative Control	A (Enterobacter cloacae)	-6.254*	.9742	.000	-8.173	-4.335
		B (Klebsiella pneumoniae)	-3.921*	.9742	.000	-5.840	-2.001
		C (Kluyvera cryocrescens)	-6.187*	.9742	.000	-8.106	-4.268
		AB	-7.454*	.9742	.000	-9.373	-5.535
		AC	-6.387*	.9742	.000	-8.306	-4.468
		BC	-5.754*	.9742	.000	-7.673	-3.835
		ABC	2.446*	1.0814	.025	.316	4.576
		Positive Control	.638	1.0292	.536	-1.390	2.665
Root_Length	A (Enterobacter cloacae)	B (Klebsiella pneumoniae)	-5.480	3.0876	.077	-11.562	.602
		C (Kluyvera cryocrescens)	-.163	3.0876	.958	-6.245	5.919
		AB	-.870	3.0876	.778	-6.952	5.212

		AC	1.970	3.0876	.524	-4.112	8.052
		BC	-.730	3.0876	.813	-6.812	5.352
		ABC	-1.713	3.4520	.620	-8.513	5.087
		Positive Control	-1.365	3.2748	.677	-7.816	5.086
		Negative Control	2.365	3.2041	.461	-3.946	8.677
	B (Klebsiella pneumoniae)	A (Enterobacter cloacae)	5.480	3.0876	.077	-.602	11.562
		C (Kluyvera cryocrescens)	5.317	3.0876	.086	-.765	11.399
		AB	4.610	3.0876	.137	-1.472	10.692
		AC	7.450*	3.0876	.017	1.368	13.532
		BC	4.750	3.0876	.125	-1.332	10.832
		ABC	3.767	3.4520	.276	-3.033	10.567
		Positive Control	4.115	3.2748	.210	-2.336	10.566
		Negative Control	7.845*	3.2041	.015	1.534	14.157
	C (Kluyvera cryocrescens)	A (Enterobacter cloacae)	.163	3.0876	.958	-5.919	6.245
		B (Klebsiella pneumoniae)	-5.317	3.0876	.086	-11.399	.765
		AB	-.707	3.0876	.819	-6.789	5.375
		AC	2.133	3.0876	.490	-3.949	8.215
		BC	-.567	3.0876	.855	-6.649	5.515
		ABC	-1.550	3.4520	.654	-8.350	5.250
		Positive Control	-1.202	3.2748	.714	-7.653	5.249
		Negative Control	2.528	3.2041	.431	-3.783	8.840
	AB	A (Enterobacter cloacae)	.870	3.0876	.778	-5.212	6.952

		B (Klebsiella pneumoniae)	-4.610	3.0876	.137	-10.692	1.472
		C (Kluyvera cryocrescens)	.707	3.0876	.819	-5.375	6.789
		AC	2.840	3.0876	.359	-3.242	8.922
		BC	.140	3.0876	.964	-5.942	6.222
		ABC	-.843	3.4520	.807	-7.643	5.957
		Positive Control	-.495	3.2748	.880	-6.946	5.956
		Negative Control	3.235	3.2041	.314	-3.076	9.547
	AC	A (Enterobacter cloacae)	-1.970	3.0876	.524	-8.052	4.112
		B (Klebsiella pneumoniae)	-7.450*	3.0876	.017	-13.532	-1.368
		C (Kluyvera cryocrescens)	-2.133	3.0876	.490	-8.215	3.949
		AB	-2.840	3.0876	.359	-8.922	3.242
		BC	-2.700	3.0876	.383	-8.782	3.382
		ABC	-3.683	3.4520	.287	-10.483	3.117
		Positive Control	-3.335	3.2748	.310	-9.786	3.116
		Negative Control	.395	3.2041	.902	-5.916	6.707
	BC	A (Enterobacter cloacae)	.730	3.0876	.813	-5.352	6.812
		B (Klebsiella pneumoniae)	-4.750	3.0876	.125	-10.832	1.332
		C (Kluyvera cryocrescens)	.567	3.0876	.855	-5.515	6.649
		AB	-.140	3.0876	.964	-6.222	5.942

		AC	2.700	3.0876	.383	-3.382	8.782
		ABC	-.983	3.4520	.776	-7.783	5.817
		Positive Control	-.635	3.2748	.846	-7.086	5.816
		Negative Control	3.095	3.2041	.335	-3.216	9.407
	ABC	A (Enterobacter cloacae)	1.713	3.4520	.620	-5.087	8.513
		B (Klebsiella pneumoniae)	-3.767	3.4520	.276	-10.567	3.033
		C (Kluyvera cryocrescens)	1.550	3.4520	.654	-5.250	8.350
		AB	.843	3.4520	.807	-5.957	7.643
		AC	3.683	3.4520	.287	-3.117	10.483
		BC	.983	3.4520	.776	-5.817	7.783
		Positive Control	.348	3.6205	.923	-6.783	7.480
		Negative Control	4.078	3.5566	.253	-2.928	11.084
	Positive Control	A (Enterobacter cloacae)	1.365	3.2748	.677	-5.086	7.816
		B (Klebsiella pneumoniae)	-4.115	3.2748	.210	-10.566	2.336
		C (Kluyvera cryocrescens)	1.202	3.2748	.714	-5.249	7.653
		AB	.495	3.2748	.880	-5.956	6.946
		AC	3.335	3.2748	.310	-3.116	9.786
		BC	.635	3.2748	.846	-5.816	7.086
		ABC	-.348	3.6205	.923	-7.480	6.783
		Negative Control	3.730	3.3850	.272	-2.938	10.398

	Negative Control	A (Enterobacter cloacae)	-2.365	3.2041	.461	-8.677	3.946
		B (Klebsiella pneumoniae)	-7.845*	3.2041	.015	-14.157	-1.534
		C (Kluyvera cryocrescens)	-2.528	3.2041	.431	-8.840	3.783
		AB	-3.235	3.2041	.314	-9.547	3.076
		AC	-.395	3.2041	.902	-6.707	5.916
		BC	-3.095	3.2041	.335	-9.407	3.216
		ABC	-4.078	3.5566	.253	-11.084	2.928
		Positive Control	-3.730	3.3850	.272	-10.398	2.938
Plant_Height	A (Enterobacter cloacae)	B (Klebsiella pneumoniae)	.613	1.0740	.568	-1.502	2.729
		C (Kluyvera cryocrescens)	-.857	1.0740	.426	-2.972	1.259
		AB	-.927	1.0740	.389	-3.042	1.189
		AC	-.317	1.0740	.768	-2.432	1.799
		BC	-2.017	1.0740	.062	-4.132	.099
		ABC	-3.760*	1.2008	.002	-6.125	-1.395
		Positive Control	-1.966	1.1392	.086	-4.210	.278
		Negative Control	2.357*	1.1145	.035	.161	4.552
	B (Klebsiella pneumoniae)	A (Enterobacter cloacae)	-.613	1.0740	.568	-2.729	1.502
		C (Kluyvera cryocrescens)	-1.470	1.0740	.172	-3.586	.646
		AB	-1.540	1.0740	.153	-3.656	.576
		AC	-.930	1.0740	.387	-3.046	1.186

		BC	-2.630*	1.0740	.015	-4.746	-.514
		ABC	-4.373*	1.2008	.000	-6.739	-2.008
		Positive Control	-2.579*	1.1392	.024	-4.823	-.335
		Negative Control	1.744	1.1145	.119	-.452	3.939
	C (Kluyvera cryocrescens)	A (Enterobacter cloacae)	.857	1.0740	.426	-1.259	2.972
		B (Klebsiella pneumoniae)	1.470	1.0740	.172	-.646	3.586
		AB	-.070	1.0740	.948	-2.186	2.046
		AC	.540	1.0740	.616	-1.576	2.656
		BC	-1.160	1.0740	.281	-3.276	.956
		ABC	-2.903*	1.2008	.016	-5.269	-.538
		Positive Control	-1.109	1.1392	.331	-3.353	1.135
		Negative Control	3.214*	1.1145	.004	1.018	5.409
	AB	A (Enterobacter cloacae)	.927	1.0740	.389	-1.189	3.042
		B (Klebsiella pneumoniae)	1.540	1.0740	.153	-.576	3.656
		C (Kluyvera cryocrescens)	.070	1.0740	.948	-2.046	2.186
		AC	.610	1.0740	.571	-1.506	2.726
		BC	-1.090	1.0740	.311	-3.206	1.026
		ABC	-2.833*	1.2008	.019	-5.199	-.468
		Positive Control	-1.039	1.1392	.363	-3.283	1.205
		Negative Control	3.284*	1.1145	.004	1.088	5.479
	AC	A (Enterobacter cloacae)	.317	1.0740	.768	-1.799	2.432

		B (Klebsiella pneumoniae)	.930	1.0740	.387	-1.186	3.046
		C (Kluyvera cryocrescens)	-.540	1.0740	.616	-2.656	1.576
		AB	-.610	1.0740	.571	-2.726	1.506
		BC	-1.700	1.0740	.115	-3.816	.416
		ABC	-3.443*	1.2008	.005	-5.809	-1.078
		Positive Control	-1.649	1.1392	.149	-3.893	.595
		Negative Control	2.674*	1.1145	.017	.478	4.869
	BC	A (Enterobacter cloacae)	2.017	1.0740	.062	-.099	4.132
		B (Klebsiella pneumoniae)	2.630*	1.0740	.015	.514	4.746
		C (Kluyvera cryocrescens)	1.160	1.0740	.281	-.956	3.276
		AB	1.090	1.0740	.311	-1.026	3.206
		AC	1.700	1.0740	.115	-.416	3.816
		ABC	-1.743	1.2008	.148	-4.109	.622
		Positive Control	.051	1.1392	.964	-2.193	2.295
		Negative Control	4.374*	1.1145	.000	2.178	6.569
	ABC	A (Enterobacter cloacae)	3.760*	1.2008	.002	1.395	6.125
		B (Klebsiella pneumoniae)	4.373*	1.2008	.000	2.008	6.739
		C (Kluyvera cryocrescens)	2.903*	1.2008	.016	.538	5.269
		AB	2.833*	1.2008	.019	.468	5.199

		AC	3.443*	1.2008	.005	1.078	5.809
		BC	1.743	1.2008	.148	-.622	4.109
		Positive Control	1.794	1.2594	.156	-.687	4.275
		Negative Control	6.117*	1.2372	.000	3.680	8.554
	Positive Control	A (Enterobacter cloacae)	1.966	1.1392	.086	-.278	4.210
		B (Klebsiella pneumoniae)	2.579*	1.1392	.024	.335	4.823
		C (Kluyvera cryocrescens)	1.109	1.1392	.331	-1.135	3.353
		AB	1.039	1.1392	.363	-1.205	3.283
		AC	1.649	1.1392	.149	-.595	3.893
		BC	-.051	1.1392	.964	-2.295	2.193
		ABC	-1.794	1.2594	.156	-4.275	.687
		Negative Control	4.323*	1.1775	.000	2.003	6.642
	Negative Control	A (Enterobacter cloacae)	-2.357*	1.1145	.035	-4.552	-.161
		B (Klebsiella pneumoniae)	-1.744	1.1145	.119	-3.939	.452
		C (Kluyvera cryocrescens)	-3.214*	1.1145	.004	-5.409	-1.018
		AB	-3.284*	1.1145	.004	-5.479	-1.088
		AC	-2.674*	1.1145	.017	-4.869	-.478
		BC	-4.374*	1.1145	.000	-6.569	-2.178
		ABC	-6.117*	1.2372	.000	-8.554	-3.680
		Positive Control	-4.323*	1.1775	.000	-6.642	-2.003

Wet_Mass	A (Enterobacter cloacae)	B (Klebsiella pneumoniae)	.503	.3761	.182	-.237	1.244
		C (Kluyvera cryocrescens)	.317	.3761	.401	-.424	1.057
		AB	-1.590*	.3761	.000	-2.331	-.849
		AC	-1.383*	.3761	.000	-2.124	-.643
		BC	-1.090*	.3761	.004	-1.831	-.349
		ABC	-1.077*	.4205	.011	-1.905	-.248
		Positive Control	-3.590*	.3989	.000	-4.376	-2.804
		Negative Control	-.553	.3903	.158	-1.322	.216
	B (Klebsiella pneumoniae)	A (Enterobacter cloacae)	-.503	.3761	.182	-1.244	.237
		C (Kluyvera cryocrescens)	-.187	.3761	.620	-.927	.554
		AB	-2.093*	.3761	.000	-2.834	-1.353
		AC	-1.887*	.3761	.000	-2.627	-1.146
		BC	-1.593*	.3761	.000	-2.334	-.853
		ABC	-1.580*	.4205	.000	-2.408	-.752
		Positive Control	-4.093*	.3989	.000	-4.879	-3.308
		Negative Control	-1.056*	.3903	.007	-1.825	-.287
	C (Kluyvera cryocrescens)	A (Enterobacter cloacae)	-.317	.3761	.401	-1.057	.424
		B (Klebsiella pneumoniae)	.187	.3761	.620	-.554	.927
		AB	-1.907*	.3761	.000	-2.647	-1.166
		AC	-1.700*	.3761	.000	-2.441	-.959
		BC	-1.407*	.3761	.000	-2.147	-.666

		ABC	-1.393*	.4205	.001	-2.222	-.565
		Positive Control	-3.907*	.3989	.000	-4.692	-3.121
		Negative Control	-.869*	.3903	.027	-1.638	-.101
	AB	A (Enterobacter cloacae)	1.590*	.3761	.000	.849	2.331
		B (Klebsiella pneumoniae)	2.093*	.3761	.000	1.353	2.834
		C (Kluyvera cryocrescens)	1.907*	.3761	.000	1.166	2.647
		AC	.207	.3761	.583	-.534	.947
		BC	.500	.3761	.185	-.241	1.241
		ABC	.513	.4205	.223	-.315	1.342
		Positive Control	-2.000*	.3989	.000	-2.786	-1.214
		Negative Control	1.037*	.3903	.008	.268	1.806
	AC	A (Enterobacter cloacae)	1.383*	.3761	.000	.643	2.124
		B (Klebsiella pneumoniae)	1.887*	.3761	.000	1.146	2.627
		C (Kluyvera cryocrescens)	1.700*	.3761	.000	.959	2.441
		AB	-.207	.3761	.583	-.947	.534
		BC	.293	.3761	.436	-.447	1.034
		ABC	.307	.4205	.467	-.522	1.135
		Positive Control	-2.207*	.3989	.000	-2.992	-1.421
		Negative Control	.831*	.3903	.034	.062	1.599
	BC	A (Enterobacter cloacae)	1.090*	.3761	.004	.349	1.831

		B (Klebsiella pneumoniae)	1.593*	.3761	.000	.853	2.334
		C (Kluyvera cryocrescens)	1.407*	.3761	.000	.666	2.147
		AB	-.500	.3761	.185	-1.241	.241
		AC	-.293	.3761	.436	-1.034	.447
		ABC	.013	.4205	.975	-.815	.842
		Positive Control	-2.500*	.3989	.000	-3.286	-1.714
		Negative Control	.537	.3903	.170	-.232	1.306
	ABC	A (Enterobacter cloacae)	1.077*	.4205	.011	.248	1.905
		B (Klebsiella pneumoniae)	1.580*	.4205	.000	.752	2.408
		C (Kluyvera cryocrescens)	1.393*	.4205	.001	.565	2.222
		AB	-.513	.4205	.223	-1.342	.315
		AC	-.307	.4205	.467	-1.135	.522
		BC	-.013	.4205	.975	-.842	.815
		Positive Control	-2.513*	.4410	.000	-3.382	-1.645
		Negative Control	.524	.4332	.228	-.330	1.377
	Positive Control	A (Enterobacter cloacae)	3.590*	.3989	.000	2.804	4.376
		B (Klebsiella pneumoniae)	4.093*	.3989	.000	3.308	4.879
		C (Kluyvera cryocrescens)	3.907*	.3989	.000	3.121	4.692
		AB	2.000*	.3989	.000	1.214	2.786

		AC	2.207*	.3989	.000	1.421	2.992
		BC	2.500*	.3989	.000	1.714	3.286
		ABC	2.513*	.4410	.000	1.645	3.382
		Negative Control	3.037*	.4123	.000	2.225	3.849
	Negative Control	A (Enterobacter cloacae)	.553	.3903	.158	-.216	1.322
		B (Klebsiella pneumoniae)	1.056*	.3903	.007	.287	1.825
		C (Kluyvera cryocrescens)	.869*	.3903	.027	.101	1.638
		AB	-1.037*	.3903	.008	-1.806	-.268
		AC	-.831*	.3903	.034	-1.599	-.062
		BC	-.537	.3903	.170	-1.306	.232
		ABC	-.524	.4332	.228	-1.377	.330
		Positive Control	-3.037*	.4123	.000	-3.849	-2.225
Dry_Mass	A (Enterobacter cloacae)	B (Klebsiella pneumoniae)	.233	.1293	.072	-.021	.488
		C (Kluyvera cryocrescens)	.263*	.1293	.043	.009	.518
		AB	-.387*	.1293	.003	-.641	-.132
		AC	-.147	.1293	.258	-.401	.108
		BC	-.233	.1293	.072	-.488	.021
		ABC	-.180	.1445	.214	-.465	.105
		Positive Control	-.511*	.1371	.000	-.781	-.241
		Negative Control	.372*	.1341	.006	.107	.636
	B (Klebsiella pneumoniae)	A (Enterobacter cloacae)	-.233	.1293	.072	-.488	.021

		C (Kluyvera cryocrescens)	.030	.1293	.817	-.225	.285
		AB	-.620*	.1293	.000	-.875	-.365
		AC	-.380*	.1293	.004	-.635	-.125
		BC	-.467*	.1293	.000	-.721	-.212
		ABC	-.413*	.1445	.005	-.698	-.129
		Positive Control	-.744*	.1371	.000	-1.014	-.474
		Negative Control	.138	.1341	.304	-.126	.402
	C (Kluyvera cryocrescens)	A (Enterobacter cloacae)	-.263*	.1293	.043	-.518	-.009
		B (Klebsiella pneumoniae)	-.030	.1293	.817	-.285	.225
		AB	-.650*	.1293	.000	-.905	-.395
		AC	-.410*	.1293	.002	-.665	-.155
		BC	-.497*	.1293	.000	-.751	-.242
		ABC	-.443*	.1445	.002	-.728	-.159
		Positive Control	-.774*	.1371	.000	-1.044	-.504
		Negative Control	.108	.1341	.421	-.156	.372
	AB	A (Enterobacter cloacae)	.387*	.1293	.003	.132	.641
		B (Klebsiella pneumoniae)	.620*	.1293	.000	.365	.875
		C (Kluyvera cryocrescens)	.650*	.1293	.000	.395	.905
		AC	.240	.1293	.065	-.015	.495
		BC	.153	.1293	.237	-.101	.408
		ABC	.207	.1445	.154	-.078	.491

		Positive Control	-.124	.1371	.366	-.394	.146
		Negative Control	.758*	.1341	.000	.494	1.022
	AC	A (Enterobacter cloacae)	.147	.1293	.258	-.108	.401
		B (Klebsiella pneumoniae)	.380*	.1293	.004	.125	.635
		C (Kluyvera cryocrescens)	.410*	.1293	.002	.155	.665
		AB	-.240	.1293	.065	-.495	.015
		BC	-.087	.1293	.503	-.341	.168
		ABC	-.033	.1445	.818	-.318	.251
		Positive Control	-.364*	.1371	.008	-.634	-.094
		Negative Control	.518*	.1341	.000	.254	.782
	BC	A (Enterobacter cloacae)	.233	.1293	.072	-.021	.488
		B (Klebsiella pneumoniae)	.467*	.1293	.000	.212	.721
		C (Kluyvera cryocrescens)	.497*	.1293	.000	.242	.751
		AB	-.153	.1293	.237	-.408	.101
		AC	.087	.1293	.503	-.168	.341
		ABC	.053	.1445	.712	-.231	.338
		Positive Control	-.278*	.1371	.044	-.548	-.007
		Negative Control	.605*	.1341	.000	.341	.869
	ABC	A (Enterobacter cloacae)	.180	.1445	.214	-.105	.465

		B (Klebsiella pneumoniae)	.413*	.1445	.005	.129	.698
		C (Kluyvera cryocrescens)	.443*	.1445	.002	.159	.728
		AB	-.207	.1445	.154	-.491	.078
		AC	.033	.1445	.818	-.251	.318
		BC	-.053	.1445	.712	-.338	.231
		Positive Control	-.331*	.1516	.030	-.629	-.032
		Negative Control	.552*	.1489	.000	.258	.845
	Positive Control	A (Enterobacter cloacae)	.511*	.1371	.000	.241	.781
		B (Klebsiella pneumoniae)	.744*	.1371	.000	.474	1.014
		C (Kluyvera cryocrescens)	.774*	.1371	.000	.504	1.044
		AB	.124	.1371	.366	-.146	.394
		AC	.364*	.1371	.008	.094	.634
		BC	.278*	.1371	.044	.007	.548
		ABC	.331*	.1516	.030	.032	.629
		Negative Control	.882*	.1417	.000	.603	1.162
	Negative Control	A (Enterobacter cloacae)	-.372*	.1341	.006	-.636	-.107
		B (Klebsiella pneumoniae)	-.138	.1341	.304	-.402	.126
		C (Kluyvera cryocrescens)	-.108	.1341	.421	-.372	.156
		AB	-.758*	.1341	.000	-1.022	-.494

	AC	-.518*	.1341	.000	-.782	-.254
	BC	-.605*	.1341	.000	-.869	-.341
	ABC	-.552*	.1489	.000	-.845	-.258
	Positive Control	-.882*	.1417	.000	-1.162	-.603

Based on observed means.

The error term is Mean Square(Error) = .251.

*. The mean difference is significant at the .05 level.

IAA readings One Way ANOVA

Descriptives

Replicates

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Isolate 1	3	.157167	.0512334	.0295796	.029896	.284438	.1186	.2153
Isolate 2	3	.292100	.0229432	.0132463	.235106	.349094	.2659	.3086
Isolate 3	3	.274667	.0072501	.0041858	.256657	.292677	.2674	.2819
Isolate 4	3	.269100	.0617164	.0356320	.115788	.422412	.2125	.3349
Isolate 5	3	.238733	.0081144	.0046848	.218576	.258891	.2329	.2480
Isolate 6	3	.274067	.0688806	.0397683	.102958	.445176	.1963	.3274
Isolate 7	3	.228500	.0173491	.0100165	.185403	.271597	.2092	.2428
Isolate 8	3	.221033	.0166533	.0096148	.179664	.262402	.2077	.2397

Isolate 9	3	.284033	.0474226	.0273795	.166229	.401838	.2295	.3156
Isolate 10	3	.199600	.0298878	.0172557	.125355	.273845	.1800	.2340
Isolate 11	3	.152300	.0130104	.0075115	.119980	.184620	.1390	.1650
Isolate 12	3	.192233	.0224068	.0129366	.136572	.247895	.1680	.2122
Isolate 13	3	.171667	.0257016	.0148388	.107820	.235513	.1440	.1948
Isolate 14	3	.185900	.0185548	.0107126	.139807	.231993	.1653	.2013
Isolate 15	3	.213467	.0373558	.0215674	.120670	.306264	.1916	.2566
Isolate 16	3	.214833	.0128165	.0073996	.182995	.246671	.2066	.2296
Isolate 17	3	.257900	.0108056	.0062386	.231058	.284742	.2473	.2689
Isolate 18	3	.231833	.0070010	.0040420	.214442	.249225	.2249	.2389
Isolate 19	3	.223233	.0728654	.0420689	.042226	.404241	.1737	.3069
Isolate 20	3	.226633	.0274201	.0158310	.158518	.294749	.2017	.2560
Total	60	.225450	.0499356	.0064467	.212550	.238350	.1186	.3349

ANOVA

Replicates

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.097	19	.005	4.088	.000
Within Groups	.050	40	.001		
Total	.147	59			