



**Characterisation, metabolite production and
biological activities of endophytes from
Artemisia afra and *Lessertia frutescens***

S Tsipinana

 **orcid.org/ 0000-0003-1522-7112**

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Promoter: Prof RA Adeleke

Co-promoter: Prof SO Amoo

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Dedication

I dedicate this work to my lovely son, Nakhane Tsipinana, who I have sacrificed spending time with for the duration of my postgraduate studies.

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ABSTRACT

Medicinal plants remain a valuable source for natural drug bioprospecting owing to their multi-target spectrum. However, their use as raw materials for novel drug synthesis has been greatly limited by unsustainable harvesting leading to decimation of their wild populations coupled with inherent low concentrations of constituent secondary metabolites. Thus, adding value to the medicinal plants research dynamics calls for adequate attention. Medicinal plant cultivation is considered as a gateway to high-quality products and commercialization. Consequently, stimulating metabolite production in medicinal plants by using endophytes as plant growth promoters has been demonstrated to be one of the most effective methods for increasing metabolite syntheses. Thus, this study aimed to isolate endophytes from *Artemisia afra* Jacq. ex Willd. var. *afra* and *Lessertia frutescens* (L.) Goldblatt & J.C. Manning subsp. *Frutescens* and determine their biological activities such as their ability to promote plant growth and enhance plant metabolite production. In order to achieve this, four objectives were formulated. Objective 1 was to evaluate metabolite variation of *Artemisia afra* (*A. Afra*) and *Lessertia frutescens* (*L. frutescens*) and determine if endophytes have an impact on the production of metabolites in these two plants. Objective 2 was to isolate, identify and characterise bacterial endophytes from *A. afra* and *L. frutescens* using both culture-based and DNA barcoding approaches. Objective 3 was to determine plant growth-promoting attributes of the endophytes isolated from *A. afra* and *L. frutescens*. Objective 4 was to examine if endophytes can accelerate the growth of *A. afra* and *L. frutescens* and enhance their metabolite production in a pot trial.

Using nuclear magnetic resonance (NMR) and 16S rDNA gene sequencing, metabolite profiles and endophytic bacterial communities of *Artemisia afra* sourced from two different environments where two cultivation methods (seed cultivation and vegetative cutting cultivation method) were used. The NMR analysis revealed that more metabolites were recovered in *A. afra* samples collected from Pretoria where vegetative cutting cultivation method was used compared to samples collected from Klerksdorp where seed cultivation was used. Additionally, plant parts and cultivation method also had an effect in bacterial endophytes' abundance and community structure. The most dominant phyla were Proteobacteria, Actinobacteria, and Firmicutes. Redundancy analysis (RDA) demonstrated a positive correlation between bacterial endophytes and metabolites from *A. afra* leaves and stems, indicating a close relationship between

metabolites and endophytes. On the other hand, the metabolite profiles and endophytic bacterial communities of *L. frutescens* plant organs were determined and there was variability in chemical profiles as well as the endophytic microbial communities across different plant organs in *L. frutescens*. On leaves, metabolites including n-acetylglucosamine positively correlated with bacterial endophytes such as *Arthrobacter*, *Xanthomonas*, *Pseudomonas* and *Kineococcus*.

Bacterial endophytes have plant growth promoting attributes that presents significant potential for sustainable agriculture. *In-vitro* capabilities of bacterial endophytes isolated from *L. frutescens* for plant growth promotion and their effect on initial seedling growth was evaluated. Using a culture-dependent approach, plant samples of *L. frutescens* were screened for bacterial endophytes. The isolated bacterial endophytes were subsequently evaluated for their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes. The obtained results revealed that many of the bacterial endophytes had potential to promote plant growth. Specifically, 86% of the endophytes possessed nitrogen-fixing, phosphate solubilizing, and Indole Acetic acid (IAA)-producing abilities, while approximately 71% were able to exhibit siderophore-producing capabilities. The endophytes exhibited significant production of essential hydrolytic enzymes, including amylase (86%), gelatinase (86%), protease (29%), lipase (43%), and D-nase (57%). The two best isolates were chosen and identified as *Bacillus licheniformis* and *Bacillus velezensis* and their plant growth-promoting properties such as their ability to enhance plant height and their ability to be used as bio-agent were further confirmed in the pot trial study as they enhanced the growth of *L. frutescens* seedlings compared to the control. On the other hand, endophytes from *Artemisia afra* sourced from two different environments under different cultivation methods were also assessed for plant growth promotion and enzyme activities. Data obtained in this study revealed that 64% of the endophytes fixed biological N, 79% solubilised insoluble tricalcium phosphate, 86% produced IAA, and 79% produced siderophores. The endophytes also demonstrated a substantial ability to produce essential extracellular enzymes (protease (57%), gelatinase (64%), d-nase (43%), amylase (57%) and lipase (36%).

The notable role of endophytes in promoting host plant growth stems from their ability to enhance nutrient acquisition, induce stress tolerance, and stimulate the biosynthesis of bioactive compounds. To evaluate the effect of *Bacillus* endophytes on *A. afra* and *L. frutescens* growth and metabolite production, a pot trial experiment was conducted, and NMR analysis was used to assess the metabolites from pure bacterial endophytes and these medicinal plants leaves after harvesting. In a pot trial experiment, isolated endophytic *Bacillus* species promoted *A. afra* and

L. frutescens growths as evidenced in a significant increase in plant height, wet and dry total, and shoot and root biomass. These medicinal plants' nutrient composition was likewise impacted by the endophytes, with a greater impact seen on macronutrients. Exploring interactions between plants and microbes presents significant potential for sustainable agriculture, providing innovative approaches to enhance crop yield while minimizing dependence on chemical interventions. These *Bacillus* endophytes also increased plant metabolite production, as more diverse metabolites were obtained from inoculated plants compared to the controls. Pure *Bacillus* endophytes *L. frutescens* and *A. afra* produced more diverse metabolites than inoculated. Endophyte inoculation presents a promising avenue for enhancing the productivity and health of medicinal plants while contributing to sustainable agricultural practices. Ongoing research and innovation in this field are essential for unlocking the full potential of endophytes in both agriculture and medicine, offering a pathway toward more resilient and effective cultivation methods.

Keywords: *Artemisia afra*, *Lessertia frutescens*, endophytes, metabolite production, plant growth-promoting, pot trial.

1. CHAPTER ONE: Introduction

1.1. Introduction

Health problems have plagued humans since the dawn of time (Takahashi et al., 2020). Inflammation and weakened immune systems are two of the most common body responses to most illnesses (Taylor et al., 2001). This is due to the fact that disease attacks often result in tissue damage, setting off a series of intracellular signalling cascades that ultimately induce inflammation (Green, 2022; Khumalo et al., 2022). Unregulated inflammation can lead to various immunopathological illnesses (Khumalo et al., 2022). Historically, a number of medicinal plants have been used as anti-inflammatory and immunity-boosting remedies. Such medicinal plants include *Haplocarpha scaposa*, *Solanum nigrum*, *Hypoxis hemerocallidea*, *Baccharis dracunculifolia* dc, and *Carapa guianensis* (Oguntibeju, 2018; Ribeiro et al., 2018).

The earliest historical records for the usage of medicinal plants extend back to 5000 years, recorded on a Sumerian clay slab from Nagpur, where hundreds of medicinal plants were used to control a range of ailments (Bhardwaj et al., 2018; Jamshidi-Kia et al., 2017). The Egyptians and Chinese are thought to have been the first to embrace medicinal plants in providing medical treatments (Jamshidi-Kia et al., 2017). Primarily used for the management of various illnesses, including fever, diarrhoea, cramps, headaches, wounds, and inflammation, their usage has subsequently evolved with scientific engagement (Palanichamy et al., 2018).

To date, the World Health Organization (WHO) estimates that more than 80% of the world's population relies on traditional medicine for various ailments, either as supplemental medicine in industrialised countries, or primary healthcare in undeveloped and emerging countries, mainly in Africa (Gurib-Fakim, 2006; Jamshidi-Kia et al., 2017). Africa has a wide-ranging plant biodiversity, with approximately 45,000 medicinal plant species used to treat a variety of health issues (Mahomoodally, 2013). Due to medicinal plants being readily available and affordable, traditional medicine is possibly the oldest and most constant in the history of health care provision in Africa, and traditional healers are the most notable individuals prescribing traditional medication to people (Salmerón-Manzano et al., 2020; Van Wyk & Prinsloo, 2018). The following are some of Africa's most popular medicinal plants:

Aloe ferox, *Aloe vera* (L.) Burm.f., *Acacia senegal*, *Agathosma betulina*, *Harpagophytum procumbens*, *Hypoxis hemerocallidea*, *Prunus africana* and *Artemisia afra* (Gurib-Fakim, 2006; Van Wyk, 2008). Africa has hugely diverse climatic conditions coupled with unequal shares of ultraviolet rays. As to be expected, diverse medicinal plants are found in different regions with unequal distribution (Mahomoodally, 2013). In South Africa alone, there are over 3 000 medicinal plant species spread across the country, with just over thirty being commercialised (Van Wyk, 2008). To mention a few, the following are some of the native and commercial medicinal plants used in South Africa: (i) *Aloe ferox* Mill. (*Xanthorrhoeaceae*) traditionally used as a laxative; (ii) *Aloe vera* (L.), a perennial plant recognisable by its succulent nature which makes it resistant to drought; (iii) *Harpagophytum procumbens* commonly known as Devil's Claw, aiding in the treatment of gastrointestinal disorders, anorexia, blood diseases, fibrositis, boils and fever (Mahomoodally, 2013; Van Wyk & Prinsloo, 2018; Van Wyk, 2008). *Lessertia frutescens* and *Artemisia afra* are among those South African medicinal plants that are notable sources of bioactive compounds utilised in the production of pharmaceutical drugs for the treatment of diseases and infections in humans (Aboyade et al., 2014; Liu et al., 2009). Furthermore, they are also processed to meet modern specifications, and made accessible in a variety of forms, such as capsules, pills, ointments, and teas (Albrecht et al., 2012).

1.2. *Artemisia afra*: Taxonomy, Morphology and distribution

Artemisia is one of the largest plant genera in the Asteraceae family, with over 500 species ranging from woody shrubs to herbaceous perennials with a large number of essential compounds of economic significance (Guerriero et al., 2018; Hussain et al., 2017). *Artemisia* species are widely distributed across the globe, pollen fossil records suggesting that they originated in Asia's arid and semi-arid regions of Xinjiang and Qinghai Provinces in China (Vallès et al., 2011; Wang, 2004; Yunfa et al., 2011). Besides China, their vast distribution includes the United States, Canada, Russia, Western Europe, and several African countries, including Southern Africa, where species such as *A. afra* are thriving. *A. afra* is widely distributed throughout Africa and in South Africa, it is one of the country's oldest medicinal plants, with consistent usage (Liu et al., 2009).

Artemisia afra var. *Jacq. ex Willd.*, commonly known as African wormwood, belongs to the Magnoliophyta division of Magnoliopsida class, subclass: Asteridae, order Asterales, Family:

Asteraceae (Liu et al., 2009). *Artemisia afra* is a perennial woody shrub distinguishable by its green or grey leaves, depending on the stage of growth, and can grow up to 2 m tall (Haile & Jiru, 2022). It normally begins to flower in late summer and continues until autumn, depending on the occurrence of rainfall, which dictates how long the vegetative stage will last (Azmeraw et al., 2019).

1.3. Phytochemical composition of *Artemisia afra* and their medicinal activities

Asteraceae is one of the leading medicinal plant families in metabolite production (Caruso et al., 2020). This could be due to the fact that Asteraceae plants are found all over the world and can thrive in a variety of climates (Pasta et al., 2022). They store energy as inulin, which allows them to synthesise a wide range of secondary metabolites (Caruso et al., 2020). Secondary metabolites are structurally diversified low-molecular weight biochemical compounds produced by almost all living species, including medicinal plants and microorganisms, through metabolic pathways descended from primary pathways as a defence response to unfavourable conditions (Gakuubi et al., 2021; Nielsen & Nielsen, 2017). Secondary metabolites are divided into a number of functional groups which include, but are not limited to alkaloids, phenolics, flavonoids, saponins, tannins, lignans, steroids, terpenoids, anthocyanins, tetralones, cardiac glycosides, and anthraquinones (Caruso et al., 2020; Guerriero et al., 2018).

Artemisia afra, for example, is rich in phytochemical components of medicinal importance because it produces both volatile and non-volatile bioactive compounds that are used for the management of a number of ailments (Ali et al., 2017; Liu et al., 2009). Such bioactive compounds commonly produced by *A. afra*, include amongst others acacetin, α -amyrin, β -amyrin, Yomogiartemin, scopoletin, camphor, eucalyptol and flavonoids (kaempferol, tamarixetin, apigenin, genkwanin, luteolin) (Kumar et al., 2021; Sotenjwa et al., 2020). Consequently, *A. afra* has long been used to treat a variety of conditions, including inflammation, cough, colds, influenza, asthma, malaria, stomach and gynaecological issues, diabetes, and occasionally in clearing the bronchial and respiratory passageways (Janačković et al., 2019; Sotenjwa et al., 2020). In order to clear the airway and respiratory ailments, fresh *Artemisia afra* leaves are traditionally gargled with or inhaled after being boiled for clearing the airway as well as treatment of throat and respiratory ailments. Components of *A. afra*, such as camphor and eucalyptol, are among the bioactive compounds that provide such

alleviation (Dailah, 2022; Taylor et al., 2001). Of vital importance is eucalyptol, a well-known anti-inflammatory bioactive compound which is primarily found in the eucalyptus plant species, yet also present in *A. afra* (Taylor et al., 2001).

Artemisia afra has recently gained some attention in studies as an antidiabetic, anticardiovascular, and anticancer agent because of its numerous functions in regulating illness (Sunmonu & Afolayan, 2013; van Loggenberg et al., 2022). Diabetes impacts more than 10% of the world's population and causes millions of fatalities worldwide (Kumar et al., 2021). Extracts from *Artemisia afra* have been found to aid in diabetes management, with improvements in blood sugar levels, decreased insulin resistance, and enhanced glucose to glycogen conversion (Du Toit & Van der Kooy, 2019; Sunmonu & Afolayan, 2013). *Lessertia frutescens* is another well-known South African medicinal plant having anticancer properties (Street & Prinsloo, 2013).

1.4. *Lessertia frutescens*: Taxonomy, Morphology and distribution

Lessertia frutescens (L.) Goldblatt & J.C.Manning (syn. *Sutherlandia frutescens* (L.) W.T.Aiton) is a leguminous shrub belonging to the Fabaceae family and is native to southern Africa: Botswana, Lesotho, Zimbabwe, Namibia, and South Africa (Aboyade et al., 2014; Zonyane et al., 2019). Even though it is available in the coastal and inland part of South Africa, *L. frutescens* is highly distributed in the Western Cape and Karoo regions of South Africa (Street & Prinsloo, 2013). *Lessertia frutescens* is a perennial plant and can grow up to 2 m. It bears oblong-shaped flowers that range in pigmentation from orange to red (seldom white) (Korth, 2021; Van Wyk & Albrecht, 2008). *Lessertia frutescens* was first utilised as a traditional medicine by the Khoisan in the 1800s. They used it to cure a variety of ailments, including gastrointestinal issues, wound dressing, cold and flu-related sickness, as well as cancer-related symptoms (van der Walt et al., 2016). Cancer bush was adopted as the common name for *L. frutescens* due to the plant's ability to control cancer-related symptoms (Van Wyk & Albrecht, 2008). Furthermore, its multiple purpose use is associated with its ability to produce a variety of bioactive compounds (Aboyade et al., 2014).

1.5. Phytochemical composition of *Lessertia frutescens* and its medicinal activities

Lessertia frutescens is used as a complementary medicine for a variety of illnesses due to its wide range of phytochemicals (Albrecht et al., 2012). The ability of *L. frutescens* to manage diverse ailments is explained by its ability to produce a variety of bioactive compounds, free amino acids, and nonprotein amino acids. Saponins, L-canavanine, L-arginine, hexadecenoic acid, pinitol, triterpenoid, γ -sitosterol, flavonoids (sutherlandin A, sutherlandin B, sutherlandin C, and sutherlandin D) and Gamma-aminobutyric acid (GABA), are examples of popular bioactive compounds of this plant (Shaik et al., 2011b; Street & Prinsloo, 2013).

Lessertia frutescens has been reported to produce bioactive compounds and enzymes with human immunodeficiency virus (HIV) and tumour inhibitory ability (Van Wyk 2008). As a result, it is one of the main two herbal remedies recommended by health practitioners alongside *Hypoxis hemerocallidea* for management of several ailments including cancer and HIV infection (Street & Prinsloo, 2013). The anticancer property of *L. frutescens* is primarily linked to its ability to produce saponins, a group of bioactive compounds with anticancer potential that are produced by a variety of leguminous plants and are predominantly found in the leaves (Van Wyk, 2008). Furthermore, the antiviral properties of *L. frutescens*, particularly for HIV and influenza, are directly related to its ability to produce L-canavanine, which has been found to treat a range of viral infections (Ojewole, 2008). Reduction in viral load and an increase in CD4 cell counts are two of the most frequently documented favourable improvements in HIV patients following the use of *L. frutescens* (Liu et al., 2005). They were also observed to improve the appetite and mood of immune-compromised patients, especially those living with HIV. Reduced stress levels can lead to mood improvement and *L. frutescens* is also believed to have stress-relieving, antimutagenic, antioxidant, and antidiabetic properties (Sergeant, 2017). The use of *L. frutescens* as antianxiety and anti-stress agents could be linked to its ability to produce GABA, a well-known neurotransmitter that prevents imbalances in impulses between nerve cells in the brain (Savage et al., 2018; Shaik et al., 2011b). Extracts from *L. frutescens* have been observed to assist in reduction of insulin resistance (Albrecht et al., 2012). The ability of *L. frutescens* to produce Pinitol, a cyclitol, which is an antidiabetic drug, represents a significant prospect for better diabetes control as it assists in controlling glycaemic. Pinitol, has also been proven to have anti-inflammatory, anticancer, and antioxidant properties in addition to being helpful in

managing diabetes (Antonowski et al., 2019). There have not been any side effects related to the use of *L. frutescens*, and thus the lower risk associated with its use makes it one of the advocated medicinal plants for future research (Aboyade et al., 2014; Street & Prinsloo, 2013).

1.6. Limitations surrounding production of drugs from medicinal plants

There has been inconsistency on the use of natural materials such as medicinal plants in drug development (Koehn & Carter, 2005). Some of these inconsistencies are due to a range of ecological, biological, regulatory, and ethical challenges that impact safety and efficacy in contemporary healthcare. These include (i) low yields of medicinal plant metabolites which fluctuate with varying environmental conditions, (ii) limited supply of medicinal plants due to over-harvesting and extinction (iii) complex trading structure and/or collection regulations and (iv) the presence of toxic heavy metals (Shasmita et al., 2018; Verma & Shukla, 2015). Therefore, alternative approaches should be considered for the improved search for new novel drugs.

1.7. Medicinal plant production and the need for different strategies for production of metabolites

Comprehending the ever-increasing demand for medicinal plants and their products is critical for determining the activities that can be restructured for the smooth flow of medicinal plants from producers to end-users. The collection of medicinal plants from the wild is vital. However, medicinal plant propagation can impose a better quality and quantity of medicinal plants leading to value-added returns and value chain strengthening. There is currently a huge gap in the study of the value chain of medicinal plants, which can have a significant effect on wide-ranging factors relating to the sustainability and safe supply of the end product (Aziz et al., 2018). Value chain strengthening is a powerful tool for promoting NHWP-related (Nature, Health, Wealth, and Power) development goals to create opportunities for people in rural areas, mainly in underdeveloped and developing countries (Hishe et al., 2016). It is therefore essential to have a standardised production method for medicinal plants that can help to improve the quality of medicinal plants and their final products. In addition, various subdivisions of the medicinal plant value chain, such as harvesting, refining, storage and packaging can be standardised in order to satisfy quality certification and safety evaluation

requirements (Masondo & Makunga, 2019). Thus, adding value to medicinal plants may lead to the improvement of the medicinal plant market value (S.-L. Chen et al., 2016). The World Health Organisation (WHO) has estimated that the trade in medicinal plants will increase to 5 trillion US dollars by 2050, putting more pressure on their demand and possibly lead to the ultimate extinction of some of these plant species (Aslam & Ahmad, 2016; Greenwell & Rahman, 2015). On an annual basis, the demand for medicinal plants is expected to increase by 14% to 25% (Booker et al., 2012).

In order to be competitive on the global market, medicinal plant products should be more versatile, creative, imperative and effective (Dzoyem et al., 2013). To achieve this, different strategies need to be applied to improve the value chain of medicinal plants. Such strategies should be capable of (i) encouraging the sustainable use and development of natural resources, (ii) improving the health and medicinal value of these plants, (iii) fostering economic growth and alleviating poverty and, iv) empowering rural producers with minimal production risks for better quality (Riisgaard et al., 2010).

As mentioned earlier, this dependence on medicinal plants for drug production has significantly accelerated environmental deterioration and the extinction of biodiversity (S.-L. Chen et al., 2016; Uzma et al., 2019). Add to this the slow growth and low supply of medicinal plant materials in the market, and a growing need for alternative ways of producing drugs efficiently to address a shortage of drug supplies and its consequential effects, has become essential. Considering that endophytes from medicinal plants have a greater potential to fulfil these needs, they can help to prevent the overharvesting and extinction of some medicinal plants by serving as the primary source of highly targeted chemotherapeutics (Golinska et al., 2015; Uzma et al., 2019).

1.8. Therapeutic functions of endophytes from medicinal plants

Several metabolites are produced by endophytic microorganisms, including bacterial and fungal endophytes (Ek-Ramos et al., 2019). Endophytes co-exist with medicinal plants, and are able to exhibit antifungal, antiviral, antibacterial and other biological properties, similar to those of their host plant (Palanichamy et al., 2018). The planet has approximately 300,000 plant species including medicinal plants of which the majority, if not all of them, has been confirmed to contain endophytes (Santoyo et al., 2016). Endophytes colonise plants through their seed, vegetative part, or from the phyllosphere and rhizosphere (Ogbe et al., 2020).

Heinrich Anton de Bary coined the term endophyte (endo=inside, phyton=plant) in the 1800s to designate any microbes that live within plant tissues (Gakuubi et al., 2021; Hyde & Soyong, 2008). Endophytes were later identified as facultative or obligatory symbiotic microorganisms, mainly bacterial and fungal species, that live in healthy internal tissues of plants without causing apparent diseases (Schulz & Boyle, 2006). Modern endophytic fungi studies can be traced back to the 19th century (Arora & Ramawat, 2017). However, fossil archives show that endophytes were associated with terrestrial plants more than 400 million years ago (Krings et al., 2007). Endophytes and their host plants have a mutualistic relationship through which the plant provides habitat for them, while they give plant tolerance to biotic/abiotic stimuli (Burrage & Jeon, 2021). Metabolites produced by endophytes can assist plants to withstand pathogenic invasion (Uzma et al., 2019).

Since the invention of antibiotic penicillin from the fungus *Penicillium* in the early 1900s by Alexander Fleming, the bioactive compounds from microbes have been demonstrated to be a significant source of relief against a variety of diseases as they possess anticancer, antioxidant, anti-immunological, antibacterial, antiviral, and antifungal properties, to name a few (Bhardwaj et al., 2015; Dias et al., 2012). Following the discovery of the first antibiotic medicine from *Penicillium* sp., another ground-breaking discovery of an anticancer bioactive chemical, taxol, produced by *Taxomyces andreanae*, has saved the lives of many individuals (Manganyi & Ateba, 2020; Tiwari & Bae, 2022). New advances have since then been aiming at the discovery of new novel drugs from microorganisms, including endophytes (Manganyi & Ateba, 2020).

Endophytes possess distinctive characteristics and a wide range of abilities, such as the capacity to produce enzymes that can catalyse a variety of chemical processes that cannot be replicated synthetically in the lab by the use of chemical formulations (Uzma et al., 2019). Endophytes, particularly fungi, also have exceptional biotransformation ability. Thus, they play an important role in drug development in the biotransformation of compounds to less toxic variants, without compromising their biological and pharmacological properties (Suryanarayanan et al., 2012). Furthermore, their ability to be genetically and physiochemically modified to meet the standards needed for a greater and more abundant synthesis of secondary metabolites is another aspect of their distinctiveness (Thomford et al., 2018; Uzma et al., 2019).

Natural bioactive compounds from endophytes are regarded as the most versatile since they are renewable, less resistant, and have a wide range of structural diversity. They are also toxic free, making them a safer and cheaper option for drug development (Manganyi & Ateba, 2020; Uzma et al., 2019). It is not surprising that endophytes produce less toxic or non-toxic bioactive compounds, as they live in symbiosis with plants without causing any apparent diseases. This indicates that endophytes have low or no cell toxicity, otherwise, tissue damage would ensue (Kumar et al., 2014). Moreover, biosynthesis of novel metabolites by endophytes remains a greater alternative because plants are constantly invaded by various types of endophytes from time to time, pointing to the possibility of new strains with new bioactive compounds being found (Dias et al., 2012; Tiwari & Bae, 2022). However, some gene clusters that are responsible for metabolite production are mostly silent under laboratory conditions (Tiwari & Bae, 2022). Nonetheless, diverse techniques, including co-culturing, mutagenesis, and genetic engineering, have been demonstrated as capable of activating silent gene clusters (Sinha et al., 2019; Thomford et al., 2018). In addition, it is essential to employ various methods, such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometers that can aid in the discovery of new compounds, rather than limiting one's attention to molecules that are already recognised to have applications. The adventure of employing high throughput screening of metabolites from microbes has increased the importance of endophytes in drug discovery (Dias et al., 2012; Tiwari & Bae, 2022).

1.9. Use of endophytes for improvement of human health and food security

Good food and proper diet have been recognised as one of the tactics that can be utilised to delay the development of various non-transmissible chronic diseases (NTCD) such as diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer (Espín et al., 2007; Takahashi et al., 2020). Thus, the combination of nutraceutical and pharmacological therapy may yield desirable effects in terms of improving human health (Takahashi et al., 2020). Nutraceuticals, often known as pharma-foods, are foods that have the ability to prevent, delay, manage, or heal certain health conditions (Daliu et al., 2019). In addition to its nutritional value of supplying the body with essential nutrients, nutraceuticals have medicinal benefits as they assist in managing certain diseases that are not yet curable by reducing the intensity and spread of sickness (Nazir et al., 2019). As a result, they can be employed as a prevention method by lowering the risk of pathological long-term disorders (Daliu et al., 2019).

The presence of non-nutrient bioactive compounds such as terpenoids with high antimicrobial activity in plant-derived meals is linked to their capacity to reduce the onset or severity of chronic illness (Espín et al., 2007). Consequently, a variety of plant-derived nutraceuticals have been proposed to include immune-modulating agents, sometimes better tolerated than certain pharmaceutical therapies (Di Sotto et al., 2020). Immunomodulators strengthen the immune system by boosting the body's response to cell or tissue damage or by reducing aberrant immunological responses to specific attacks (Di Sotto et al., 2020).

Despite the fact that plant-derived foods contain less bioactive molecules with biological activity than pharmaceutical medications, their long-term use may have significant health benefits (Espín et al., 2007). If plants contain extremely low quantities of pharmacological bioactive components, enhancement strategies could then be applied.

Furthermore, because they have fermentation ability, several microbes, primarily fungi, have been employed in the food industry for the production of nutraceuticals (Strobel et al., 2004; Takahashi et al., 2020). Among these fungi are *Penicillium*, *Aspergillus*, and *Fusarium* species (Takahashi et al., 2020). Endophytes with high productivity suitable for industrial fermentation are regarded as the best for large-scale metabolite production due to their positive resistance potential under various pressures (Banyal et al., 2021). In addition, changing to the agricultural cropping system that allows pre-placement of synthetic foods with pharma food which are naturally produced could reduce the load on healthcare systems worldwide (Daliu et al., 2019).

1.10. Endophytes in agricultural industry: Plant growth-promoting attributes

Although agriculture is the global largest economic sector, crop production suffers a great loss due to abiotic and biotic stresses, leading to the loss of yield and crop productivity (Alvin et al., 2014). Resistance of pests and diseases to currently available insecticides and herbicides has exacerbated the situation. Consequently, many synthetic agrochemicals are scheduled to be phased out because of their severe environmental impact (Strobel et al., 2004). In addition, over-dependence on chemical fertilisers is one of the major problems faced by agricultural sectors as it leads to soil degradation (Kumar et al., 2019). Soil infertility and climate change are also two of the major challenges faced by the agriculture industry, mainly in developing and underdeveloped countries (Mabrouk et al., 2018). This has put more pressure on crop production in addition to the demands of an ever-increasing

human population (Alvin et al., 2014). The use of rapid, ideal, economic and reproducible sustainable strategies that enable increased agricultural productivity for better crop production are more needed than before (Alvin et al., 2014; Choi & Shin, 2023). For instance, regarding the issues with overly dependence on chemical compounds, there are fascinating possibilities for replacing the usage of synthetic agrochemicals with the exploitation of endophytes. Antimicrobial compounds from endophytes can help to control human pathogens as well as plant pathogens (Kumar et al., 2014). It has, therefore, been suggested that some of the functional bioactive substances produced by endophytes can be used in the production of agrochemicals (Tiwari & Bae, 2022).

Additionally, leguminous plants play a vital role in climate change mitigation as their use can result in reduced application of nitrogen-based fertilisers which are known to have a negative impact on climate (Stagnari et al., 2017). Due to its cost-effectiveness and environmental friendliness, the use of leguminous plants which helps with biological nitrogen fixation has been deemed necessary to supplement chemical fertilisers in crop production (Hossain et al., 2016). *Lessertia frutescens*, just like any other medicinal plant, harbours a variety of endophytic microbes with different essential functions. As mentioned earlier, *L. frutescens* is a leguminous plant, and thus forms an association with *Rhizobia* microbes that help increase plant growth through biological nitrogen fixation (Mabrouk et al., 2018). Furthermore, endophytes actively stimulate plant growth directly through phosphate solubilisation, indole acetic acid and siderophore production (Ghasemnezhad et al., 2021). Siderophores' principal function is to acquire insoluble iron from iron rich environments and convert it to soluble iron complexes, making it accessible for plant usage (Roskova et al., 2022). Siderophores are crucial in sustainable agriculture and crop production because they impart ecological fitness to plants under abiotic and biotic stress conditions, primarily by safeguarding plant resistance to diseases, insects, pests/nematodes, and assisting with iron acquisition (Dhuldhaj & Pandya, 2021). Endophytes further provide protection for plants against pests and diseases and heavy metal deterioration, as these collectively, when not well taken care of, can affect plant health and result in stunted plant growth (Uzma et al., 2019). Thus, endophytes have been identified as natural resources that promote plant growth (Choi & Shin, 2023) and adoption of plant growth-promoting endophytes as biofertilizers could be one of the most advantageous and cost effective strategies for combating issues related to climate change and food security (Arora & Ramawat, 2017). Biofertilizers serve as a better alternative for nourishment as they are renewable sources of nutrients (Fadiji & Babalola, 2020).

Despite all the aforementioned advantages, endophytes with medicinal potential are thought to be underutilised in terms of discovering new potential bioactive compounds and agrochemicals. More research on the formulation, development of novel technologies and methodologies is needed for employing these endophytes in the agricultural and medical sectors in view of the drastically increased (Shasmita et al., 2018). Due to their untapped niches, endophytes have excellent prospects as biological repositories of bioactive compounds, with the potential to create new paths for the development of novel drugs and agrochemicals.

1.11. Aim

This study aimed to isolate endophytes from *Artemisia afra* and *Lessertia frutescens* and determine their biological activities such as their ability to promote plant growth and enhance plant metabolite production.

1.12. Objectives

1. To evaluate metabolite variation from metabolite profiles of *Artemisia afra* and *Lessertia frutescens* and determine if the associated bacterial endophytes of *A. afra* and *L. frutescens* impact on the production of the metabolites by these plants;
2. To isolate, identify and characterise bacterial endophytes from *A. afra* and *L. frutescens* medicinal plants using both culture-based and DNA-barcoding approaches;
3. To screen isolated bacterial endophytes obtained from *A. afra* and *L. frutescens* for their plant growth-promoting attributes;
4. To evaluate the potential of selected endophytic bacterial isolates on the growth and metabolite production of *A. afra* and *L. frutescens* in a greenhouse pot trial experiment.

1.13. Hypothesis

Endophytes promote *Artemisia afra* and *Lessertia frutescens* medicinal plant growth and enhance their metabolite production.

2. CHAPTER TWO: LITERATURE REVIEW

This chapter reviews research on the historical applications of medicinal plants, the synthesis of secondary metabolites from medicinal plants, and valuable attributes of plant-based natural drugs. Limitations surrounding the use of medicinal plants for drug production has been discussed and the possible solution revolving the intervention of endophytes which are harboured by these medicinal plants to improve those limitations. Limitations such as extinction of medicinal plants, their contamination by heavy metals, vulnerability to environmental factors and how endophytes can help in red-tapping those limitations. This chapter also covers, endophytes as an important source of bioactive compounds and also means of improving secondary metabolite production by endophytes such as strain improvement, mutagenesis, genetic engineering, molecular breeding techniques, co-culturing and medium optimization. Limitations of secondary metabolites production by the endophytes and also prospects of the endophytes-medicinal plants secondary metabolite production were covered to end this chapter. This chapter was published in *Frontiers in Plant Science*. Tsipinana, S., Hussein, S., Alayande, K.A., Raslan, M., Amoo, S. and Adeleke, R., 2023. Contribution of endophytes towards improving plant bioactive metabolites: a rescue option against red-taping of medicinal plants. *Frontiers in Plant Science*, 14, p.1248319. <https://doi.org/10.3389/fpls.2023.1248319>.

ABSTRACT

Medicinal plants remain a valuable source for natural drug bioprospecting owing to their multi-target spectrum. However, their use as raw materials for novel drug synthesis has been greatly limited by unsustainable harvesting leading to decimation of their wild populations coupled with inherent low concentrations of constituent secondary metabolites per unit mass. Thus, adding value to the medicinal plants research dynamics calls for adequate attention. In light of this, medicinal plants harbour endophytes which are believed to be contributing towards the host plant survival and bioactive metabolites through series of physiological interference. Stimulating secondary metabolite production in medicinal plants by using endophytes as plant growth regulators has been demonstrated to be one of the most effective methods for increasing metabolite syntheses. Use of endophytes as plant growth promoters

could help to ensure continuous supply of medicinal plants and mitigate issues with fear of extinction. Endophytes minimize heavy metal toxicity in medicinal plants. It has been hypothesized that when medicinal plants are exposed to harsh conditions, associated endophytes are the primary signalling channels that induce defensive reactions. Endophytes go through different biochemical processes which lead to activation of defence mechanisms in the host plants. Thus, through signal transduction pathways, endophytic microorganisms influence genes involved in the generation of secondary metabolites by plant cells. Additionally, elucidating the role of gene clusters in production of secondary metabolites could expose factors associated with low secondary metabolites by medicinal plants. Promising endophyte strains can be manipulated for enhanced production of metabolites, hence, better probability of novel bioactive metabolites through strain improvement, mutagenesis, co-cultivation, and media adjustment.



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EDITED BY
Sergio de los Santos Vilalobos,
Instituto Tecnológico de Sonora (ITSON),
Mexico

REVIEWED BY
Rahul Jain,
University of Amsterdam, Netherlands
Sharada Mallubhotla,
Shri Mata Valishno Devi University, India

*CORRESPONDENCE
Rasheed Adeleke
✉ Rasheed.Adeleke@nwu.ac.za

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Contribution of endophytes towards improving plant bioactive metabolites: a rescue option against red-taping of medicinal plants

Sinawo Tshipinana¹, Samah Hussein², Kazeem A. Alayande¹,
Mai Raslan², Stephen Amoo^{1,3} and Rasheed Adeleke^{1*}

¹Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa, ²Department of Biotechnology and Life Sciences, Faculty of Postgraduate Studies for Advanced Sciences, Beni-Suef University, Beni-Suef, Egypt, ³Agricultural Research Council – Vegetables, Industrial and Medicinal Plants, Roodeplaat, Pretoria, South Africa

Medicinal plants remain a valuable source for natural drug bioprospecting owing to their multi-target spectrum. However, their use as raw materials for novel drug synthesis has been greatly limited by unsustainable harvesting leading to decimation of their wild populations coupled with inherent low concentrations of constituent secondary metabolites per unit mass. Thus, adding value to the medicinal plants research dynamics calls for adequate attention. In light of this, medicinal plants harbour endophytes which are believed to be contributing towards the host plant survival and bioactive metabolites through series of physiological interference. Stimulating secondary metabolite production in medicinal plants by using endophytes as plant growth regulators has been demonstrated to be one of the most effective methods for increasing metabolite syntheses. Use of endophytes as plant growth promoters could help to ensure continuous supply of medicinal plants, and mitigate issues with fear of extinction. Endophytes minimize heavy metal toxicity in medicinal plants. It has been hypothesized that when medicinal plants are exposed to harsh conditions, associated endophytes are the primary signalling channels that induce defensive reactions. Endophytes go through different biochemical processes which lead to activation of defence mechanisms in the host plants. Thus, through signal transduction pathways, endophytic microorganisms influence genes involved in the generation of secondary metabolites by plant cells. Additionally, elucidating the role of gene clusters in production of secondary metabolites could expose factors associated with low secondary metabolites by medicinal plants. Promising endophyte strains can be manipulated for enhanced production of metabolites, hence, better probability of novel bioactive metabolites through strain improvement, mutagenesis, co-cultivation, and media adjustment.

KEYWORDS

over-harvesting, secondary metabolites, endangered species, heavy metal contaminant, co-cultivation

2.1. INTRODUCTION

Several clinical conditions, ranging from acute to chronic, have long plagued mankind since the dawn of time (Takahashi et al., 2020). Traditionally, medicinal plants have been used as remedies to sustain human health and well-being (Palanichamy et al., 2018). Many of such plants are essential sources and reservoirs of bioactive metabolites used in production of pharmaceuticals for therapeutic purposes. However, several of these medicinal plants have been red taped and classified as threatened species by different conservative authorities (S.-L. Chen et al., 2016) due to unabated demand, aggressive wild harvesting, habitat degradation, and ultimate fear of extinction. Mass production of bioactive compounds from medicinal plants often requires a high volume of biomass. The quality and quantity of naturally synthesized bioactive compounds fluctuate with varying environmental growth conditions, compounded by the global climate change (Shasmita et al., 2018; Venugopalan & Srivastava, 2015). Thus, there is a growing need for sustainable alternative templates or sources for efficient production of drugs to meet the ever-increasing demands (Asmita & Sashirekha, 2016). Specifically, different strategies have been explored on improving quality and quantity of bioactive secondary metabolites associated with medicinal plants (Bourgaud et al., 2001). Moreover, the global challenge of antibiotic resistance coupled with recurrent, new and re-emerging life-threatening infectious diseases necessitates dedicated search for novel therapeutic and prophylactic agents (Golinska et al., 2015). A deliberate attention has therefore been focused on the metabolites produced by varieties of bacterial and fungal endophytes (Ek-Ramos et al., 2019). The endophytes co-exist with medicinal plants and as well exhibit pharmacological activities similar to those of the host plants (Palanichamy et al., 2018). This positions endophytes as potential resources for bioactive natural products which could eventually play a significant role in the discovery of new drugs (Manandhar et al., 2019). They are known for their unique ability to influence the quality of bioactive secondary metabolites produced by their hosts, hence, a better understanding of the interactions between medicinal plants and their symbiont endophytes is paramount (Jia et al., 2016). Furthermore, the complex nature of secondary metabolite production pathways could be a limiting factor for diversity of natural products (Thomford et al., 2018). Advanced technologies can enable the continuous manufacturing of high-valued bioactive compounds with short production cycles (Marchev et al., 2020). Sensitivity of new analytical instrument for compound identification, elucidation and quantification has greatly increased. This allows for study of

important bioactive compounds even at very low concentrations and boosting the likelihood of discovering new compounds that could have escaped detection due to limitations from the previous analytical techniques. Slow-growing trees and shrubs produce most of the high-valued and often demanded plant-derived pharmaceuticals, but at very low rate both in the *in vivo* field environments and *in vitro* tissue culture set-ups. Therefore, application of modern biotechnology would definitively play a critical role in the production of therapeutic plant-based metabolites at industrial and commercial scale, while ensuring protection of nature reserves (Isah et al., 2018). Synthesis of diverse varieties of phytochemicals have been observed in undifferentiated plant tissue cultures under controlled laboratory condition while induced with elicitors or feeding precursors. However, this has not been without limitations. For instance, introduction of the genes that are involved in biosynthesis of artemisinin in the heterologous hosts ended up in a very low yield (Guerriero et al., 2018). Secondary metabolite biosynthesis involves a conglomerate of genes, enzymes, and metabolic pathways, most of which are not well understood due to their complexity (Castillo et al., 2013). For instance, genes are sometimes not expressed in a functional state which makes it impossible to determine how much of a role they play in biosynthesis of secondary metabolites (Kroymann, 2011). In addition, gene-metabolites networks remain unclear because most functional genes in medicinal plants remain unknown and this constitutes a major constraint in the upscaling of metabolite production (Yang et al., 2014). Consequently, many metabolic pathways are not discovered due to the unknown functional genomic (Yang et al., 2014). Currently there are only a few assembled genomes of medicinal plants that address the function of genes involved in the synthesis of secondary metabolites (Chakraborty et al., 2018). In addition, plant cytochrome P450s enzymes, for example, are one of the keystone enzymes for the biosynthesis of secondary metabolites such as terpenoids, glucosinolates, phenylpropanoids, and alkaloids, yet a number of their catalytic functions remains unknown (Giddings et al., 2011). Plants contain a large number of P450 genes, and identifying a P450 that catalyzes a specific metabolic transformation in plants remains difficult due to P450 gene similarities and unknown catalytic activities (Nguyen & Dang, 2021). *Catharanthus roseus*, for example, is one of the sources of anti-cancer drugs, and produces compounds such as the alkaloid tabersonine. However, only 5 *Catharanthus roseus* plant P450s (cinnamate 4-hydroxylase, flavonoid 3', 5'-hydroxylase, secologanin synthase, geraniol 10-hydroxylase, and tabersonine 16-hydroxylase) have been successfully characterized for the biosynthesis of plant secondary metabolites such as alkaloids (Chakraborty et al., 2018; Giddings et al., 2011). This review, therefore, highlights importance of medicinal plants in folklore remedy

and how modern techniques can be employed to improve their biomass and quality of bioactive secondary metabolites they produce through exploring and enhancing contributions of the symbiont endophytes in support of the host plants. This is with a view to reducing threat of extinction due to over-harvesting of the medicinal plants, thereby supporting genuine conservation efforts for sustainable planet.

2.2. Medicinal plants in traditional healing systems

Medicinal plants contain several compounds of therapeutic and prophylactic importance; hence, man have relied on them traditionally in the management and prevention of different ailments ranging from mild to more serious medical conditions (Hussein & El-Anssary, 2019). Most of these plants are limited in distribution and characterized with localized indigenous knowledge associated with their applications. In terms of abundance, China and India are the top two nations with the most medicinal plants, followed by Colombia, United States and then South Africa (S.-L. Chen et al., 2016). A growing number of people in developing countries rely on medicinal plants for their primary healthcare (Jain et al., 2019). The historic records on the use of medicinal plants for the treatment of ailments dated back to ~5000 years ago on a Sumerian clay slab from Nagpur (Bernardini et al., 2017; Süntar, 2020). It contained information on over 100 plant-derived pharmaceuticals and oils obtained from various herbal plants (Süntar, 2020; Wanjari & Wanjari, 2019). These medicinal plants are mostly used in their raw state, either by infusion or decoction (Süntar, 2020). Studies on the extraction and identification of chemical components from these therapeutic plants dated back to 1800s, and morphine was the first biochemical compound to be extracted from plants (Bernardini et al., 2017; Süntar, 2020). In addition, extracts from medicinal plants have proven to be the backbone for modern drug syntheses while their bioactive secondary metabolites are primarily responsible for this effectiveness (Hussein & El-Anssary, 2019; Jain et al., 2019).

2.3. Production of secondary metabolites and their medicinal importance

All plants undergo primary metabolism, while the metabolites produced are crucial for their growth and development (Sinha et al., 2019), and serve as precursors for the synthesis of secondary metabolites (Nielsen & Nielsen, 2017). For instance, carbon assimilated during photosynthesis serves as a substrate for secondary metabolism. The slow plant development

under stress conditions often increases production of secondary metabolites as more carbon would be available for this purpose (van Wyk & Prinsloo, 2020). It has also been noted that plants containing large contents of nitrogen tends to be prime producers of nitrogenous secondary metabolites such as alkaloids (Puri et al., 2018). Most secondary metabolites in plants are produced as defence mechanism in response to unfavourable conditions (Cardoso et al., 2019). Secondary metabolites can be divided into a few classes based on their functional groups, and these include alkaloids, phenolics, flavonoids, saponins, tannins, lignins, steroids, terpenoids, anthocyanins, tetralones, cardiac glycosides, and anthraquinones (Guerriero et al., 2018). Plants and microorganisms utilise three pathways for secondary metabolite biosynthesis; shikimate, polyketide, and mevalonate (Burrasoni & Jeon, 2021; Kumar et al., 2014). Shikimate is primarily responsible for the synthesis of aromatic amino acid compounds, whereas polyketide and mevalonate pathways are mainly responsible for generation of molecules having medicinal properties (Kumar et al., 2014).

The largest group of secondary metabolites is alkaloids, and they are found in almost 20% of vascular plants (Mahajan et al., 2020). Alkaloids are hydrophilic and are usually stored in the vacuoles (L. Yang et al., 2018). They are mainly produced through decarboxylation of amino acids such as tryptophan, tyrosine, histidine, lysine and ornithine using different pathways (Hussein & El-Anssary, 2019); (Burrasoni & Jeon, 2021). They have a number of therapeutic applications including anticancer and antimalarial activities (Burrasoni & Jeon, 2021; Keshri et al., 2021). It has been discovered that there are more than 20,000 alkaloids that can be extracted from plants. They are also present in a variety of microbes, with *Bacillus* species being notable producers of alkaloids (Burrasoni & Jeon, 2021). The second largest group is phenol and are produced via shikimate pathway. They are best known as dietary metabolites with a wide range of therapeutic applications such as anti-inflammatory, anti-allergic, anti-cancer, anti-viral, antioxidant and anti-ulcer properties (Burrasoni & Jeon, 2021; Jamwal et al., 2018). Phenolic compounds also play a vital role in plant pigmentation (S.-Q. Yang et al., 2018). Terpenes, which are another prevalent group, are categorized into three main subgroups, monoterpenes, sesquiterpenes and di-terpenes based on the number of carbon isoprene units they contain (Awuchi, 2020). Terpenes are normally biosynthesised from acetyl-CoA and/or glycolytic intermediates and are lipophilic. There are two enzymatic pathways, mevalonic acid and methylerythritol 4- phosphate pathways that are involved in the production of terpenes (Hussain et al., 2012). Artemisinin is a well-known sesquiterpene metabolite produced by *Artemisia* species and it has been found to be effective in controlling

cancer, malaria, and several infectious diseases (Guerriero et al., 2018; Pandey & Pandey-Rai, 2016). Taxol is also a diterpenoid anticancer medication that is commonly used to suppress the growth of various malignancies (Yan et al., 2018), and it was first extracted from *Taxus brevifolia* (Guerriero et al., 2018). Thereafter, it was recommended that terpene compounds be included in daily human dietary as a prophylactic measure against a variety of diseases (Palanichamy et al., 2018). All these secondary metabolites have contributed significantly to the development of medications from medicinal plants.

2.4. Uniqueness and valuable attributes of plant-based natural drugs

Over the years, there has been rising global demand for natural products from medicinal plants and herbs which has contributed to their enhanced market value (Williams et al., 2013). The increased demand for medicinal plants is linked to increasing rate of communicable and non-communicable chronic diseases such as diabetes, cardiovascular, neurodegenerative, and cancer diseases (Espín et al., 2007; Takahashi et al., 2020). Pathogenic microorganisms are getting more resistant to antibiotics leading to multi-drug resistant strains becoming a global public health crisis (Mohammadi et al., 2020). Antibiotic resistance is estimated to cause more than 700,000 fatalities worldwide annually, with a prediction that the trajectory could be doubled by 2050 if proper measures are not implemented (Ye et al., 2020). That being the case, there is a rising demand for novel therapeutic agents to tackle infections, and natural products have gained popularity in combating multi-drug resistance challenges (Ye et al., 2020). Despite a global drop in malaria-related deaths in recent years, sub-Saharan African countries continue to have the highest rate of malaria-related fatalities (Okell et al., 2023). *Plasmodium falciparum* has developed resistance to several antimalarial medications, and this is prevalent mainly in Southeast Asian and sub-Saharan countries (Dadgostar, 2019). However, natural products such as artemisinin, have exhibited significant efficacy against multidrug-resistant strains of malaria parasites with minimal side effects (Gupta et al., 2020; Mohammadi et al., 2020). Drugs of natural origin have demonstrated high level of potency and cost effectiveness (Banyal et al., 2021). They constitute almost half of the available medications (Banyal et al., 2021). Despite the strong demand for natural-based drugs, the rate of discovery of novel metabolites is quite low (Gupta et al., 2020). There are more than half a million medicinal plants globally in which most of them have not been exploited for their therapeutic potentials (Anand et al., 2019; Dar et al., 2017). Most of the commercialised high-valued secondary

metabolites with minimal or no toxicity are of plant origin (Nomura et al., 2018). Attempts to identify and synthesize drugs from medicinal plants have reduced drastically (Scotti et al., 2017). Some of the main reasons for this include (i) medicinal plant population decimation leading to the extinction of some species, (ii) generally low quantity of secondary metabolites produced by medicinal plants, and (iii) access to modern and sophisticated analytical equipment for novel discovery from the crude components. Undoubtedly, there are great potentials for more drug discoveries because only ~15% of the existing medicinal plants have been explored for their potential as a source of therapeutic agents (Mathur & Hoskins, 2017). Hence, adding value to the medicinal plant research dynamic would be another way to ensure the continuous use of medicinal plants in the search for new medications. There have been a paucity of investigations into the elements that influence medicinal plant secondary metabolite production, as well as prospective tactics for increasing their production. For example, medicinal plants harbour a variety of endophytes capable of producing secondary metabolites similar to those of their host plants. Thus, new paradigms including discovery of endophyte roles in the survival and defence tactics of their host plants are essential.

2.5. Endophytes intervention towards improving limitations associated with medicinal plant use

Endophytes as symbiont microbial community in the tissue of plants, offer a number of inherent potential benefits towards sustaining the mutual association (Figure 2.1). If consciously explored, the contributions by endophytes could become manipulative templates which could help mitigate the impact of over-harvesting due to over-reliance on medicinal plants, most importantly in folklore remedy. This could reduce the fear of extinction that has prompted strict regulations (red tapping) by the conservationists.

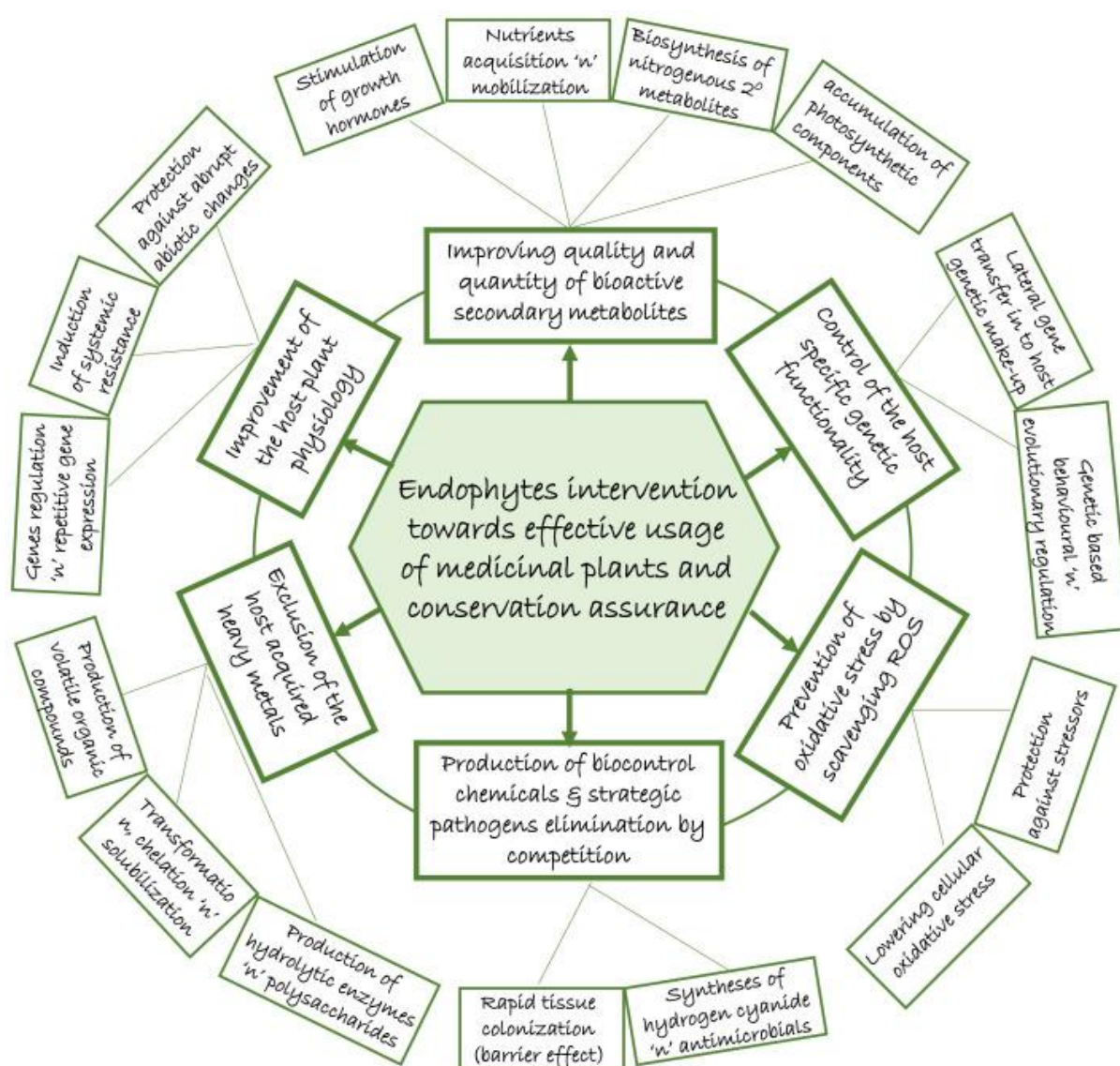


Figure 2.1: Potentials of endophytes in mitigating the over-reliant impact on medicinal plants

2.5.1. Endophytes ameliorating factors responsible for medicinal plants extinction

Flowering medicinal plants range from 50,000 – 80,000 among which about 15,000 of them are threatened by extinction as a result of over harvesting, deforestation and recurrent wildfires (S.-L. Chen et al., 2016; Llauroadó Maury et al., 2020). Transition from subsistence to commercial trading of medicinal plants has been identified as one of the primary causes of their overharvesting from the wild (Williams et al., 2013). It is also encouraged by the high demand due to growing human population, as huge quantities are required to meet the market demand. It has been projected that loss of several species of medicinal plants is at its peak with possible loss of a number of potential drugs. This threat has been known for decades, but

not much work has been done to save the plant biodiversity (S.-L. Chen et al., 2016; Shafi et al., 2021). Climate change is another factor affecting the distribution and abundance of medicinal plants with high capability to cause extinction (Gairola et al., 2010). According to reports from the Intergovernmental Panel on Climate Change (IPCC), global average temperatures have risen and this will have a significant impact on plant diversity (Anderson & Song, 2020). Each degree Celsius rise in average temperature can have a negative impact on medicinal plant distribution, as these changes are likely to increase plant mortality and extinction risk (Tangjitman et al., 2015). The impact of climate change on certain plants becomes more severe as some plants take longer to adapt to changes in environmental conditions (Akula & Ravishankar, 2011). Plant conservation is critical because it seeks to preserve plant species, especially those that are endangered, in order to ensure a steady supply of essential products while also improving plant biodiversity (Das et al., 2016; Llauroadó Maury et al., 2020). A variety of measures have been put in place with the intention of conserving nature and guaranteeing a stable supply of vital and vulnerable medicinal plants for drug production (Guerriero et al., 2018). Using *in-situ* and *ex-situ* conservation strategies are among the most effective ways to preserve medicinal plants (S.-L. Chen et al., 2016; Kadam & Pawar, 2020). Some medicinal plants are site- and environment-specific in their ability to produce significant secondary metabolites, and they might not be able to do so under controlled environments (Wani et al., 2021). In such cases, it is advised to practice *in-situ* conservation of such medicinal plants, which means preserving medicinal plants in their natural habitats (Rajpurohit & Jhang, 2015). *Ex-situ* conservation refers to the preservation of medicinal plants away from their natural habitats (Wani et al., 2021). Unfortunately, several of these techniques have shown to be insufficient for improving the quality and quantity of secondary metabolites from medicinal plants, although adequate for plant conservation. Finding techniques to improve medicinal plants bioactive chemical production is of utmost importance (Awasthi et al., 2011; Isah et al., 2018). It has been noted that a combination of compatible arbuscular mycorrhizal fungi, *Glomus mosseae* and *Bacillus subtilis* endophytic microbes increased the production of artemisinin content in the leaves of *Artemisia annua* L (Awasthi et al., 2011). In addition, endophytes are essential growth promoting agent in plants (Golinska et al., 2015). They are a group of microorganisms, mainly bacteria and fungi, that colonize the inter- and intracellular tissues of plants without causing apparent diseases (Gouda et al., 2016). Endophytic microbes directly enhance plant growth through acquisition and mobilization of essential nutrients by fixing biological nitrogen, solubilizing insoluble phosphate, and producing siderophores (Cueva-Yesquén et al., 2021; Santoyo et al., 2016).

They further stimulate production of hormones such as indole acetic acid (IAA), gibberellin, cytokinins, ethylene and abscisic acid (Ghasemnezhad et al., 2021; Yan et al., 2018). In a study conducted by (Khalil et al., 2021), fungal endophytes (*Penicillium crustosum* EP-2, *Penicillium chrysogenum* EP-3, and *Aspergillus flavus* EP-14) isolated from *Ephedra pachyclada* leaves demonstrated plant growth promoting characteristics such as IAA production. Endophytes indirectly enhance plant growth by protecting plants from biotic and abiotic stressors. For instance, siderophores produced can enhance plant adaptability and tolerance to stress conditions caused by diseases, insects, pests/nematodes while assisting with nutrient acquisition (Dhuldhaj & Pandya, 2021; X. Sun et al., 2022). Endophytes have been proven to increase the biosynthesis of nitrogen-containing secondary metabolites such as alkaloids by altering the biochemistry of medicinal plants through nutrient intake and exchanges (Chadha et al., 2015). It has been postulated that nitrogen-fixing endophytes contribute greatly into increasing production of such secondary metabolites by medicinal plants (Tör et al., 2009). (Zhang et al., 2013) investigated the ability of *Mycena* sp. to enhance *Anoectochilus formosanus* production of flavonoid and kinsenoside in a greenhouse environment. (Song et al., 2017) also noted that using *Bacillus altitudinis* KX230132.1 as an elicitor greatly boost the content of ginsenoside, a bioactive component found in ginseng. Improved plant biomass could be associated with improved production of secondary metabolites. This is premised on the fact that about 90% of plant dry matter are derived from photosynthetic carbon fixation, and organic matters build-up by photosynthesis which serves as the precursors for secondary metabolite production (Li et al., 2023). Endophytes aid in the accumulation of photosynthetic components of the plant by enhancing the growth of medicinal plants, resulting in a high biosynthesis of secondary metabolites by plants (Gupta & Chaturvedi, 2019; Z. Li et al., 2023). Moreover, several studies have reported degrees of influence the environmental and biological stressors as well as developmental factors have on the timing of flowering in plants (Jagadish et al., 2016; Kazan & Lyons, 2016). Plants often accelerate the flowering process as an indication of stress escape strategy (Bernal et al., 2011), and medicinal plants are not an exception. Synthesis of bioactive secondary metabolites which is the hallmark of the medicinal plants are often triggered in reaction to these stress factors. Interactions between endophytes and the host medicinal plants are equally believed to trigger flowering processes (Khan A. L. et al., 2017). The flowering stage of some medicinal plants is critical to the developmental state with notable impact on the synthesis of secondary metabolites, and production of secondary metabolites is expected to be at its peak (Li Y. et al., 2020). Thus, during this time, harvesting medicinal plants for

therapeutic purposes is recommended, depending on the metabolite accumulation site and the plant part(s) used for medicinal purposes.

2.5.2. Endophytes as a shield for medicinal plants against undesirable environmental impacts

Medicinal plants produce low metabolites, which is usually about less than 1% of plant dry weight depending on plant's physiological and developmental stage (Guerriero et al., 2018). Unfavourable environmental conditions result in reduced chances for plant survival (Kliebenstein & Osbourn, 2012). Consequently, environmental changes and/or climate change induced stress are the main determinants for medicinal plant secondary metabolite biosynthesis fluctuations (Verma & Shukla, 2015). In addition, medicinal plant prevalence differs under different climatic conditions and their ability to produce certain metabolites is largely affected by climate variables such as temperature and rainfall (Das et al., 2016; Z. Li et al., 2023). Understanding the biological controls of secondary metabolism in relation to changing climate is essential (Akula & Ravishankar, 2011). Though medicinal plants may evolve defense mechanisms to cope with high or low temperatures, such mechanisms are often insufficient, resulting in unstable production of secondary metabolites (Chadha et al., 2015; Y. Li et al., 2020). For instance, during extremely low temperature, plants channel their metabolism energy through the development of cryo-protective compounds such as sugar alcohols, soluble sugars, and low-molecular nitrogenous compounds at the expense of essential secondary metabolites (Thakur et al., 2019). On the other hand, elevated temperatures are known to promote the production of some secondary metabolites while reducing the production of others, mainly volatile ones, due to volatilization (Gairola et al., 2010). Furthermore, temperature influences some of the primary metabolic processes of plants, such as photosynthesis, respiration, transpiration, and dry matter partitioning, all of which are prerequisites for secondary metabolism (Nahar et al., 2015). The adaptation process that medicinal plants go through under various conditions also causes changes in plant morphology, anatomy and physiological processes (Li T. et al., 2020). All these have an impact on the accumulation of secondary metabolites, therefore, depending on the pathways and response genes that were engaged under various conditions, different metabolites can be biosynthesized by medicinal plants (Li T. et al., 2020). Medicinal plant associated endophytes on the other hand, are shielded from abrupt changes in the environment (Papik et al., 2020; W. Wu et al., 2021). Consequently, it has been hypothesized that when medicinal plants are

exposed to harsh conditions, the harbouring endophytes are the primary signalling channels that induce a defensive reaction (Singh et al., 2019). Endophytes go through different biochemical process which leads to the activation of their defence mechanisms including those of their host plants (Ogbe et al., 2020). These processes include the synthesis of antioxidants, phytohormones, and activation of stress-induced genes, all of which play a part in secondary metabolite synthesis (Ogbe et al., 2020). In order to trigger the response in plants, endophytic signal transduction pathway first binds extracellular signaling molecules and ligands on the cell surface or inside the cell (Jin et al., 2016). Thus, using activating signal transduction pathways, endophytic microorganisms influence genes involved in the generation of secondary metabolites by the plant cells (Z. Li et al., 2023). Moreover, they help medicinal plants flourish by lowering cellular oxidative stress brought on by biotic and abiotic stress factors (Khan A. N. et al., 2017). The ability of medicinal plants to withstand biotic and abiotic stresses might not be from the plants themselves but rather from the associated endophytic microbes (Chadha et al., 2015). Extreme climatic conditions can also result in disease, insect, and pathogen infestations, and these biotic stressors have substantial impact on the quality and quantity of secondary metabolites produced by medicinal plants. Pathogenic microbes in medicinal plants induce infection and offer a significant risk to patients (Chadha et al., 2015; Slama et al., 2021). However, controlling some plant pathogenic diseases such as vascular wilt is challenging (Yadeta and Thomma, 2013). On the other hand, the continued use of chemical pesticides to combat plant pathogens has led to the emergence of pesticide-resistant pathogenic strains (Hawkins et al., 2019). Medicinal plants activate reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are the primary plant defense responses to combat entry of both beneficial and non-beneficial microorganisms (Kapoor et al., 2019). However, endophytes are capable of degrading both the ROS and RNS through enzymatic actions in order to pave way for the infestation and colonization of their host plants (Chadha et al., 2015). Plant cell walls, including those of medicinal plants, get re-reinforced shortly after endophytic colonization, and this aids in preventing pathogenic infestation. Endophytes also shield medicinal plants from pathogenic attack through a phenomenon known as the “barrier effect,” which occurs due to their widespread and intense replication in the colonized plants, leaving little to no space for non-beneficial microorganisms (Mengistu, 2020). The use of endophytes as a biological control measure has been recognized to be most effective in controlling plant diseases and pests as endophytes have the ability to antagonize pathogens (Mohamad et al., 2018). An in-vitro experiment conducted by Mohamad et al. (2020) revealed that *Bacillus* and *Enterobacter*

species isolated from *Thymus vulgaris* were able to significantly promote growth of tomato (*Solanum lycopersicum* L.) under saline conditions (NaCl supplemented). The strains were also able to antagonise *Fusarium oxysporum*. The ability of the tomato to withstand stressful conditions was attributed to the plant's enzymatic activity which was believed to have been initiated by those endophytic strains. In addition, the ability of those endophytes to assist tomato with tolerance to stress was due to their ability to produce a variety of compounds such as nonadecane, tetracosane, cyclohexanecarboxylic acid, 2-, which are antimicrobials. Endophytes can also control pathogens through a variety of mechanisms, including (i) the synthesis of bio-control chemicals such as siderophore, hydrogen cyanide, and antibacterial components, (ii) competing for important resources, and (iii) induction of systemic resistance via activation of stress signalling hormones like jasmonate and ethylene (Kushwaha et al., 2020; Slama et al., 2021). This was evident in an experiment conducted (Karthikeyan et al., 2012) under saline conditions, where *Achromobacter xylosoxidans* endophyte was able to enhance growth, reduced level of ethylene, and increased metabolic production of antioxidant enzymes by *Catharanthus roseus*. (Yang et al., 2015) also reported induced systemic resistance by *Bacillus amyloliquefaciens* isolated from *Ginkgo biloba* which inhibits pepper phytophthora blight. In another study, hydrogen cyanide producing bacterial endophytes isolated from *Panax ginseng* belonging to *Xanthomonadaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Micrococcaceae*, and *Bacillaceae*, successfully controlled plant fungal pathogens, *Botrytis cinerea* and *Cylindrocarpum destructans* (Hong et al., 2018). Restricting access to iron by microbial pathogens appears to be a promising strategy for combating infectious diseases in plants (Mohamad et al., 2018). This is often accomplished through microbial production of siderophores. Siderophores are small iron (Fe^{3+}) chelating ligands with less than 10 kDa molecular weight and are produced by different microorganisms under iron-deficient conditions (Roskova et al., 2022). The endophytes with high affinity for iron are excellent siderophore producers because they scavenge iron from the surroundings, thus making it unavailable for the pathogens. Hydroxamate is one of the most abundant siderophore secreted by various species of fungi and bacteria (Dhuldhaj & Pandya, 2021). Certain extracellular enzymes produced by the endophytes such as dehydrogenase, β -glucanase, amylases, gelatinase, cellulases, lipases, pectinase, proteinase, and chitinases also protect plants from pathogenic attack through microbial cell wall degradation (Kushwaha et al., 2020; Slama et al., 2021). They further help in strengthening plants and endophytes cell wall boundaries which ultimately build resistance against the pathogenic microbes (Fadiji & Babalola, 2020).

2.5.3. Endophytes reducing uptake of heavy metal contaminants by the plants

Heavy metal toxicity is one of the major problems affecting the use of medicinal plants as raw materials. Heavy metals are usually transmitted from the soil to the plants, affecting the overall health of the plants and their ability to produce important secondary metabolites (Street, 2012). Their presence in non-tolerant plants often lead to a decrease in enzyme activity of the plants which is the backbone for production of secondary metabolites (Sharma & Kumar, 2021). Heavy metal toxicity causes plants to accumulate more ROS (Zheng et al., 2023). Even though ROS are produced as normal by-products of metabolism, different conditions can result to excessive production of ROS which exceeds the defense mechanisms, leading to plant oxidative stress and ultimate cell death (Sharma et al., 2012). Although ROS can result in increased secondary metabolite production, excessive ROS can have a deleterious impact on secondary metabolite production through damage to primary metabolites such as lipids, proteins and nucleic acid compounds, which are the building blocks for secondary metabolites (Sharma et al., 2012). Endophytes on the other hand, are known to aid in the elimination of ROS in plants by scavenging active oxygen systems, which are typically triggered when plants are stressed (Zheng et al., 2023). Endophytes limit the uptake of harmful heavy metals by plants and/or immobilize them through a variety of mechanisms, including transformation, chelation, solubilization, and precipitation (Durand et al., 2021; Singh et al., 2020). For instance, they use a mechanism called biosorption, which includes a number of procedures like ion exchange, electrostatic contact, precipitation, and redox reaction, to capture these dangerous heavy metals and absorb them into their cell walls (Kushwaha & Kashyap, 2021). Endophytes also protect medicinal plants from heavy metals by producing volatile organic compounds (VOCs) such as terpenoids and phenylpropanoids (Slama et al., 2021). Moreover, endophytes produce a wide range of valuable hydrolytic enzymes which can sequester various organic and inorganic compounds (Liu et al., 2021; Suryanarayanan et al., 2012). Several microbes have been reported for degrading and immobilizing hazardous heavy metals to less-toxic forms (Kushwaha & Kashyap, 2021; Priyadarshini et al., 2021). Microbial enzymes have therefore been demonstrated to have outstanding unique features that many chemical catalysts might not have. Endophytes help medicinal plants with improvement of physiological responses and immune activity (Khan A. N. et al., 2017). Endophytic 1-aminocyclopropane- 1-carboxylic acid deaminase (ACC), for example, aids plant tolerance under stress condition by counteracting ethylene inhibitory

effects while enhancing plant growth and development (Kushwaha et al., 2020; Zheng et al., 2023). Lastly, even though heavy metals can enter through other parts of the plant, the root is regarded as their main entry point. Fungi have been proven to be the most prominent in heavy metal and other toxic compounds remediation. The hyphae from fungal microbes help in amelioration of heavy metals through cell wall thickening and increased surface area, which assist in improved absorption of those toxic elements to their cell wall (Nandy et al., 2020; Sharma & Kumar, 2021). Endophytic cell walls also synthesize polysaccharides such as hydroxyl, carboxyl, and amino groups, which function as a barrier to metal ions by increasing the binding sites with positive metal compounds (Zheng et al., 2023).

2.5.4. Endophytes controlling gene clusters in the host plants for biosynthesis of novel active agents

Finding new novel bioactive compounds to treat diverse ailments is a fundamental goal of all drug discovery initiatives. To combat newly emerging and old incurable diseases, new bioactive compounds must be discovered. Despite the strong demand for natural drugs from medicinal plants, the rate of discovery of novel metabolites for treatment of diseases and infections is on decline (Gupta & Chaturvedi, 2019). In the past few years, there has been high probabilities of extracting already known therapeutic components with little to no luck in discovering new compounds for potential new drug discovery from medicinal plants (Li and Vederas, 2009; (Dias et al., 2012). The extraction of new and sophisticated metabolites is limited when using the traditional extraction methods only, hence the problems with repeated rediscovery of the same secondary metabolites predominates (Roopashree & Naik, 2019) Medicinal plants contain complex compounds often in relatively small quantities that may be difficult to extract/isolate and identify, hence their capability may be under-investigated (Thomford et al., 2018). Production of novel natural drugs requires development of innovative multidisciplinary drug discovery methods beyond traditional techniques (Li & Lou, 2018; Zhang & Elliot, 2019). For instance, the employment of high-throughput and molecular models have proven to be effective in the discovery and identification of bioactive secondary metabolites from both plants and microbes (Prasanna, 2022; Tawfike et al., 2019). Historically, identification of secondary metabolites from plants and microorganisms have been reliant on approaches such as traditional culture-based methods, biochemical screening employing Gas Chromatography-Liquid Chromatography/ Mass Spectrometry (GC/LC-MS), High-performance liquid chromatography (HPLC), and NMR (Tiwari & Bae, 2022).

However, it has been demonstrated that integrating low- and high-throughput genomic approaches has the ability to preserve the therapeutic novelty of medicinal plants and they can aid in supplying a direction for the optimization of secondary metabolite production for higher yields (Süntar, 2020). However, there is a shortage of studies on the potential use of genome mining approaches to estimate the pathways for biosynthesis of novel secondary metabolites by medicinal plants (Rivera-Chávez et al., 2022). The interaction between endophytes and plants is complex and sometimes misunderstood, but evaluating, identifying, and characterizing genes involved in their mutual association can be helpful in unravelling the underlying mechanisms between the two (Isah, 2019). Plant-endophyte symbiosis is driven by cohabitation and coevolution which ultimately leads to a stronger mutualism that becomes engraved with influence on the expression of certain traits in the genetic makeup of both partners (Mengistu, 2020). These endophytes can obtain and transfer certain genes horizontally with the host plants under specific metabolic pathways during colonisation (Gohain et al., 2020). After endophytes colonize their host plant, a number of genes regulate their behavior, resulting in changes in their genotypic features as well as that of the plants (Yan et al., 2019). Thus, there is high likelihood that some of the secondary metabolites extracted from medicinal plants are due to medicinal plants interaction with endophytes (David et al., 2015; W. Wu et al., 2021). Genomic based approach gives insight on various generic information, physiological, biological and evolutionary interactive processes of the plants (Sharma & Shrivastava, 2016). Understanding plants endophytes physiological interactions and evolutionary relatedness can also assist in elucidating the relationship between certain metabolites and their therapeutic effectiveness (Atanasov et al., 2015). This can aid in the formulation of targets that focus solely on the relevant trends of secondary metabolites produced by plants and their associated endophytes (Li & Lou, 2018).

Comparing genomic traits of closely related endophytes with different functional roles obtained from the same plant is of most importance as this can help in determining their distinctive adaptability and evolutionary trend (Kaul et al., 2016). This can also help in comprehending different gene clusters that are responsible for different production of secondary metabolites (Kaul et al., 2016; Li & Lou, 2018). Metagenomics can as well aid in the discovery of novel biocatalysts and metabolic pathways by revealing previously unknown genomes (Bosamia et al., 2020). Genomic approach would help predicting whether metabolites produced by plants and microbes are due to novel gene clusters (Atanasov et al., 2021; Sinha et al., 2019). Different genomic techniques can be used to predict the direct

biosynthetic gene clusters (BGCs) that encode the biosynthesis of secondary metabolites in microbial genomes (Keller, 2019). The archetypal cluster is the most significant as it contains the genes that are responsible for secondary metabolite synthesis (Rivera-Chávez et al., 2022). Following the identification of critical gene clusters for secondary metabolite production, it is imperative to discover limiting pathways for enhancing their biosynthesis, and thereafter eliminate them while modifying the pathways responsible for the metabolites of interest (Dutta et al., 2009). This could be through repetitive gene expression, deletion of unfavourable and competitive genes, and/or introduction of new genes required for better bioactive compound biosynthesis (Li & Lou, 2018; Pham et al., 2019). Deleting a competitive pathway helps in saving useful cellular resources for better and sufficient production of high quality secondary metabolites (Pham et al., 2019); Li T. et al., 2020). This helps in creating conditions that are conducive for the gene of interest which is responsible for the production of the desired metabolite(s) (Venugopalan & Srivastava, 2015). Synergism also can improve the possibilities of discovering novel compounds and boost therapeutic effectiveness of drugs. The possible identification of synergistic bioactive compounds under the umbrella of systems biology can usher this generation into the new era of drug discovery. It may be simple to combine and grow many endophytes and facilitate their interaction, which increases the likelihood of synthesising a variety of bioactive compounds (Sun et al., 2013). With medicinal plants, it may not be possible to combine two or more plants to produce a new bioactive compound. Nonetheless, the substances produced by the individual plants can be combined. Synergistic endophytes can then be used as biological elicitors for cultivation of medicinal plants in a controlled environment.

2.6. Metabolite yield enhancement strategies by the endophyte

In general, microorganisms are susceptible to genetic manipulation for desired performance. Different techniques have been successfully applied in the attempt to enhance secondary metabolite production by endophytes, and these include strain improvement, mutagenesis, co-cultivation, and medium optimization (Kumar et al., 2014) (Table 2.4).

2.6.1. Strain improvement

The process of strain improvement involves genetically modifying microbial strains with the aim of enhancing their capabilities for a wide range of applications. This involves interactive genetic modifications and fermentation techniques (Ademakinwa et al., 2017; Barrios-Gonzalez et al., 2003). The process would not only bring about elevated product yield, but also focus on enhancing and creating new genetic traits, such as eliminating unwanted co-metabolites, improving nutrient utilization, and modifying cellular morphology to enhance oxygen transfer during fermentation or to facilitate downstream processing (Barrios-Gonzalez et al., 2003). It also offers several advantages such as modifying product ratios, by-products removal, product excretion enhancement, increasing tolerance to high product concentrations, reduction in fermentation time, and high production of native or foreign products through genetic recombination (Li P. et al., 2012).

2.6.2. Mutagenesis

The classical technique of induced mutagenesis has been successfully applied in strains improvement and increasing production of microbial metabolites that are of commercial significance. This approach has contributed significantly to the progress made in strain improvement (El-Sayed et al., 2019). Mutagens are chemical compounds such as hydroxylamine, nitrosoguanidine, methyl methanesulfonate, ethyl methanesulfonate, nitrosoguanidine, ethyl methyl sulfonate, sodium nitrite and diethyl sulfate, as well as physical forms of radiation such as ultraviolet (UV) light or X-rays. These mutagens have the ability to cause permanent and inheritable changes (mutations) in the microbial genome (Khan et al., 2020; Ma et al., 2011). When selecting for improved mutants, there are two approaches: random selection for the desired feature or target selection based on specific characteristics that differ from the previous genotype of interest (Zhang et al., 2018).

2.7. Endophytes as an important source of bioactive compounds

Dependence on medicinal plants for production of significant bioactive compounds for the development of novel drugs has a few drawbacks, including variation in secondary metabolites produced due to seasonal, spatial, and environmental conditions (Shasmita et al., 2018; van Wyk & Prinsloo, 2020). Additionally, some medicinal plants have long generation

time to reach maturity while some are strictly seasonal and thus unavailable during the off-season (Isah, 2019; Verma & Shukla, 2015). A considerable quantity of medicinal plants is required for the production of high yield secondary metabolites, however, due to rapid changes in climate and other factors, it is becoming more difficult to have a consistent supply of medicinal plants all year long. Finding a quick, economical, and reproducible solution for producing these compounds of pharmaceutical value is of utmost importance (Asmita & Sashirekha, 2016; Choi & Shin, 2020). Novel strategies proposed for production of secondary metabolites should be more versatile, effective, and capable of promoting sustainable use of natural resources and as well support economic growth (Dzoyem et al., 2013; Riisgaard et al., 2010). Endophytes appear to be a feasible alternative for production of these key bioactive compounds. Endophytes are not only fast growing and inexhaustible, but they have also been classified as one of the sustainable natural resources since they encourage green processes (Choi & Shin, 2020). Thus, exploring them for different therapeutic properties is crucial. In addition, large-scale growth of endophytes in a fermenter under control conditions can result in an inexhaustible supply, which would ensure steady supply of the secondary metabolites. Furthermore, due to rapid development of mycelial biomass, production of bioactive compounds from fungal endophytes offers bigger benefits (Banyal et al., 2021; Parulekar Berde et al., 2020). Endophytes can produce secondary metabolites with various and distinct structures from that of their host plants, thus, suggesting a host-independent model of producing metabolites (Caruso et al., 2020; Egamberdieva et al., 2017).

Endophytes have independent capacity to synthesize cyclic peptides of great pharmaceutical value (Ludwig-Müller, 2015). For instance, endophytes use dependent non-ribosomal peptide synthesis (NRPS) and independent NRPS pathways for additional production of bioactive compounds (Dhuldhaj & Pandya, 2021; Swayambhu et al., 2021). This has highlighted the potential of endophytes as a long-term pipeline for novel drug production and exploration to combat not only infectious diseases but also non-infectious illnesses like cancer, diabetes, arthritic conditions, and cardiac issues (Toghueo, 2019). Endophytes-based pharmaceuticals are attracting attentions due to their structural diversity, multi-target therapeutic actions and low cost of productions (Alvin et al., 2014). They are often found to be significantly safe, especially in cancer treatments, with minimal side effects on normal cells (Banyal et al., 2021). Substantial efforts have been carried out on the development of bioactive compounds from endophytic microbes and their application in the management of different medical conditions (Tables 2.1, 2.2 and 2.3).

2.7.1. Fungal and bacterial endophytes

Endophytic fungi are essential sources of valuable bioactive compounds linked to most of the commonly used antibiotic and anticancer drugs, among which are vinblastine, camptothecin, hypericin, paclitaxel, diosgenin and podophyllotoxin (Gouda et al., 2016; Kumar et al., 2014). The penicilllenols, derived from *Penicillium* sp., are cytotoxic to a variety of cell lines. Taxol, derived from the endophytic fungi *Taxomyces andreanae*, is the most potent and successful anticancer medication to date. Clavatol (*Torreya mairei*), sordaricin (*Fusarium* sp.), jesterone (*Pestalotiopsis jesteri*), and javanicin (*Chloridium* sp.) are all known to have potent antibacterial and antifungal activities against a wide range of pathogens. Pestacin, derived from *Microsporum* sp. has very powerful antioxidant properties (Gouda et al., 2016; Kumar et al., 2014). *Pestalotiopsis neglecta* BAB-5510 and *Cupressus torulosa* are endophytic fungi considered to be a promising source of phenols, flavonoids, terpenoids, alkaloids, and tannins (Pimentel-Elardo et al., 2010). Capsaicin, a bioactive compound prevalent in red and chilli peppers, is a pain reliever and an anti-cancerous agent applied in the treatment of human malignancy. Capsaicin is produced by *Alternaria alternata*, an endophytic fungus identified from *Capsicum annum* (Ke Zhao et al., 2011). Moreover, actinomycetes particularly *Streptomyces* sp., have been identified as a promising source of bioactive metabolites (Golinska et al., 2015; Kai Zhao et al., 2011). To date, over 140 genera of actinomycetes have been identified, but only a small fraction of them produce the majority of known essential antibiotics (Pimentel-Elardo et al., 2010). *Streptomyces* are known to be rich in many bioactive compounds such as munumbicins (A and B), naphthomycin (A and K), clethramycin, coronamycin, cedarmycin (A and B), saadamycin, and kakadumycins. *Streptomyces rochei* CH1, an endophytic actinomycete of *Cinnamomum* sp., showed significant antibacterial activity against various pathogens like *Aeromonas caviae*, *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* (Roy & Banerjee, 2015).

Table 2.1 Anti-cancer compounds produced by endophytic microorganisms

Host Plants	Endophytes	Metabolites produced	References
<i>Capsicum annuum</i> L.	<i>Alternaria alternata</i>	Alkaloid, capsaicin	(Devari et al., 2014)
<i>Nothapodytes foetida</i>	<i>Entrophospora infrequens</i>	Camptothecin	(Puri et al., 2005)
<i>Panax ginseng</i>	<i>Burkholderia</i> sp.	Saponin: Ginsenoside Rg3	(Singh & Dubey, 2018)
<i>Taxodium distichum</i> , <i>Taxus wallichiana</i>	<i>Pestalotiopsis microspora</i>	Paclitaxel or Taxol	(Parulekar Berde et al., 2020)
<i>Panax ginseng</i>	<i>Burkholderia</i> sp.	Saponins: Ginsenoside Rg3, Ginsenoside Rg2	(Fu et al., 2017)
<i>Zingiber officinale</i> (Ginger)	<i>Streptomyces aureofaciens</i>	4-arylcoumarins	(Taechowisan et al., 2007)
<i>Cinnamomum cassia</i>	<i>Streptomyces cavourensis</i>	Bafilomycin	(Vu et al., 2018)
<i>Camptotheca acuminata</i>	<i>Trichoderma atroviridae</i>	Camptothecin	(Kumar et al., 2014)
<i>Taxus</i> sp.	<i>Fusarium chlamydosporum</i>	Kaempferol	(Chaturvedi et al., 2014)
Mangrove	<i>Fusarium</i> sp. (No. DZ27)	Beauvericin	(Tao et al., 2015)
<i>Markhamia platycalyx</i>	<i>Aspergillus flocculus</i>	4hydroxymellein, 5hydr oxymellein, diorcinol, botryoisocoumarin A	(Tawfike et al., 2019)
<i>Anvillea garcinii</i>	<i>Fusarium chlamydosporium</i>	Fusarithioamide benzamide derivative	(Ibrahim et al., 2016)
<i>Miquelia dentata</i> Bedd. (Icacinaceae)	<i>Lysinbacillus</i> sp, <i>Bacillus cereus</i>	Camptothecine	(Shweta et al., 2013)
<i>Isodon eriocalyx</i>	<i>Streptomyces</i> sp. YIM66403	Anthracyclin	(Li et al., 2015)
<i>Taxus cuspidata</i>	<i>Periconia</i> spp.	Periconicin A and B	(Kim et al., 2004)
Mangrove	<i>Streptomyces</i> sp.	Indolocarbazoles	(Xu et al., 2014)

Table 2.2: Antifungal compounds from endophytic microorganisms

Host Plants	Endophytes	Metabolites produced	References
<i>Moringa oleifera</i>	<i>Nigrospora</i> sp. LLGLM003	Mullein, Griseofulvin, 8-dihydorramulosin	(Zhao et al., 2012)
<i>Fragaria bodenii</i>	<i>Pestalotiopsis jesteri</i>	Jesterone	(Li & Strobel, 2001)
<i>Camellia sinensis</i>	<i>Pestalotiopsis fici</i>	Ficipyrone A	(Liu et al., 2013)
<i>Taxus mairei</i>	<i>Aspergillus clavatonanicus</i>	Clavatul patulin	(Zhang et al., 2008)
<i>Senna spectabilis</i>	<i>Phomopsis</i> sp.	Cytochalasin H	(Chapla et al., 2014)
<i>Ephedra fasciculata</i>	<i>Xylaria</i> sp	Sordaricin (17)	(Chen et al., 2018)
<i>Garcinia dulcis</i>	<i>Eutypella scoparia</i> PSU-D44	Scoparasin B	(Pongcharoen et al., 2006)
<i>Glycyrrhiza uralensis</i>	<i>Bacillus atrophaeus</i> , <i>Bacillus mojavenensis</i>	1,2 bezenedicarboxyl acid, Methyl ester, Decanodioic acid, bis(2-ethylhexyl) ester	Mohamad et al. (2018)

Table 2.3: Antibacterial compounds from endophytic microorganisms

Host plants	Endophytes	Metabolites produced	References
<i>Taxus cuspidata</i>	<i>Periconia</i> sp.	Periconicin A and B	(Kim et al., 2004)
<i>Quercus</i> sp.	<i>Cytonaema</i> sp.	Cytonic acids A and B	(Guo et al., 2000)
<i>Polygonum senegalense</i>	<i>Phomopsis longicolla</i>	Dicerandrol A, dicerandrol B, dicerandrol C	(Lim et al., 2010)
<i>Urospermum picroides</i>	<i>Ampelomyces</i> sp.	Altersolanol A	(Aly et al., 2008)
<i>Salicornia bigelovii</i> Torr	<i>Fusarium tricinctum</i>	Enniatin B	(Zhang et al., 2015)

<i>Ephedra fasciculata</i>	<i>Fusarium tricinctum</i>	Fusartricin	(Zhang et al., 2015)
<i>Kennedia nigriscans</i>	<i>Streptomyces</i> NRRL 30562	Munumbicins	(Castillo et al., 2002)

2.7.2. Genetic engineering

In order to enhance secondary metabolites, various genetic methods are employed in strain improvement. These include amplification of secondary metabolite gene clusters, cloning of regulatory genes, inactivation of competing pathways, disruption or amplification of regulatory genes, manipulation of secretory mechanisms, and expression of a suitable heterologous proteins. These are accomplished through application of different techniques namely; (i) transposition mutagenesis, (ii) target deletions and duplications by genetic engineering and (iii) genetic recombination by protoplast fusion (Genilloud, 2018). Recent additions to these techniques include genomics, transcriptome, proteomics, metabolic engineering analyses, and whole genome shuffling (Adrio & Demain, 2006).

2.7.3. Molecular breeding techniques

Techniques of DNA shuffling and molecular breeding involve *in vitro* homologous recombination i.e., mimicking the natural process of recombination (Gunatilaka, 2006). These methods include both the recombination of DNA fragments and the controlled introduction of point mutations at a low rate (Bode et al., 2002; Paranagama et al., 2007). Genome shuffling of similar genes from different species or strains yields significant improvements, unlike site-directed mutagenesis which is more expensive and time consuming (Patten et al., 1997). An updated approach of this method involves breeding a population with high genetic variability i.e., DNA family shuffling. By starting with four cephalosporinase genes, this method led to a significant increase in cephalosporinase activity ranging from 240 to 540 folds (Raimi & Adeleke, 2021). In contrast, when each of these genes was shuffled independently, only an eight-fold improvement was achieved. Whole genome shuffling is a new approach for strain improvement that combines the benefit of multi-parental crossover enabled by DNA shuffling with the recombination of whole

genomes. According to (Halpin et al., 2001), this technique increased the production of tylosin in *Streptomyces fradiae*.

Table 2.4: Examples of successful strain improvement strategies

Compound	Type of strain improvement strategy	Original organismis	Yield (folds)	References
6'-deoxy-bleomycin Z	UV, mutagenesis and ribosome engineering	<i>Streptomyces flavoviridis</i> G-4F12	7.00	(Zhu et al., 2018)
Vinblastine	gamma irradiation	<i>Alternaria alternata</i>	3.98	(El-Sayed, 2021)
Glycoside digoxin	Gamma irradiation	<i>Epicoccum nigrum</i>	5.00	(El-Sayed et al., 2020)
Taxol	UV irradiation, Nitrous acid Ethidium bromide	<i>Aspergillus</i> sp.	1.10	(Khan et al., 2020)
Paclitaxel	UV, gamma irradiation	<i>Aspergillus fumigatus</i> , <i>Alternaria tenuissima</i>	1.22 1.24	(El-Sayed et al., 2019)
Tanshinone IIA	UV, NaNO ₂	<i>Emericella foeniculicola</i> TR21	1.46	(Ma et al., 2011)
Tanshinone IIA	Genomic shuffling	<i>Emericellafoeniculicola</i>	11.07	(Zhang et al., 2018)
Lovastatin	Genomic shuffling	<i>Fusarium</i> sp. ALAA20	11.55	(El-Bondkly et al., 2021)

2.7.4. Co-culturing

Microbial co-cultivation, also called mixed fermentation, refers to the process of cultivating two or more microorganisms in a single confined environment. When endophytes are isolated and cultured in axenic monocultures to produce the desired product, repeated culturing may lead to the loss of access to the specific microenvironment of the host plant to which the endophyte has adapted. This loss of access may result in the silencing of the endophyte's secondary metabolite genes, and standard culture conditions may not be sufficient to activate the expression of these genes (Somjaipeng et al., 2016; Venugopalan et al., 2016). Moreover, typical cultural methods might not be sufficient to activate the hidden biosynthetic gene clusters observed in endophytes, which could lead to either redundancy in terms of

identifying new metabolites or a decrease in the amount of generated metabolites. Based on whole genome sequencing techniques, the quantity of genes encoding biosynthetic enzymes in various fungi and bacteria surpasses the number of secondary metabolites that are currently recognized in these microorganisms. Co-culturing is regarded as a highly effective ecological method that involves replicating the natural environment of the host in a laboratory setting, which includes both competition and coexistence with other endophytic communities. As shown in Table 2.5, this approach can have a positive effect on the production of particular metabolites. By fermenting a mixture of microbes, it is possible to stimulate the expression of silent biosynthetic genes that may result in the creation of novel secondary metabolites, often through the use of signaling molecules such as auto-regulators, quorum-sensing molecules and siderophores (Bertrand et al., 2014; Marmann et al., 2014). A different interpretation proposes that this phenomenon may be connected to the creation of enzymes that stimulate the production of the metabolite precursor generated by the producing strain leading to the active metabolites (Abdelmohsen et al., 2015). Alternatively, the inducing strain might prompt epigenetic changes in the producing strain. To fully comprehend these interactions between endophytes, as well as between endophytes and host plants, a thorough knowledge of their ecological relationships is necessary (Wakefield et al., 2017).

2.7.5. Medium optimization

The initial phase in carrying out a substantial metabolite synthesis is medium optimization, which remains a highly scrutinized process and has several challenges associated with it. In the past, traditional methods were used for media optimization which were costly, time-consuming, and involved numerous experimental procedures with less accuracy. However, with the advent of sophisticated mathematical and statistical techniques, such as artificial neural networks and genetic algorithms, media optimization has become more dynamic, productive, cost-effective, and dependable in yielding results (Franco-Lara et al., 2006). The production of Zofimarin, a potent antimicrobial agent derived from *Xylaria* sp. *Acra* L38, was increased by 8 times through the optimization of carbon and nitrogen sources by utilizing orthogonal array and Plackett Burman designs (Chaichanan et al., 2014). Similarly, in the case of taxol, a renowned anticancer bioactive substance obtained from *Fusarium mairei* UH23, the yield was increased by 10.2% through optimization of carbon and nitrogen sources and the fermentation period, making use of single factor experiment (Wen-yi, 2008). Camptothecin, which is a prominent anticancer agent of the 21st century, is produced by

Trichoderma atroviride (Egbuna et al., 2020). Through the use of the single factor method, by optimizing the medium composition, fermentation time (including elicitor and adsorbent addition), and the production of camptothecin was increased by 50 to 75 times (Pu et al., 2013). Optimisation of medium parameters has been proven to enhance production of secondary metabolites by microorganisms (Table 2.6). For instance, modifying the carbon and nitrogen sources, and trace elements through the use of factorial design and RSM, the yield of exopolysaccharide (EPS) from *Berkleasmium* sp. Dzf12 was also improved by 6.29 times (Li J. et al., 2012).

Table 2.5: Effect of co-cultivation strategy on product yield and discovery of novel metabolites from endophytes

Products	Original species	Co-culturing strain	Yield enhancement	References
Luteoride D G methyipseurotin	pseurotin 11-O- <i>Aspergillus fumigatus</i> MR2012	<i>Streptomyces leeuwenhoekii</i> C34 & C58	New metabolites	(Wakefield et al., 2017)
Taxol	<i>Paraconiothyrium</i> SSM001	<i>Alternaria alternata</i>	3 fold	(Soliman & Raizada, 2013)
Taxol	<i>Fusarium</i>	<i>Taxus</i> suspension cells	38 fold	(Li et al., 2009)
Enniatins A1 and B1	<i>Fusarium tricinctum</i>	<i>Bacillus subtilis</i> 168 trpC2	78 fold	(Wätjen et al., 2009)
Enniatins A1, B1, and B (cyclic depsipeptides)	<i>Bacillus subtilis</i> 168 trpC2	<i>Fusarium tricinctum</i>	78 fold	(Ola et al., 2013)
Taxol	<i>Taxus chinensis</i> var. <i>Taxus cuspidate</i>	<i>Fusarium mairei</i>	38 fold	(Li et al., 2009)
Cercosporin	<i>Cercospora</i> sp	<i>Bacillus velezensis</i> B04 <i>Lysinibacillus</i> sp. B15	4.89 fold	(Zhou et al., 2021)
Hypocrellin A	<i>Shiraia</i> sp	<i>P. fulva</i> , <i>P. putida</i> , and <i>P. parafulva</i>	3.25 fold	(Ma et al., 2019)
<u>Camptothecine</u>	<u><i>Colletotrichum fructicola</i></u> UK1	<i>Corynespora cassiicola</i> SUK2	4 fold	(Bhalkar et al., 2016)
Bisvertinol, Koninginin A, Mevastatin, 1-	<i>Trichoderma atroviride</i> SG3403	<i>Bacillus subtilis</i> 22	Increased metabolites	²⁰ (Tang et al., 2020)

naphthylacetic acid					
Citrinin	<i>Aspergillus sydowii</i> EN-534	<i>Penicillium</i> EN-535	<i>citrinum</i>	Novel citrinin analogues	ten (X. Wang et al., 2022)
Chrysophaein, resveratrol, chrysophanol, emodin, physcion		<i>Aspergillus</i> sp.		3.85-fold	(Ding et al., 2018)

2.7.6. Limitations of secondary metabolites production by the endophytes

Table 2.6: Effect of changed parameter on yield of metabolite of different microorganisms

Product	Producing Strain	Optimized parameters	Yield (folds)	References
Vitexin	<i>Dichotomopilus funicola</i> Y3	Medium composition	4.59	(Gu et al., 2018)
Epothilone B	<i>Aspergillus fumigatus</i>	Sucrose, tryptone and incubation time	2.8 - 3.0	(El-Sayed, 2021)
Palmarumycin C ₁₃	<i>Berkleasmium</i> sp. Dzf12	Glucose, peptone and yeast extract conc opt	2.50	(Zhao et al., 2013)
Exo-polysaccharide	<i>Berkleasmium</i> sp. Dzf12	C and N sources	6.29	(Li et al., 2012)
Beauvericin	<i>Fusarium redolens</i> Dzf2	C and N sources, initial pH	1.27	(Tao et al., 2015)
Zofimarin	<i>Xylaria</i> sp. Acra L38	C and N sources, temperature, fermentation period	8.00	(Chaichanan et al., 2014)
Antibiotics	<i>Streptosporangium</i>	Aeration (gas flow), agitation system and stirrer speed	20.00	(Pfefferle et al., 2000)
Taxol	<i>Nodulisporium sylviforme</i>	pH, temperature, agitation rate,	1.15	(Ke Zhao et al., 2011)
Lovastatin	<i>Aspergillus terreus</i>	B-group vitamins, MgSO ₄ and sodium acetate.	42.00	(Ravuri & Shivakumar, 2020a)
Lovastatin	<i>Meyerozyma guilliermondii</i>	Zinc sulphate, histidi, tween-8 and B-group vitamins	11.40	(Ravuri & Shivakumar, 2020b)

Palmarumycin C13	<i>Berkleasium</i> sp. Dzf12	Metal ions	6.00	(Mou et al., 2013)
Camptothecin	<i>Fusarium oxysporum</i> NFX06	Glucose, peptone and MgSO ₄	1.02	(Musavi et al., 2015)
Camptothecin	<i>Fusarium solani</i>	Initial pH ,temperature and agitation speed	152.00	(Venugopalan et al., 2016)
Camptothecin	<i>Trichoderma atroviride</i> LY357	Medium composition, fermentation time, pH, temperature, agitation rate	50 - 75	(Pu et al., 2013)
Vincamine	<i>XM-J2</i>	Medium composition and pH	1.40	(Yin & Sun, 2011)

It is a well-established concept that microbial metabolisms are tightly connected to the selection of culture media and other conditional parameters (Kusari et al., 2012). Considering the scale of complexity in the endophyte-endophyte and endophyte-plants interactions in their ecological niche, choice of appropriate growth support media and varying morphological and physiological responses of the endophytic microbes in different culture media remain a major pitfall. For instance, (Paranagama et al., 2007) demonstrated a significant switch in the secondary metabolites produced by endophytic *Paraphaeosphaeria quadrisepata* after the tap water used in the preparation of the growth media was substituted with distilled water. The production of radicicol instead of chaetochromin A was also reported when the agar medium was replaced with broth during *in vitro* laboratory culture of the *Chaetomium chiversii*. Inefficiency of endophytes in the biosynthesis of certain molecules of interest when grown in different culture media in laboratories creates a wide gap in the understanding of full potential of the endophyte biosynthetic capability (Toghueo et al., 2020). In addition, when endophytes are isolated from respective host plants to be screened for their biosynthetic active metabolite capabilities, there might be substantial loss of great potentials which were built on inherent ecological chemical interactions while functioning within their niche. Most isolated endophytes are often discarded as incompetent endophytes after *in vitro* screening based on the observed potential as individual microbe. This results in losing out on whole lots of desired important metabolites which can only be synthesized optimally in synergy with endophytic community while interacting with the host plant tissues (Kusari et al., 2012). Moreover, due to undiscovered genes or unknown enzyme pathways, endophytes heterologous gene expression makes it even more difficult to precisely link secondary metabolite biosynthetic pathways with specific enzymatic activities (Medema et al., 2021).

Understanding the full potential of endophytes in biosynthesis of secondary metabolites could be further hindered by linear gene functions, gene co-expression, and gene duplication as well as various kinetics and characteristics of enzymes involved (Isah et al., 2018). As much as omics techniques like transcriptomics, metabolomics, and proteomic analyses are helpful in revealing the secondary metabolite-producing and/or non-metabolites producing microbes, they are not without limitations (Paz et al., 2017). It is still a difficult task to deeply comprehend the relationship between proteomics and metabolomics patterns in an endophyte (Isah et al., 2018).

2.8. Prospects of the endophytes-medicinal plants secondary metabolite production

The major limiting factors in the supply of medicinal plants for drug discovery include conservation regulatory measures, offseason periods and environmental variations, pathogens attack, heavy metal contamination, and low production of secondary metabolites. Hence, a deep understanding of how endophytes enhance and regulate secondary metabolite production by the host plants is vital to the desired up-scaling of therapeutic values of medicinal plants, because endophytes are the primary drivers of the host plant resistance to abiotic and biotic stress. The interaction between the two symbiotic partners is informed by very strong synergy rooted from their genetic makeup (Mengistu, 2020). Understanding the balance between plant defense and microbial virulence may also offer better outcomes in unlocking the full potential of medicinal plants and endophytes for secondary metabolite production. Investigating possibility of chemical elicitors influence on the full capacity of endophytes in biosynthesis of secondary metabolite appears to be a viable solution. Chemical elicitors, for instance, could induce epigenetic changes in endophytes, and thereby activate most silent genes (Pacheco-Tapia et al., 2022). The use of small molecular weight compounds such as proteasome and histone deacetylase could be useful in rectifying limitations regarding inadequate production of secondary metabolites by endophytes (Xue et al., 2023). Eliciting increased bioactive secondary metabolite production in medicinal plants by the endophytes has been demonstrated to be an effective approach. Combination of endophytic genes that encode a specific biosynthetic pathway for a secondary metabolite via hybrid pathways could also be effective in overcoming gene-based secondary metabolite constraints. For example, the synthesis of terpenoid secondary metabolites, which have anti-cancer properties, from actinomycetes bacteria was successfully increased with a biosynthetic

hybrid isoprenoids (Gallagher et al., 2010). Lastly, chemo-taxonomic information and molecular phylogenetic data can also be used to better understand the relationship between certain metabolites and their therapeutic effectiveness (Atanasov et al., 2015). This would aid the formulation of particular targets that focus solely on the relevant trends of secondary metabolites produced by plants and their associated microbes (Li & Lou, 2018).

2.9. CONCLUSION

Low secondary metabolite production by medicinal plants, inconsistent supply of medicinal plants due to extinction, seasonality, and environmental variations, medicinal plant contamination, and re-discovery of already known secondary metabolites from medicinal plants with known functions are some of the major factors influencing the pharmaceutical industries' derailed continued use of medicinal plants in the search for novel drugs. Stimulating secondary metabolite production in medicinal plants by using endophytes as plant growth regulators has been shown to be one of the most effective methods for increasing metabolites in an in-vitro setting. Endophytes can transfer new genes to medicinal plants, which can aid in the activation of already existent genes while boosting the development of new secondary metabolites, as genes and enzymes are intermediates in the production of secondary metabolites. This can assist to avoid concerns with secondary metabolite rediscovery, and the entire process can be launched by better understanding the genetic impact of endophytes on medicinal plant secondary metabolite production using genomic-based approaches combined with low and high-throughput methodologies. Endophytes appear to be a feasible alternative for production of these key bioactive compounds. The ability of endophytes to produce metabolites that are similar to those of their host plants in a short amount of time has led to classification of them as one of the sustainable natural resources for drug production and they are fast-growing and inexhaustible.

3 CHAPTER THREE: CHEMO-DIVERSITY AND BACTERIAL ENDOPHYTES IN *ARTEMISIA AFRA* AND *LESSERTIA FRUTESCENS*

This chapter covers the results from Objective 1, which was to evaluate the metabolite variation of *Artemisia afra* and *Lessertia frutescens*. Additionally, the study determined the impact of the associated bacterial endophytes of *A. afra* and *L. frutescens* on metabolite production by these plants. This chapter is divided into two sections (A and B), presenting the findings from *A. afra* in Section A and the findings from *L. frutescens* in Section B.

SECTION A: Cultivation method and plant organs as potential determinants of chemo-diversity and bacterial endophytic community in *Artemisia afra*.

ABSTRACT

Using nuclear magnetic resonance (NMR) and 16S rDNA gene sequencing, this chapter investigated the metabolite profiles and endophytic bacterial communities of *Artemisia afra*. The *A. afra* samples were sourced from two different environments where two cultivation methods were used: seed cultivation and vegetative cutting cultivation. To investigate the relationship between metabolites and bacterial endophytic community structure, Redundancy analysis (RDA) was used to establish the correlation between the genera of the most abundant endophytes and the dominating metabolites on *A. afra* leaves and stems. The NMR analysis revealed variations in metabolites from *A. afra* parts, as well as variations in samples collected from different regions under different cultivation methods. More metabolites were recovered in samples collected from Pretoria, which used the vegetative cutting cultivation method, compared to samples collected from Klerksdorp, where the seed cultivation method was used. Therefore, the vegetative cutting cultivation method is recommended as the best method for better metabolite production by medicinal plants such as *Artemisia afra*. Plant parts, cultivation method, and environment also influenced the abundance and community structure of bacterial endophytes. The most dominant phyla were Proteobacteria, Actinobacteria, and Firmicutes. *Pseudomonas*, *Pantoea*, *Curtobacterium*, *Lactobacillus*, *Allorhizobium-neorhizobium-Pararhizobium-Rhizobium*, *Staphylococcus*, *Williamsia*, *Cellulomonas*, and *Rhodococcus* were the most abundant genera from *A. afra*. Redundancy

analysis (RDA) demonstrated a positive correlation between bacterial endophytes and metabolites from *A. afra* leaves and stems, indicating a close relationship between metabolites and endophytes.

3.1. INTRODUCTION

Over the years, the reduction of medicinal plants in the wild has become more common as a result of overgrazing, overharvesting, and the unregulated commercialisation of medicinal plants (Van Wyk & Prinsloo, 2018). On the other hand, the market for medicinal plants is rising sharply worldwide, with an estimated annual value of R2.9 billion in South Africa alone (Nwafor et al., 2021). Medicinal plants, such as *Artemisia afra*, are among the oldest medicinally important indigenous plants of Southern Africa, possessing various health benefits (Koul et al., 2017). For instance, *Artemisia afra* leaf infusion has long been used to treat and control malaria, as well as a number of other illnesses, including colds, coughs, influenza, pneumonia, sore throat, and menstrual issues. Furthermore, leaf extracts from *A. afra* have been shown to possess a range of biological properties, such as antimicrobial action against several pathogens (Koul et al., 2017). Its potential to control or treat a variety of chronic ailments is connected with the variety of bioactive chemicals it contains (Van Wyk, 2008). Researchers have thus concentrated on profiling *A. afra* to discover novel bioactive compounds with potential applications in the medicine sector (Fennell et al., 2004). Due to medicinal plants' therapeutic properties and the increasing demand for quality-assured plant materials for developing pharmaceutical products, medicinal plant cultivation has been highlighted as a viable alternative (Nwafor et al., 2021; Tanga et al., 2018). Various studies have stressed the sustainable use and conservation of medicinal plants, and several recommendations have favoured cultivation as a better approach to preserve medicinal plants while providing sustainable products (Tanga et al., 2018). Many commercialised, high-valued pharmacological metabolites with minimum or no toxicity are of plant origin, acquired from controlled environments such as nurseries (Nomura et al., 2018). As a result, adding value to the medicinal plant research dynamic would be another approach to ensure the ongoing use of medicinal plants in the quest for novel medicines. Some medicinal plants are site- and environment-specific in their ability to synthesise important metabolites, and they may be unable to do so under diverse environments (Wani et al., 2021). In such instances, it is recommended to perform *in-situ* conservation of these medicinal plants (Rajpurohit & Jhang, 2015). Additionally, numerous studies have documented the extent to which the environment

influences the production of metabolites by medicinal plants (Jagadish et al., 2016; Kazan & Lyons, 2016). Environmental stresses have been identified as the main contributors to differences in metabolite synthesis by medicinal plants (Verma & Shukla, 2015). On the other hand, cultivation of medicinal plants in a controlled environment offers an opportunity to alleviate inherent challenges, such as plant misidentification, contamination, batch-to-batch product variability, and low quality and quantity of metabolites (Máthé & Máthé, 2006). Medicinal products derived from medicinal plants are generally examined for quality assurance to ensure their safety before commercialization (Tanga et al., 2018). The assurance of medicinal plant quality, which helps mitigate issues associated with medicinal plant safety, and the conservation of biodiversity are some advantages of cultivating medicinal plants (Nwafor et al., 2021). Thus, medicinal plant cultivation is considered a gateway to high-quality products and commercialization. Regarding medicinal plant conservation through cultivation, nature reserves and nurseries have been identified as ideal strategies for maintaining medicinal plant efficacy (Kasso & Balakrishnan, 2013). *Ex-situ* cultivation, which includes seed germination and *in-vitro* cloning (such as tissue cultures), has been recommended as one of the cultivation strategies for medicinal plants (Montenegro de Wit, 2017). One of the popular propagation techniques is seed propagation because it is cost-effective (Tanga et al., 2018). Furthermore, vegetative propagation is another widely utilised cultivation strategy in which a small fragment of the plant acts as a propagule and develops into a new plantlet (Megersa, 2017). Vegetative propagation is faster, and some commonly used vegetative methods include grafting, budding, and cuttings. It has been established that cuttings retain the genetic diversity of the parent plant, making them the most popular vegetative cultivation technique (Dumroese et al., 2016). Additionally, vegetative propagation using cuttings allows species to be multiplied by cloning, which contributes to the uniformity and safety of plant populations (Waman et al., 2019). Different cultivation methods are utilized with the primary objective of enhancing metabolite production by medicinal plants. The quantity and quality of metabolites from medicinal plants are also heavily influenced by plant genetics and the ecological environment (Nguyen et al., 2023). Moreover, plant metabolites and the plant microbiome (mainly endophytes) have a major impact on the overall health of the plant (Pang et al., 2021). Understanding the impact of endophytes on their host's metabolite production is becoming increasingly important for improving metabolite production by medicinal plants (Nguyen et al., 2023). When medicinal plants become colonized, they activate defense mechanisms to prevent endophytic invasion. Plant structural hardening caused by these defense mechanisms can result in the biosynthesis of

metabolites (Pathak et al., 2022). Even though endophytes have been demonstrated to influence the accumulation of metabolites in medicinal plants (Z. Li et al., 2023), research on their influence on cultivated medicinal plants remains largely unknown (Tanga et al., 2018). Thus, this study aimed to investigate the metabolite profiles and endophytic bacterial communities of *Artemisia afra* sourced from two different environments where two cultivation methods were used (seed cultivation and vegetative cutting cultivation method).

3.2. METHODS

3.2.1. *Artemisia afra* collection

Medicinal plant cultivation is regarded as the best alternative for preserving medicinal plants and addressing challenges linked to their safety. Furthermore, the flowering stage of medicinal plants is critical, having a significant impact on the synthesis of medicinal plant metabolites, and metabolite production is expected to be at its highest (Li Y. et al., 2020). Thus, it is recommended to harvest medicinal plants for therapeutic purposes during this time, and the medicinal plants for this study were collected during their flowering stages. *Artemisia afra* for this study was collected from two locations with the goal of comparing two cultivation methods: seed cultivation and vegetative (cuttings) cultivation. The seed-propagated *Artemisia afra* plants were obtained from Eden Nursery in Klerksdorp, North-West Province of South Africa, in April 2021. *A. afra* cultivation practices included seed sowing, and the plants were watered daily or as needed during their growth period. The cuttings-propagated *Artemisia afra* plants were obtained from the Agricultural Research Council Roodeplaat, Vegetable, and Industrial and Medicinal Plants (ARC-VIMP), located in Pretoria, Gauteng Province of South Africa, in April 2021. The plants were initiated from cuttings and were irrigated regularly to establish in the field. The samples were collected in three different positions in the same field in Pretoria at ARC-VIMP (at the beginning, in the middle, and at the end of the field). These positions resulted in sample IDs such as (BL 1, 2, 3 = leaf samples from the beginning, ML 1, 2, 3 = leaf samples from the middle, EL 1, 2, 3 = leaf samples from the end, BS 1, 2, 3 = stem samples from the beginning, MS 1, 2, 3 = stem samples from the middle and ES 1, 2, 3 = stem samples from the end). The plant samples were collected and transported on ice to the laboratory at North-West University, Potchefstroom campus, where they were surface sterilized. The plants were first rinsed with tap water and then separated into segments for effective surface sterilisation. The surface sterilisation procedure followed the method outlined by Palanichamy et al. (2018). The plant

parts were submerged in 95% ethanol for 30 seconds, then in a 2.5% sodium hypochlorite solution for 5 minutes. Afterwards, the sodium hypochlorite was neutralised by washing with 70% ethanol for 1 minute, followed by three rinses with double-distilled water to remove any remaining substances. To assess the effectiveness of the surface sterilisation, small portions of each plant part were plated on nutrient agar and incubated at 37 °C for 24 to 48 hours. The absence of microbial growth indicated that the sterilisation process was successful. The plant segments (stems and leaves) were then air-dried in a sterile environment and subsequently crushed using a pestle and mortar.

3.2.2. Plant extraction and NMR analysis

Extraction of plant metabolites was conducted following the method by (Kim et al., 2010). The powdered leaf and stem samples (50 mg) of all *Artemisia afra* were extracted with 750 µl methanol-d₄ (CH₃OH-d₄) and 750 µl of potassium dihydrogen phosphate (KH₂PO₄) buffer in deuterium water (D₂O) (pH 6.0) containing 0.01% (w/w) tri-methyl-silane-propionic acid (TSP). The mixture was vortexed for 1 min, ultrasonicated for 20 mins, and then centrifuged for 20 mins (10,000 rpm). Samples were then filtered through a 0.22 µm syringe filter. Finally, the filtrates (500 µl) were transferred to a 5 mm Norell standard NMR tube. All the proton NMR spectra were acquired using a 600 MHz NMR spectrometer (Varian Inc, CA, USA). Gradient shimming was used to improve the magnetic field homogeneity prior to all acquisitions. All spectra were Fourier-transformed, phase and baseline corrected manually.

3.2.3. Multivariate data analysis

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), while correction of phasing and baseline, normalisation and peak alignment were done manually on the ¹H NMR spectrum. All processed data were divided into 0.04 ppm bins, representing 0–10 ppm and converted to Excel CSV file format for pattern recognition multivariate data analysis. Transformed data were statistically analysed, and all imported data were Pareto scaled in the soft independent modeling of class analogy (SIMCA) software (SIMCA, Version 14.0, Umetrics, Umeå, Sweden). Similar and non-similar samples were statistically discriminated using Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structure Discriminant Analysis (OPLS-DA). Annotations of compounds were performed using the Chenomx software (NMR suite, version 10.1) and published NMR data.

3.2.4. Sequencing of amplified genomic region

Genomic DNA was extracted from *A. afra* leaves and stem using the PowerPlant Pro DNA Isolation Kit (Qiagen, Germany), following the manufacturer's instructions. The DNA molecules were amplified for 16S V3/V4 rDNA region (341F CCTAYGGGRBGCASCAG; 806R GGACTACNNGGGTATCTAAT). Amplicon polymerase chain reaction (PCR) was carried out on mixtures containing 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 µM of each primer and 10ng target DNA, under the following PCR condition for 30 cycles; 98 °C for 1 min (initial denaturing step), 10 s at 98 °C, 30 s at 50 °C, and 7230 s at °C, and a final extension at 72 °C for 5 mins. The integrity of amplicons was verified, and approximate size (bp) ascertained using gel electrophoresis (2% (w/v) agarose concentration and 1x TAE buffer). The amplicons were purified using Qiagen Gel Extraction Kit (Qiagen, Germany). The sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit (Cat No. E7645), and the libraries were quantified using the Qubit® 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. The libraries were then sequenced using the Illumina NovaSeq platform, generating 250 bp paired-end reads.

3.2.4. 16S rDNA metabarcoding data analysis

Bioinformatics analysis. The sequenced reads were demultiplexed and checked for quality control using FastQC -v 0.11.5 (Babraham Institute, UK). The low-quality regions at the beginning of the reads (~ 20 bp) were trimmed using Trimmomatic software version 0.38 (Bolger et al., 2014). Reads with an average nucleotide base quality score of less than 15 (Phred Q score) were eliminated and thereafter clustered into amplicon sequence variants (ASVs) using the DADA2 denoiser (Callahan et al., 2016) with default parameters in the Quantitative Insight into Microbial Ecology (ver. 2) (QIIME2) software (Bolyen et al., 2019). The denoised reads were then classified using a trained classifier (V3–V4 region) of the Silva 16S rDNA reference (Klindworth et al., 2013). ASVs assigned to chloroplasts and mitochondria (at the family taxonomic level) were eliminated. Additionally, the ASV count table was then rarefied to an even depth across samples before computing alpha diversity metrics. Further downstream analysis and plotting were carried out using MicrobiotaProcess, an R package for the analysis, visualisation and biomarker discovery of Microbiome (Xu et al., 2023), in conjunction with other R packages such as phyloseq, ggplot2, tidyverse, vegan, coin, and reshape2.

3.3. RESULTS

3.3.1. Metabolites from *Artemisia afra* medicinal plant

Figure 3.1 shows the overall ^1H NMR spectra of several metabolites found in *Artemisia afra* leaf and stem samples from both cultivation methods. *A. afra* leaf samples had more peaks than stem samples, suggesting that the leaves may contain more metabolites than the stem. The entire ^1H NMR spectra (δ 0–8 ppm) of the *A. afra* leaf and stem fingerprints revealed three distinct spectra regions: the sugar region of δ 3.0–5.5 ppm, which was quite complex due to high signal overlapping, the region from δ 6.0 to 8 ppm, which corresponds to aromatic compound signals, and the region from δ 0.7 to 3.1 ppm, which displays resonance signals associated with amino acids and organic acids. Resonance signals of sugars were clearly dominant in the leaves and stem extracts of *A. afra*, followed by amino acids, organic acids, and very few aromatic chemicals. The list of annotated metabolites found in the extracts of *Artemisia afra* leaves and stems is presented in Tables 3.1 and 3.2, respectively.

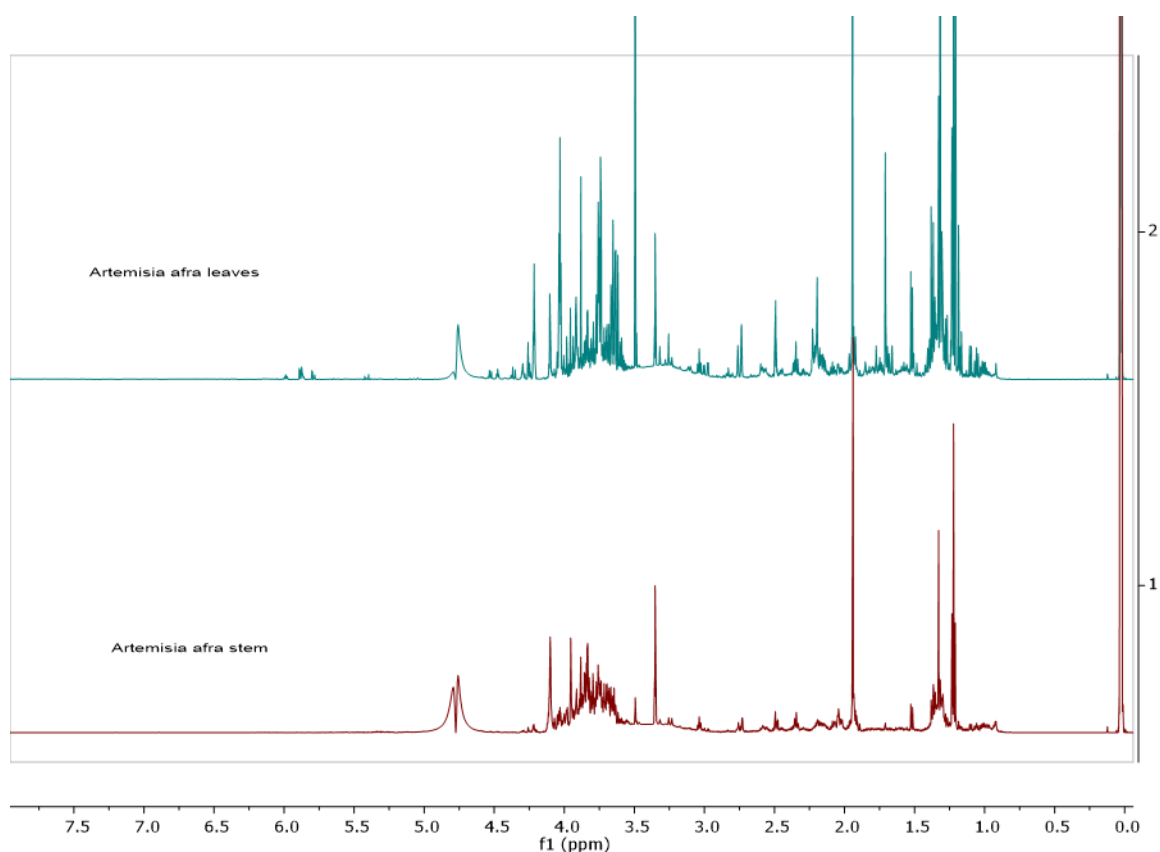


Figure 3.1: ^1H NMR representative spectra of collective *Artemisia afra* leaves and stem samples collected from Pretoria and Klerksdorp

Figure 3.2 shows the ^1H NMR spectra of several metabolites found in *A. afra* leaves from Pretoria and Klerksdorp samples. The Pretoria samples represent the cuttings cultivation

method, while the Klerksdorp samples represent the seed-cultivation method. There are noticeable similarities between the metabolic profiles of leaf samples collected from Pretoria, where the vegetative cutting method was utilized, and Klerksdorp, where the seed-cultivation method was utilized. This may suggest that comparable compounds are present in samples taken in various locations under different cultivation methods

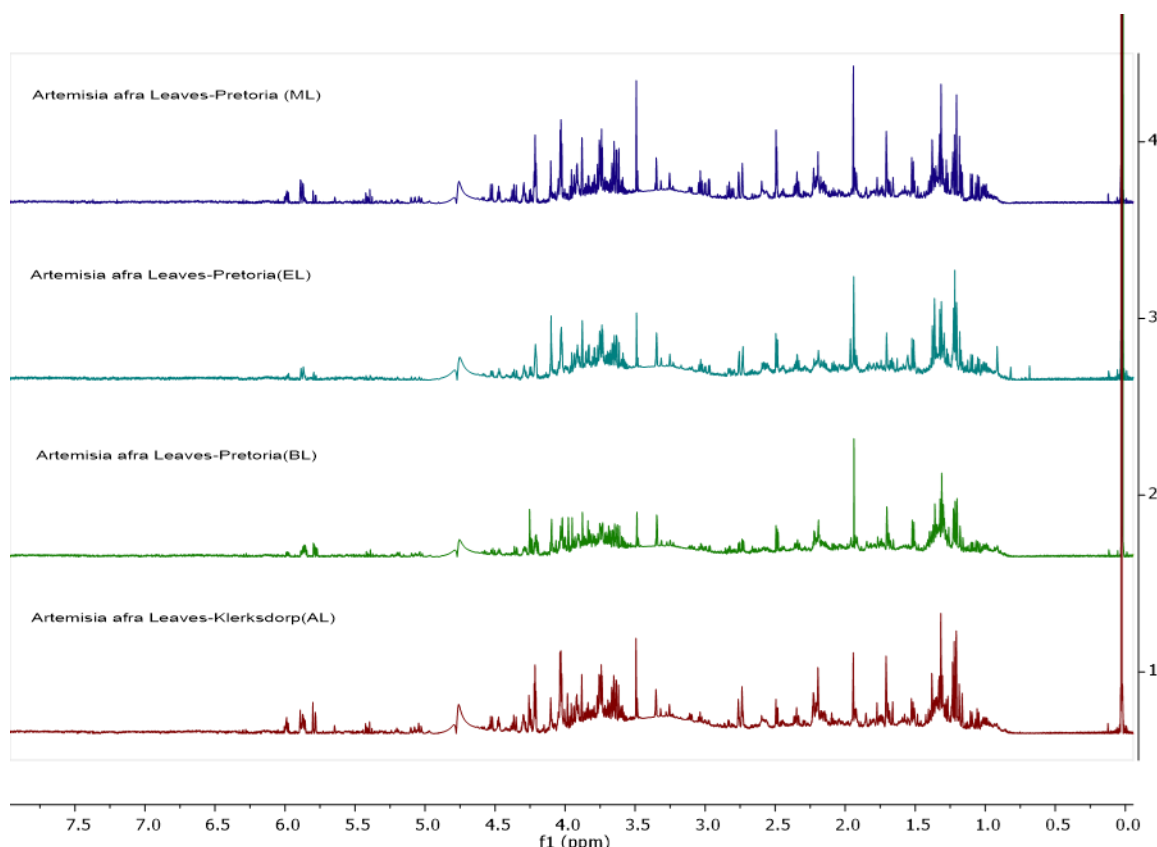


Figure 3.2: ^1H NMR representative spectra of *Artemisia afra* leaf samples from Pretoria where vegetative cutting method was utilized and Klerksdorp, where seed-cultivation method was utilized.

Table 3.1 shows the most prevalent crude metabolites synthesised by *A. afra* leaf samples from Pretoria (where the vegetative cutting method was utilized) and Klerksdorp (where the seed-cultivation method was utilized). More compounds were recovered from *A. afra* leaf samples using the vegetative cutting method compared to the seed-cultivation method. Ornithine and arginine were among the alpha amino acids recovered from *A. afra* leaf samples from Klerksdorp, along with the amino acids proline, *trans*-4-Hydroxy-L-proline, and tryptophan. Recovered sugars included fucose, xylose, and sucrose, along with the aromatic compound 3-phenyllactate and many other organic compounds such as purines (2'-deoxyadenosine, 2'-deoxyguanosine, and 2'-deoxyinosine). In addition, alpha amino acids

(pipecolate, ornithine, and leucine) and other amino acids (*trans*-4-hydroxy-L-proline, 2-aminoadipate, homocysteine, and tryptophan) were found in *A. afra* samples from Pretoria. Some sugar compounds, such as UDP-glucose, were present. There were also ibuprofen and the aromatic compound Thymol. Biotin, inosine, cellobiose, pantothenate, and arabinose were among other organic compounds annotated from *A. afra* leaf samples from Pretoria.

Table 3.1: Chenomx-assisted annotation metabolites from *Artemisia afra* leaf samples under different cultivation methods

<i>Artemisia afra</i> plant part and cultivation methods	Metabolites	Concentration (mM)	Chemical shifts (ppm)
<i>Artemisia afra</i> leaf samples from seed cultivation method in Klerksdorp	Ornithine	1.6370	1.94 (m)
	Arginine	6.3982	1.92 (m), 1.72 (m)
	Proline	39.1638	3.33(m)
	Trans-4-Hydroxy-L-proline	11.0220	3.40 (m), 3.47 (d)
	Tryptophan	6.5291	3.48 (m), 3.30 (m)
	Fucose	32.0158	5.23 (m), 4.18 (m), 4.01 (q), 3.96 (m), 3.36 (m)
	Xylose	7.9361	4.21 (s)
	Sucrose	6.6826	3.47 (s)
	UDP glucose	2.7214	4.19 (m)
	3-Phenyllactate	1.1325	4.26 (m)
	Biotin	18.0338	4.61 (m), 3.35 (m)
	1,6-Anhydro- β -D-glucose	2.8713	4.63 (d)

	2'-Deoxyadenosine	16.9588	4.65 (m), 4.18 (m), 3.84 (d)
	2'-Deoxyguanosine	1.6131	4.61 (m)
	2'-Deoxyinosine	3.9624	4.64 (m)
	Cellobiose	4.7606	4.66 (d), 3.74 (m)
	Carnitine	1.9301	3.49 (m)
	Melatonin	5.2991	3.47 (m)
<i>Artemisia afra</i> leaf samples vegetative cutting cultivation method in Pretoria	Pipecolate	2.2226	1.65 (m)
	Ornithine	6.6324	1.93 (m)
	Leucine	1.3426	2.34 (m)
	2-Aminoadipate	14.1556	1.91 (s)
	Homocysteine	6.4083	2.11 (m), 2.18 (m), 3.87 (m)
	Tryptophan	19.1156	3.29 (m)
	Trans-4-Hydroxy-L-proline	15.2770	4.66 (m), 3.47 (d)
	Xylose	32.3928	3.31 (m), 3.33 (s)
	Galactose	7.6674	4.07 (m), 3.48 (s)
	Maltose	4.6377	4.33 (d)

	Sucrose	4.4630	3.47 (s)
	Ribose	3.4630	4.92 (d), 4.86 (d), 4.20 (m), 3.85 (t), 3.74 (d), 3.60 (dd)
	Fucose	4.7698	5.27 (d), 5.22 (d), 4.19 (dd), 4.07 (m), 4.03 (s), 3.56 (s)
	UDP-galactose	4.9021	4.19 (m)
	UDP-glucose	5.4859	3.87 (m), 3.78 (d), 3.54 (q)
	Thymol	1.4542	3.16 (m)
	Ibuprofen	2.0637	2.46614 (d)
	Glucose-6-phosphate	2.4412	4.64 (d), 3.92 (m)
	Saccharopine	8.8406	1.93 (m), 1.50 (m), 1.45 (m)
	Riboflavin	2.8929	5.12 (m), 4.95 (d)
	N-Carbamoyl- β -alanine	3.3282	6.23 (m), 3.29 (m)
	Hippurate	1.3658	8.52 (m)
	Biotin	5.3928	4.61(m), 3.35 (t)
	Inosine	24.9996	4.76 (m)

	Cellobiose	15.8382	4.66 (d), 3.32 (m)
	Pantothenate	1.0912	3.98 (s)
	Arabinose	10.8474	4.11 (m), 4.09 (m)
	Cytidine	1.3792	4.29 (q)
	Cholate	4.1764	2.05 (m), 2.01 (m), 1.7 (dd), 1.72 (m)

NB: Peak multiplicity (s = singlet; d = doublet; t = triplet; dd = doublet of doublet; q = quartet; m = multiplet).

Figure 3.3 shows the ^1H NMR spectra of several metabolites found in *A. afra* stem samples from Pretoria (where the vegetative cutting method was utilized) and Klerksdorp (where the seed-cultivation method was utilized). As seen from the entire ^1H NMR spectra (δ 0–8 ppm), stem extracts of *A. afra* from Pretoria where vegetative cutting method was utilized, had higher resonance signals of sugars (δ 3.0–5.5 ppm), amino acids and organic acids (δ 0.7 to 3.1 ppm) compared to those from Klerksdorp, where seed-cultivation method was utilized.

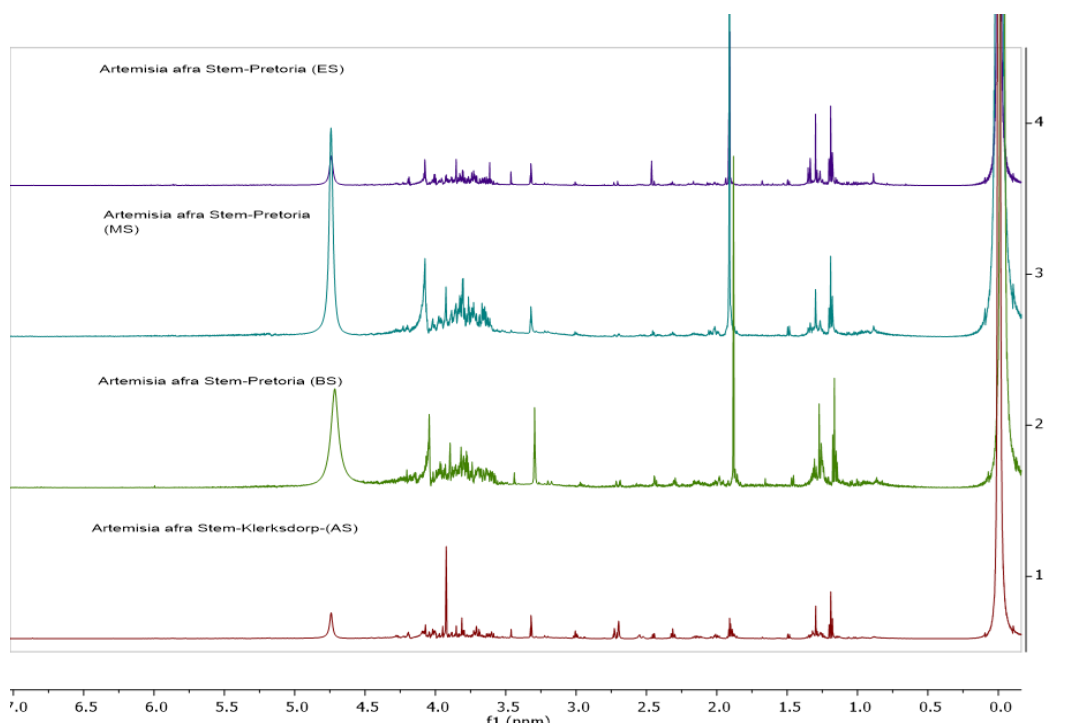


Figure 3.3: ^1H NMR representative spectra of *Artemisia afra* stem samples from Pretoria (where the vegetative cutting method was utilised) and Klerksdorp (where the seed-cultivation method was utilized)

Table 3.2 shows the most prevalent crude metabolites generated by *A. afra* stem samples from Pretoria, where the vegetative cutting method was utilised, and Klerksdorp, where the seed-cultivation method was utilised. Amino acids (proline), sugar (xylose), and a few organic compounds (guanosine and N-acetylglucosamine) were annotated from the stem of *A. afra* from the seed-cultivation method from samples collected in Klerksdorp. Under the vegetative cutting method in Pretoria, *A. afra* stem samples, more metabolites were recovered, including alpha compounds such as arginine, alanine, and γ -glutamylphenylalanine, and sugar compounds (fucose, xylose, lactose, and ribose). Some other organic compounds recovered included indole-3-lactate, cholate, and cellobiose.

Table 3.2: Chenomx-assisted annotations metabolites of *Artemisia afra* stem samples from different cultivation methods (seed cultivation method in Klerksdorp and vegetative cutting cultivation method in Pretoria)

<i>Artemisia afra</i> plant part and cultivation methods	Metabolite	Concentration (mM)	Chemical shifts (ppm)
<i>Artemisia afra</i> stem samples from seed cultivation method in Klerksdorp	Proline	10.9697	3.31 (m)
	Xylose	10.6012	4.21 (s)
	1,3-Dimethylurate	9.9658	3.29 (m)
	Guanosine	16.1593	4.74 (m)
	2-Phosphoglycerate	6.9504	3.89 (d), 4.47 (m), 3.89 (d), 3.81 (s)
	N-Acetylglucosamine	7.3943	8.18 (m)
<i>Artemisia afra</i> stem samples from vegetative cutting cultivation method in Pretoria	Arginine	1.0084	1.91 (m)
	Alanine	1.4959	3.78 (m)
	2-Aminobutyrate	3.4779	1.92 (m)
	Homoserine	13.8209	2.15 (m)

	5-Hydroxylysine	1.6936	1.90914 m
	γ -Glutamyl phenylalanine	1.6727	7.29 (d)
	Proline	46.1326	3.05 (m)
	Tryptophan	23.3556	3.30 (m)
	Fucose	46.4370	4.07 (m)
	Xylose	10.8678	4.21 (d), 3.92 (m), 3.62 (q)
	Lactose	14.8099	4.66 (d), 3.29 (m)
	Glucose	5.4751	3.45 (m)
	Ribose	2.5067	4.92 (d), 4.86 (s) 3.99 (m), 3.92 (dd), 3.75 (d)
	Mannose	5.3092	4.89 (s)
	Galactose	2.2474	4.58 (d), 4.08 (m)
	Cholate	1.5134	2.02 (m)
	Biotin	19.2858	4.61 (m), 3.35 (t)
	Indole-3-lactate	1.0099	9.99 (s)
	N-Acetylglutamine	1.5522	1.91 (m)

	Nicotinamide N-oxide	28.2821	8.36 (m)
	2'-Deoxyadenosine	8.6400	4.65 (m)
	Cellobiose	11.8102	4.66 (m), 3.32 (m)
	Glucose-6-phosphate	6.7264	4.64 (d)

NB: Peak multiplicity (s = singlet; d = doublet; t = triplet; dd = doublet of doublet; q = quartet; m = multiplet).

3.3.2. Multivariate data analysis with SIMCA for *Artemisia afra* medicinal plant leaves under different cultivation methods

To perform unsupervised principal component analysis (PCA), Q2 value was 0.91 and R2X value was 0.96 and 5 components were used to describe the data. To perform a supervised orthogonal partial least squares-discriminant analysis (OPLS-DA), Q2 value was 0.79 and R2X value was 0.96 and 3 components were used to describe the data. An unsupervised PCA plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *A. afra* samples. The principal component model was characterised by R2 X (cum) 0.96, indicating that 96% of the variation in the data was explained by the model and a 91% predictive ability (Q2 (cum) 0.91). AL 1, AL2, AL 3 and AL 4 are leaf samples from Klerksdorp, where the seed-cultivation method was utilised. Pretoria samples, where the vegetative cutting method was utilized, were collected in three different positions in the same field (at the beginning, in the middle and at the end of the field), thus having sample ID such as (BL 1, 2, 3 = leaf samples from the beginning, ML 1, 2, 3 = leaf samples from the middle, EL 1, 2, 3 = leaf samples from the end). The PCA score plot (Figure 3.4A) demonstrates the presence of two clustered groups, the largest of which, covering the majority of the data, is grouped along the negative axis. The seed cultivation samples collected in Klerksdorp were closer together, evenly divided between the positive and negative on the left side of the PC plot, and were chemically distinct from the other samples. The vegetative cutting cultivation samples collected from Pretoria are spread on the left side of the PCA. These observations initiated the use of hierarchical cluster analysis (HCA) to further investigate the chemical variability of the samples. The dendrogram obtained from the HCA shows two main branches (groups) (Figure 3.4B), confirming the grouping pattern observed in Figure 4A. The first branch (group), colored green, consists of samples using the seed cultivation method collected in Klerksdorp, and the second branch, in blue, consists of samples using the vegetative cutting cultivation method collected from Pretoria. An OPLS-DA model was then used to observe the main grouping from the HCA. Discriminant analysis (contribution) was done to reveal contributing compounds that are associated with these groupings. An OPLS-DA model was characterised by a 96 % variation R2X (cum) 0.96 and a predictive ability of 79% Q2 (cum) 0.79. The samples were classified in four distinctive groups. Group 1 (in green) comprised of samples from seed cultivation method collected from Klerksdorp (AL4, AL1, AL2, AL3), Group 2 (in blue) comprised the samples from vegetative cutting cultivation method obtained from Pretoria collected at the end of the sampling field (EL1,

EL2, EL3, EL4), Group 3 (in red) comprised of the samples from the beginning of the field (BL1, BL2, BL3, BL4), while Group 4 (in yellow) comprised of samples from the middle of the field (ML1, ML2, ML3, ML4) (Figure 3.4D).

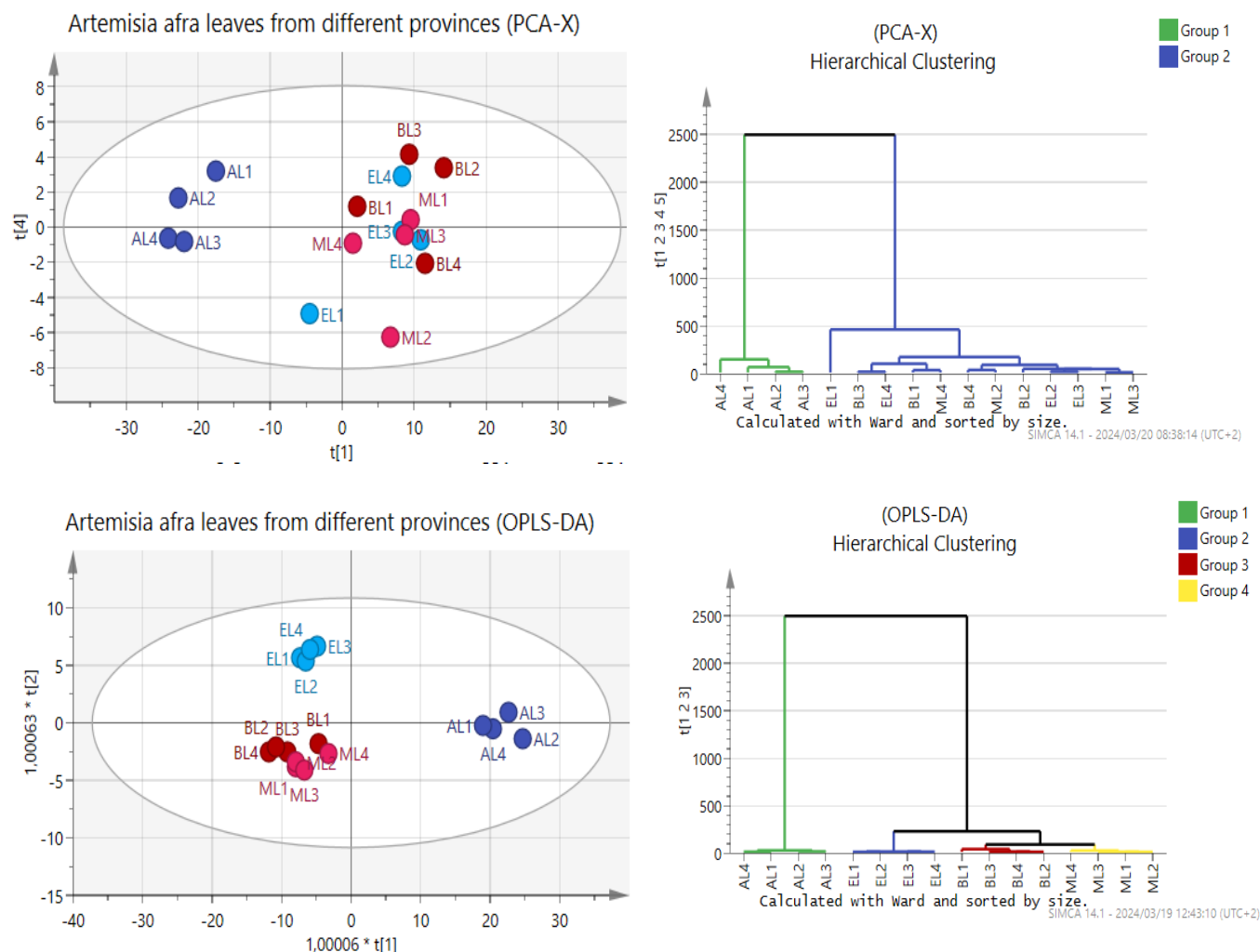


Figure 3.4: (A) Principal Component Analysis (PCA) scatter plot of *Artemisia afra* leaf samples, (B) Hierarchical clustering Analysis (HCA) dendrogram indicating inter- and intrapopulation variation with two distinguished groups, (C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plot and (D) HCA dendrogram constructed from OPSLS-DA of *A. afra* leaves samples

3.3.3. *Artemisia afra* stem samples from different cultivation methods

To perform unsupervised PCA, Q2 value was 0.83 and R2X value was 0.96 and 5 components were used to describe the data. To perform a supervised OPLS-DA, Q2 value was 0.51 and R2X value was 0.65 and 3 components were used to describe the data. An unsupervised PCA plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *A. afra* stem samples. The principal component model was characterised by R2 X (cum) 0.96 indicating that 96% of the variation in the data was

explained by the model and an 83% predictive ability (Q^2 (cum) 0.83). AS 1, AS2, AS 3 and AS 4 are stem samples from Klerksdorp, where seed-cultivation method was utilized. Pretoria samples, where vegetative cutting method was utilized, were collected in three different positions in the same field (at the beginning, in the middle and at the end of the field), thus having sample ID such as (BL 1, 2, 3 = leaf samples from the beginning, ML 1, 2, 3 = leaf samples from the middle, EL 1, 2, 3 = leaf samples from the end, BS 1, 2, 3 = stem samples from the beginning, MS 1, 2, 3 = stem samples from the middle and ES 1, 2, 3 = stem samples from the end).

The PCA score plot (Figure 3.5A), indicates that the samples were dispersed. These observations initiated the use of hierarchical cluster analysis (HCA) to further investigate the chemical variability within the samples. The dendrogram obtained from the HCA shows two main branches (groups) (Figure 3.5B). The stem samples from these cultivation methods were not confined into distinctive individual groups, indicating that they might be containing similar compounds. First grouping comprised of samples from seed cultivation methods collected from Klerksdorp (ES2, AS3, AS4, AS1, AS2) and the second grouping comprised of all the samples from vegetative cultivation methods of samples collected from Pretoria (BS1, BS2, BS3, MS4, MS2, MS3, MS1, BS4, ES3, ES1 and ES4) (Figure 3.5 B).

An OPLS-DA model (Figure 3.5C) was then used to observe the main grouping from the HCA. Discriminant analysis (contribution) was done to reveal contributing compounds that are associated with these groupings. An OPLS-DA model was characterised by a 65% variation R^2X (cum) 0.65 and a predictive ability of 51% Q^2 (cum) 0.51. The samples were classified in three groups. Group 1 (ES2, AS4, AS3, AS1, AS2), comprised all stem samples under seed cultivation methods collected from Klerksdorp and one sample under vegetative cutting cultivation method collected from Pretoria (ES2) collected at the end of the sampling field. Group 2 comprised one sample under vegetative cutting cultivation method from Pretoria collected at the beginning of the sampling field, while Group 3 (BS2, BS3, MS4, MS2, MS3, MS1, BS4, ES3, ES1, ES4) comprised the samples under vegetative cutting cultivation method from Pretoria collected from the beginning, middle, and end of the sampling field (Figure 3.5D).

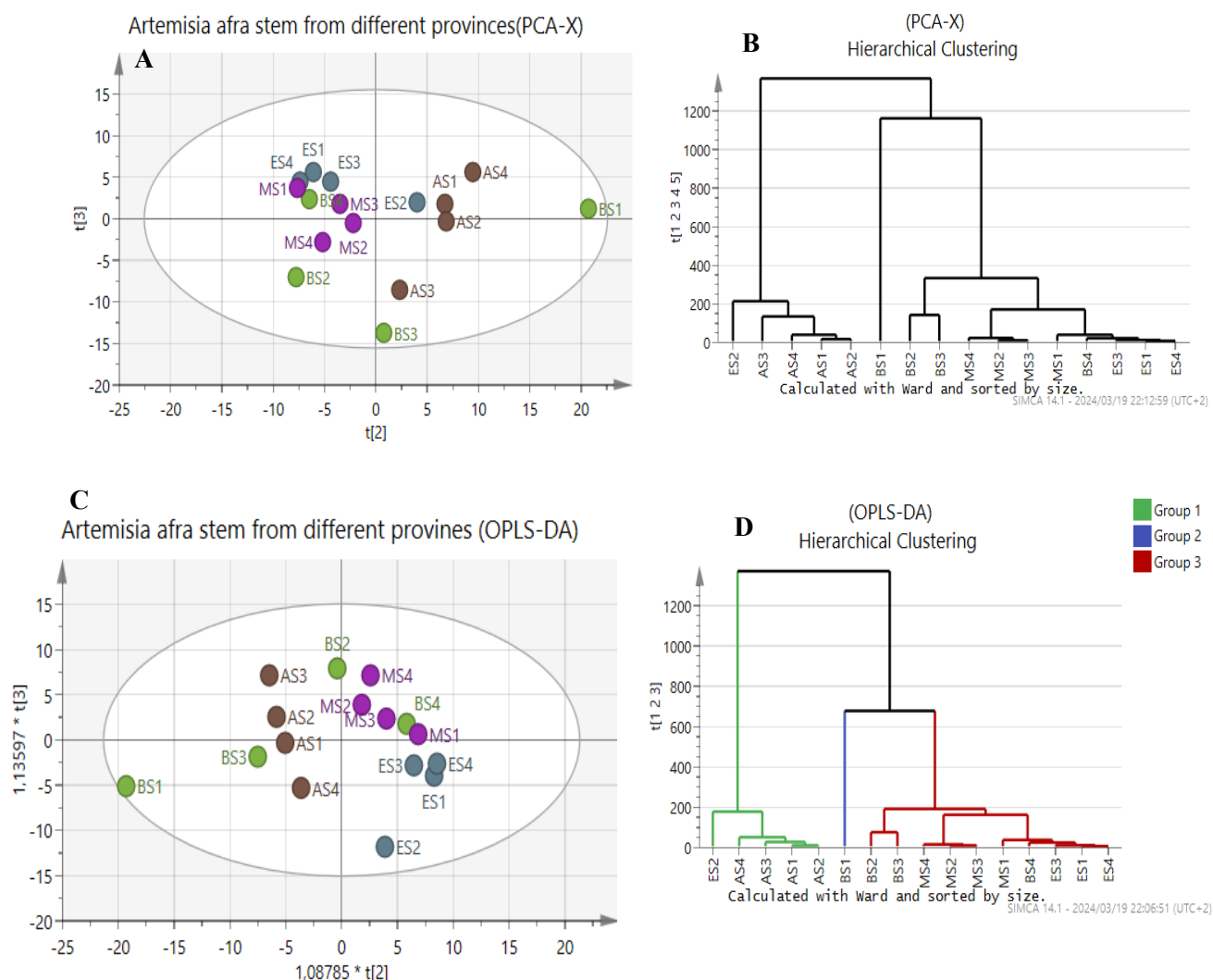


Figure 3.5 (A) Principal Component Analysis (PCA) scatter plot; (B) Hierarchical clustering Analysis (HCA) dendrogram indicating inter- and intrapopulation variation with distinguished groups; (C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plot, and (D) HCA dendrogram constructed from OPLS-DA, revealing compounds responsible for clustering of the populations within *A. afra* stem samples.

3.3.4. Comparison between *Artemisia afra* leaf and stem samples under different cultivation methods in different environments

To perform unsupervised PCA, the Q2 value was 0.93 and R2X value was 0.99 while 11 components were used to describe the data. To perform a supervised OPLS-DA, the Q2 value was 0.77 and R2X value was 0.87, while five components were used to describe the data. An unsupervised PCA plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *A. afra* leaf and stem samples. The principal component model was characterised by R2 X (cum) 0.93, indicating that 93% of the variation in the data was explained by the model and a 99% predictive ability (Q2 (cum) 0.99).

The AL 1, AL2, AL 3 and AL 4 are leaf samples from Klerksdorp, where seed-cultivation method was utilized. Pretoria samples, where vegetative cutting method was utilized, were collected in three different positions in the same field (at the beginning, in the middle and at the end of the field), thus having sample ID such as (BL 1, 2, 3 = leaf samples from the beginning, ML 1, 2, 3 = leaf samples from the middle, EL 1, 2, 3 = leaf samples from the end). AS 1, AS2, AS 3 and AS 4 are stem samples from Klerksdorp, where seed-cultivation method was utilized. Pretoria samples, where vegetative cutting method was utilized, were collected in three different positions in the same field (at the beginning, in the middle and at the end of the field), thus having sample ID such as (BL 1, 2, 3 = leaf samples from the beginning, ML 1, 2, 3 = leaf samples from the middle, EL 1, 2, 3 = leaf samples from the end, BS 1, 2, 3 = stem samples from the beginning, MS 1, 2, 3 = stem samples from the middle and ES 1, 2, 3 = stem samples from the end).

The PCA score plot (Figure 3.6A) shows that the leaf and stem samples were sparsely distributed, with some lying on the negative side of the plot. These observations initiated the use of hierarchical cluster analysis (HCA) to further investigate the chemical variability within the samples. The dendrogram obtained from the HCA shows three main branches (groups) (Figure 3.6B). The first branch (group) consists of two leaf samples under the seed cultivation method collected from Klerksdorp (green), the second group consists of samples under vegetative cutting cultivation method collected from Pretoria (blue) and the third group consists of two stem samples under seed cultivation method collected from Klerksdorp as well as Pretoria leaf samples (red). Discriminant analysis (contribution) was done to reveal contributing compounds that are associated with these groupings. An OPLS-DA model

(Figure 3.6C) was used to observe the main grouping from the HCA. An OPLS-DA model was characterised by 87% variation R^2X (cum) 0.87 and a predictive ability of 77% Q^2 (cum) 0.77. The samples were classified in five groups. Group 1 comprised of stem samples under vegetative cutting cultivation method collected from Pretoria; Group 2 also consisted of a group of stem samples under vegetative cutting cultivation method collected from Pretoria; Group 3 comprised leaf samples from both Pretoria and Klerksdorp; Group 4 consisted of one stem and one leaf sample from Pretoria; Group 5 contained leaf samples from Pretoria (Figure 3.6D).

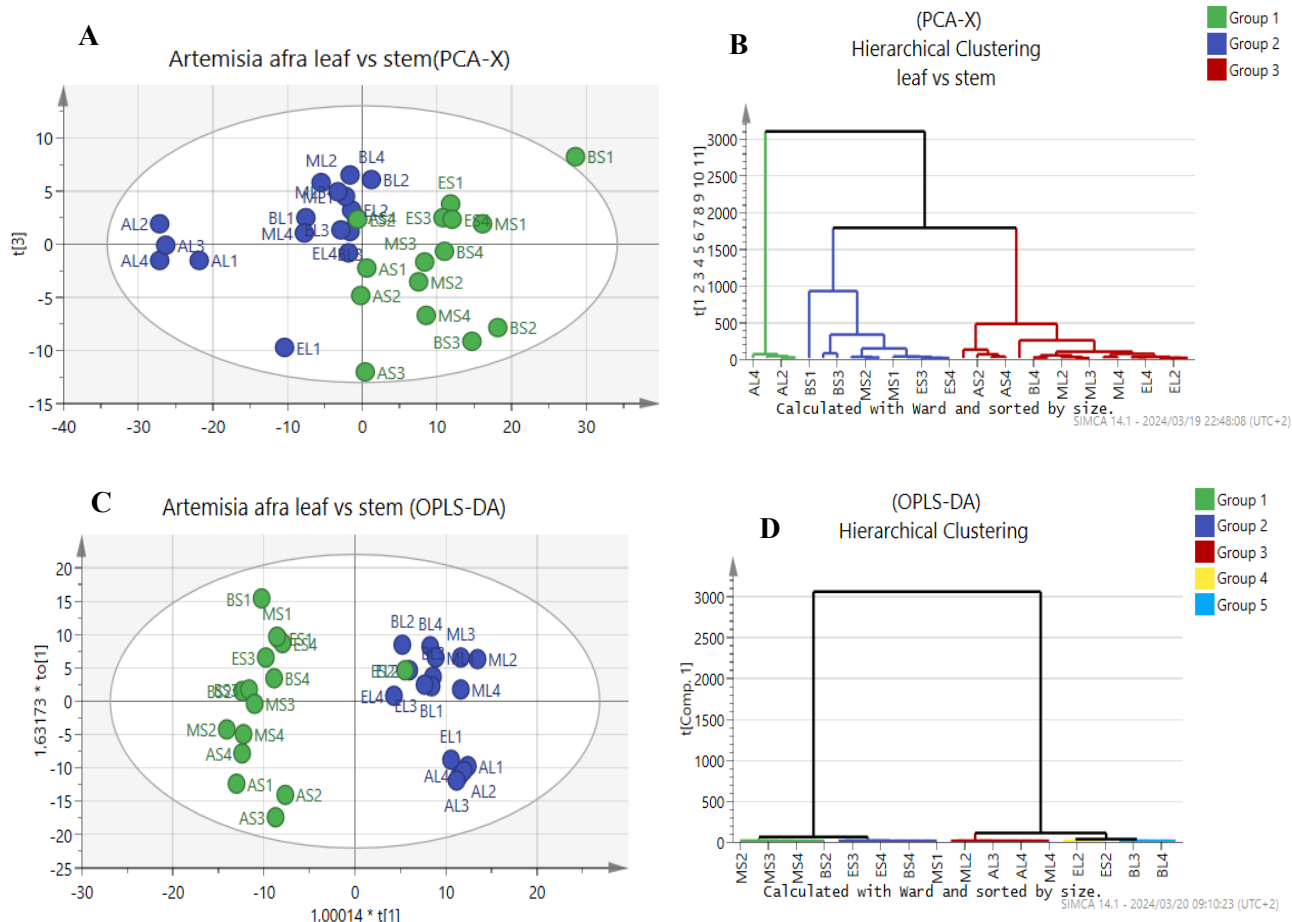


Figure 3.6: (A) Principal Component Analysis (PCA) Scatter plot (B) Hierarchical Clustering Analysis (HCA) dendrogram indicating inter- and intrapopulation variation with distinguished groups, (C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plot and (D) HCA dendrogram constructed from OPSLS-DA, of *A. afra* leaf vs stem samples

Figure 3.7 illustrates the statistical model validation for OPLS-DA discriminant model. The model's dependability and degree of overfitting were determined by looking at the intercept of the fitting line created by the estimated values R^2X and Q^2 for all samples on the y coordinate axis. The higher the value of Q^2 , the more predictive the model is, and the higher the value of R^2X , the more robust the explanatory ability. With a Q^2 regression line intercept of -1.41 (Figure 3.7A), the developed OPLS-DA discriminant model was shown to be well predictive and not overfit. The predictive residual was cross-validated (p -value < 0.05) and the receiver operating characteristic (ROC) which computes the area under the curve (AUC) was shown (Figure 3.7B)

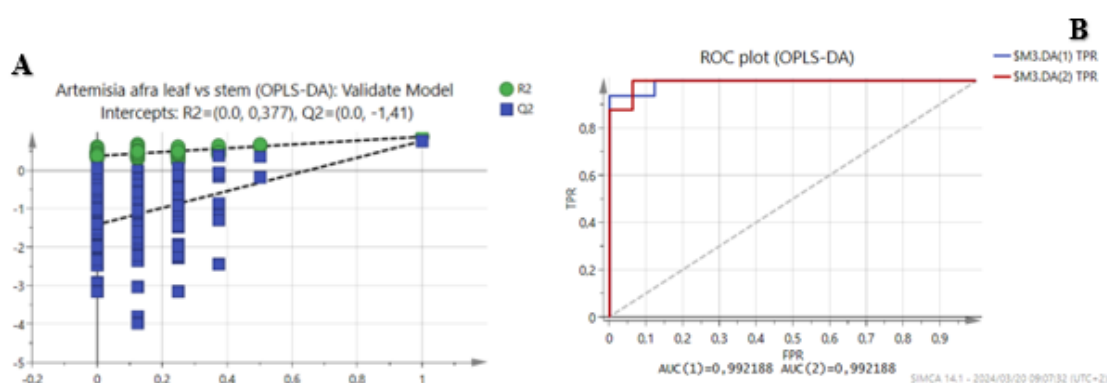


Figure 3.7: Illustration of statistical model validation (A) of the OPLS-DA model and diagnostic performance through ROC (AUC = 0.992) analysis. The Q^2 intercept was -1.41, which is less than 0.05, showing the model's validity.

3.3.5. 16S rDNA metabarcoding: Plant organ and cultivation method as possible drivers of endophytic bacterial community distribution of *A. afra*

Figures 3.8 and 3.9 illustrate Alpha diversity, which summarises the structure of the microbial community with species richness (number of taxonomic groups) indices of endophytes from *A. afra* as influenced by plant part and cultivation method from different locations. Alpha diversity indices differed between various taxonomic groups, showing that *A. afra* richness is plant part and cultivation method specific. Species with diverse communities have higher species richness. Stem samples under seed cultivation method collected from Klerksdorp and leaf samples of *A. afra* under vegetative cutting method collected from Pretoria had a high species richness, as evidenced by high observed and Chao 1 indices. In addition, leaf samples from Klerksdorp and stem samples from Pretoria showed a lower species richness, as indicated by the lowest observed and Chao 1 indices compared to other plant parts (Figure

3.8). Samples from seed cultivation method obtained from Klerksdorp had higher diversity, as shown by high Observed OTUs and Chao 1 alpha compared to samples collected from Pretoria where vegetative cultivation method was utilized for both stem and leaves (Figure 3.9).

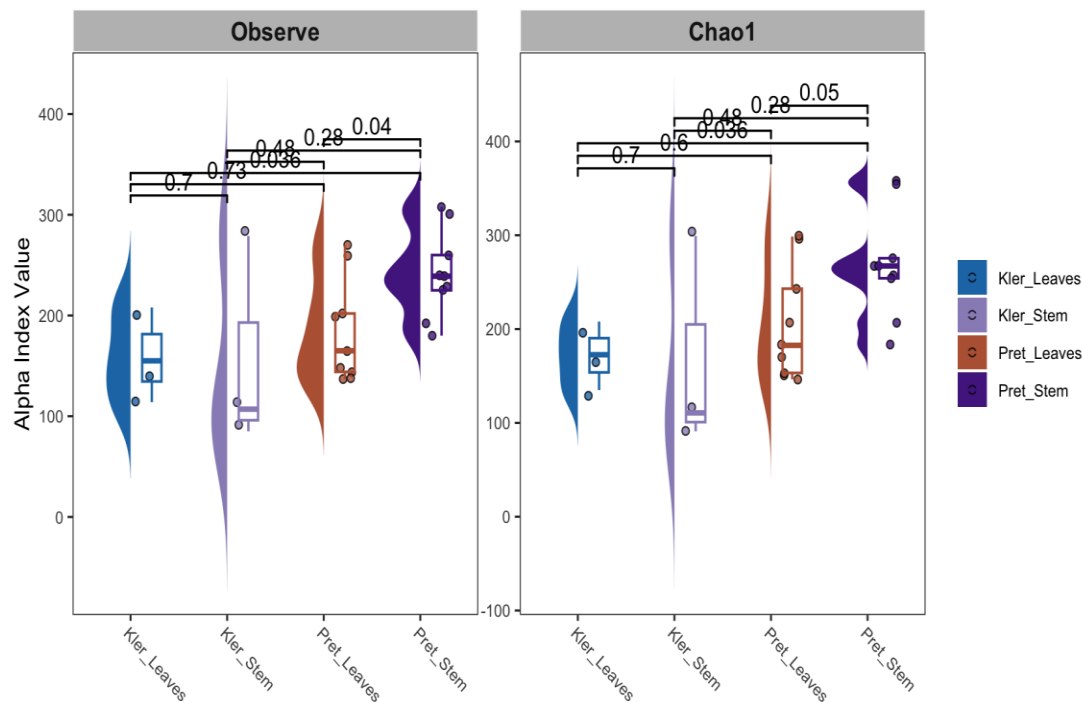


Figure 3.8: Alpha diversity, which summarises the structure of the microbial community with species richness (number of taxonomic groups) indices of endophytes from *A. afra* as influenced by plant part and cultivation method from different locations represented by observed OTUs and Chao 1 alpha diversity indices. Box plots show means and quartiles.

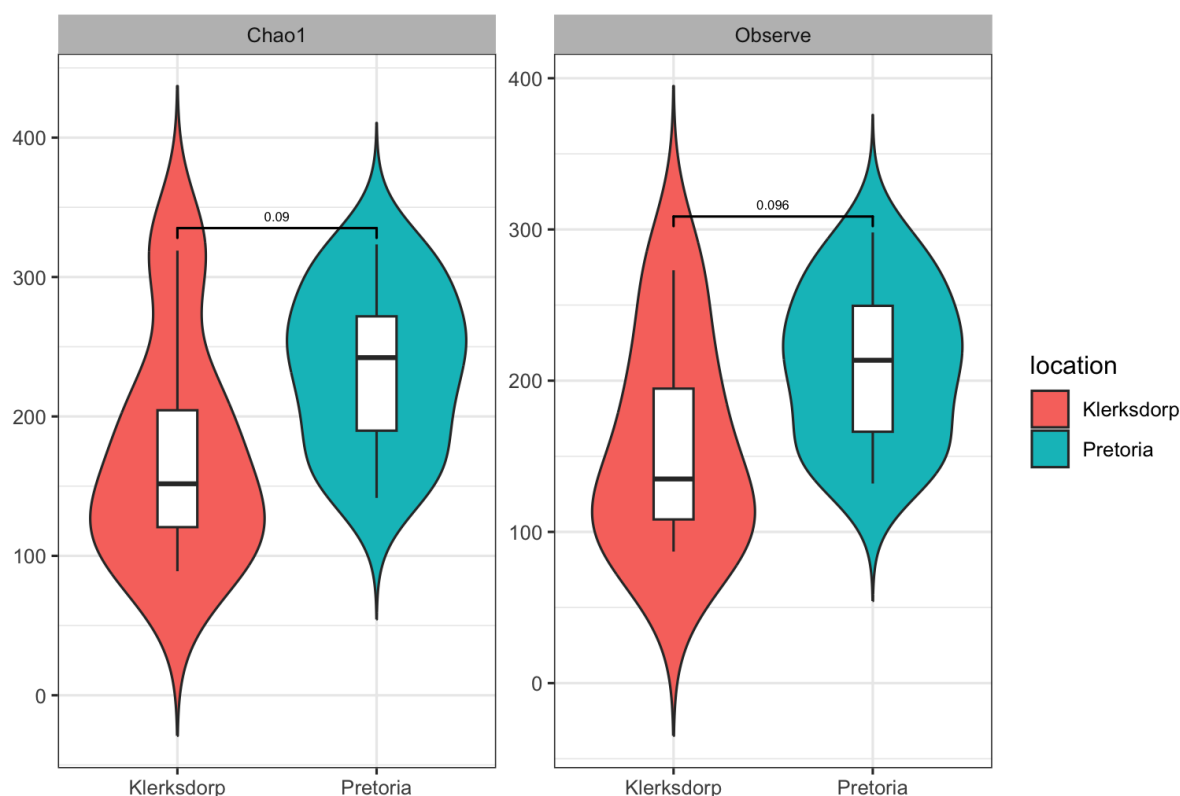


Figure 3.9: Cultivation method by location as a potential driver of *A. afra* bacterial endophytic diversity presented by Observed OTUs and Chao 1 alpha diversity indices. Box plots show means and quartiles. NB: Klerksdorp = Seed cultivation method, Pretoria = Vegetative cutting cultivation method.

Evaluation of operational taxonomic units (OTU) distribution showed that 412 OTUs were common in leaves and stem samples, 430 OTUs were unique for leaves and 559 OTUs were unique for stem samples (Figures 3.10A and B). Likewise, only 55 OTUs were common between samples from Klerksdorp where seed cultivation method was utilized and Pretoria whereby vegetative cutting cultivation method was used, having 818 OTUs unique for Pretoria under vegetative cutting cultivation method and 528 unique for Klerksdorp under seed cultivation method (Figures 3.1C and D). These findings imply that there were differences across samples based on plant sections, a trend that was also detected in the PCA (Figure 3.11).

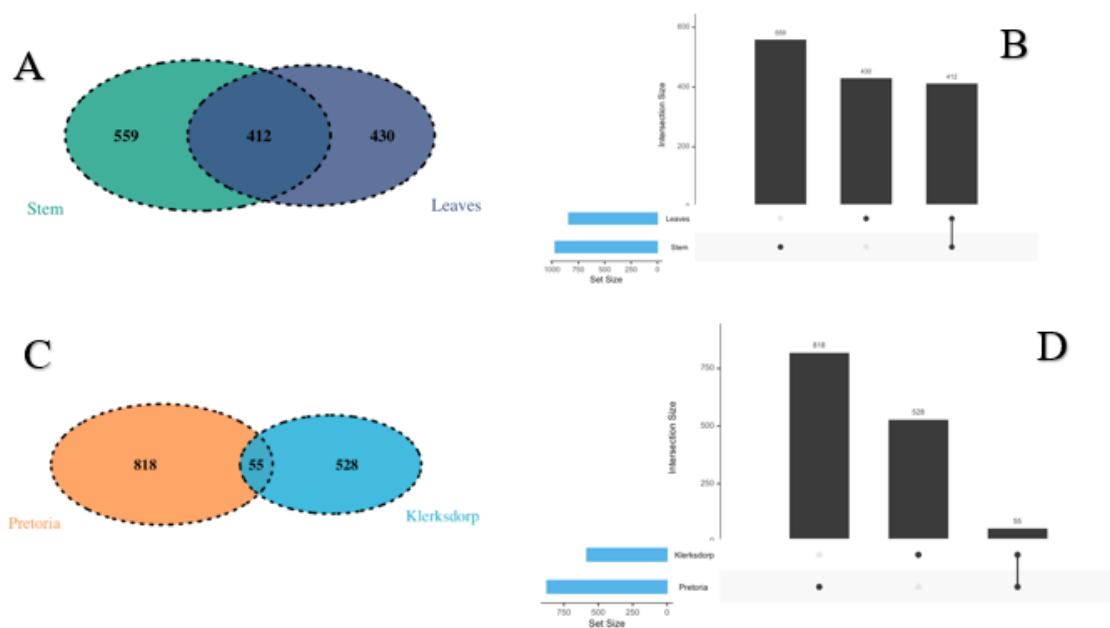


Figure 3.10: OTU distribution of *A. afra* by plant part (A and B) and location (C and D). The Venn diagram was plotted using the sum of OTUs present in each plant part and location

Bray-Curtis analysis was used to measure the dissimilarities in bacterial endophytic species composition between plant parts (stem and leaves) (Figure 3.11A) and cultivation method from two different locations (Figure 3.11B). Principal coordinate analysis (PCA) was used to visualise endophytic bacterial communities associated with plant part and cultivation method their locations. The samples varied in all parameters with 52% (PCo1) and 11.59% (PCo2) variation as the bacterial community of endophytes clustered by plant part for each location. Samples from Klerksdorp showed distinctive clustering by plant part, with *A. afra* leaf samples grouping on the negative side of the PCA and stem samples clustering on the positive side, indicating dissimilarities among the sample plant parts. Pretoria samples likewise grouped, suggesting that the community's stem and leaves were comparable (Figure 3.11A). Samples from Klerksdorp clustered on the left side of the PCA, while samples from Pretoria clustered on the right, indicating a clear location-based clustering (Figure 3.11B).

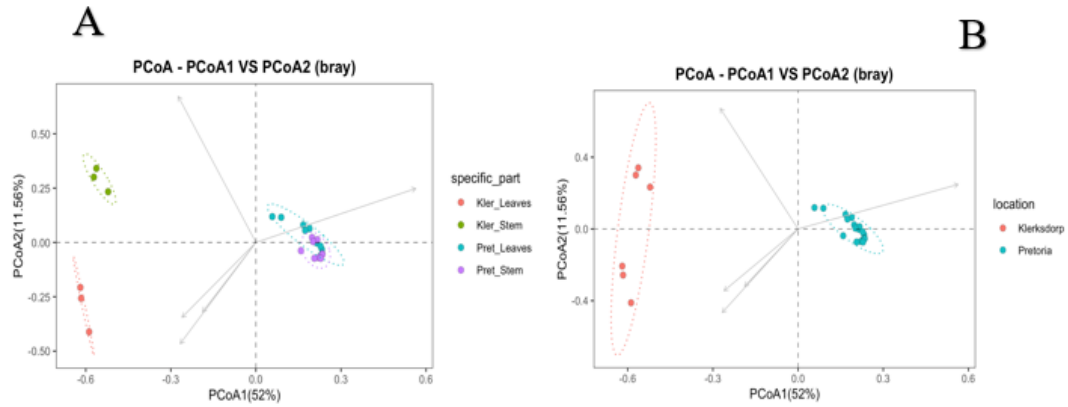


Figure 3.11: Principal component analysis (Bray-Curtis analysis) illustrating the dissimilarities in bacterial endophytic species composition between plant parts (stem and leaves) (A) and cultivation methods from different locations (B).

3.3.6. Relative abundance of bacterial endophytes from *Artemisia afra*

Figure 3.12 represents the relative abundance of top endophytic bacteria phyla from *A. afra* by plant parts. The most dominant phyla were Proteobacteria, Actinobacteria, and Firmicutes. The relative abundance of these phyla varied across plant parts. Firmicutes and Proteobacteria were the most dominating in *A. afra* leaf samples from Klerksdorp whereby seed cultivation method was utilized, while Proteobacteria proved to be the only dominating phylum in stem samples. Proteobacteria, followed by Actinobacteria, dominated in both leaf and stem samples from Pretoria whereby vegetative cutting cultivation method was utilized.

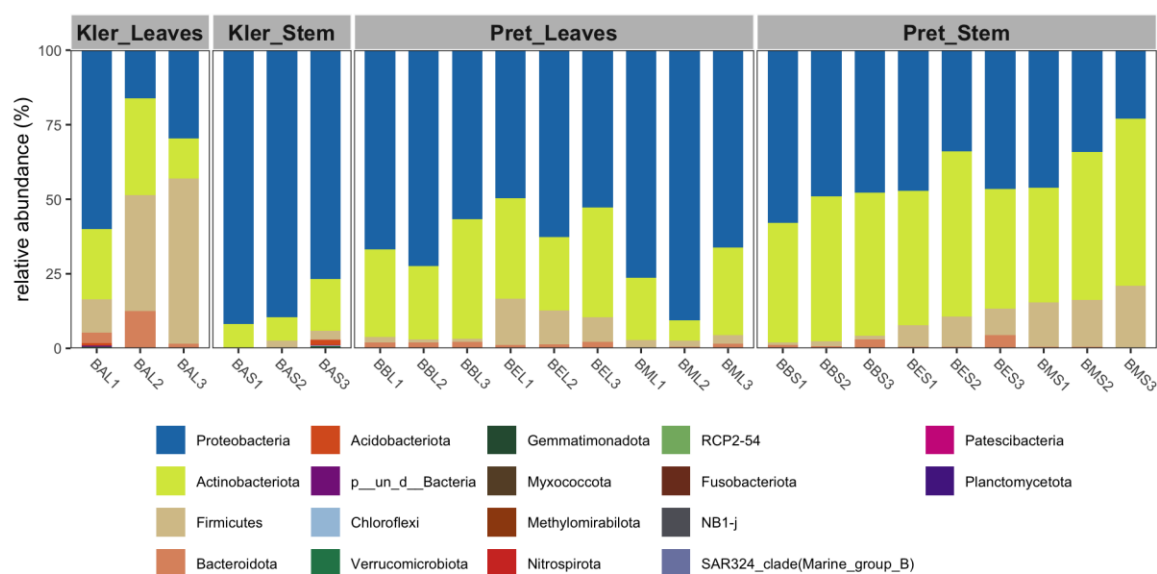


Figure 3.12: The comparative effect of plant parts on the relative abundance of *A. afra* bacteria endophytes at phyla level from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

Figure 3.13 represents the relative abundance of top endophytic bacteria phyla from *A. afra* by cultivation method in two different locations. In samples collected from Klerksdorp, seed cultivation method was utilized while in samples collected Pretoria vegetative cutting cultivation method was utilized. The most dominant phyla were Proteobacteria, Actinobacteria, and Firmicutes. The relative abundance of these phyla did not vary much across the locations. All three dominating phyla were present in most samples from both locations in varying degrees. Proteobacteria and Firmicutes mostly dominated in the Klerksdorp (seed cultivation method) samples, while Proteobacteria and Actinobacteria were dominant in the Pretoria (vegetative cutting cultivation method)

samples.

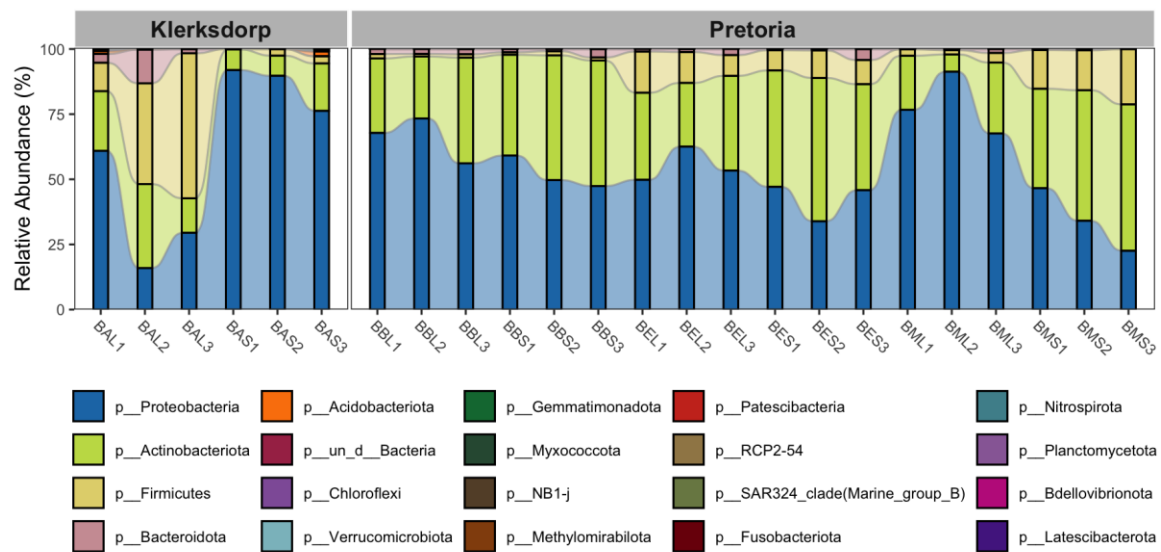


Figure 3.13: The comparative effect of cultivation method by location on the relative abundance of bacteria endophytes at phyla level in *A. afra* samples from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

Figure 3.14 illustrates the effect of plant parts on relative abundance of endophytes at genus level. *Pseudomonas*, *Pantoea*, *Curtobacterium*, *Lactobacillus*, *Allorhizobium-neorhizobium-Pararhizobium-Rhizobium*, *Staphylococcus*, *Williamsia*, *Cellulomonas*, *Rhodococcus* were the most abundant genera from *A. afra*. *Pseudomonas* was the most dominant in leaf samples, followed by stem samples from *A. afra* under vegetative cuttings proagation from Pretoria. *Pantoea* was most prevalent from the stem, followed by leaf samples from *A. afra* under seed cultivation method collected from Klerksdorp. The most prevalent bacteria in the leaf samples from *A. afra* under seed cultivation method collected from Klerksdorp were *Staphylococcus*.

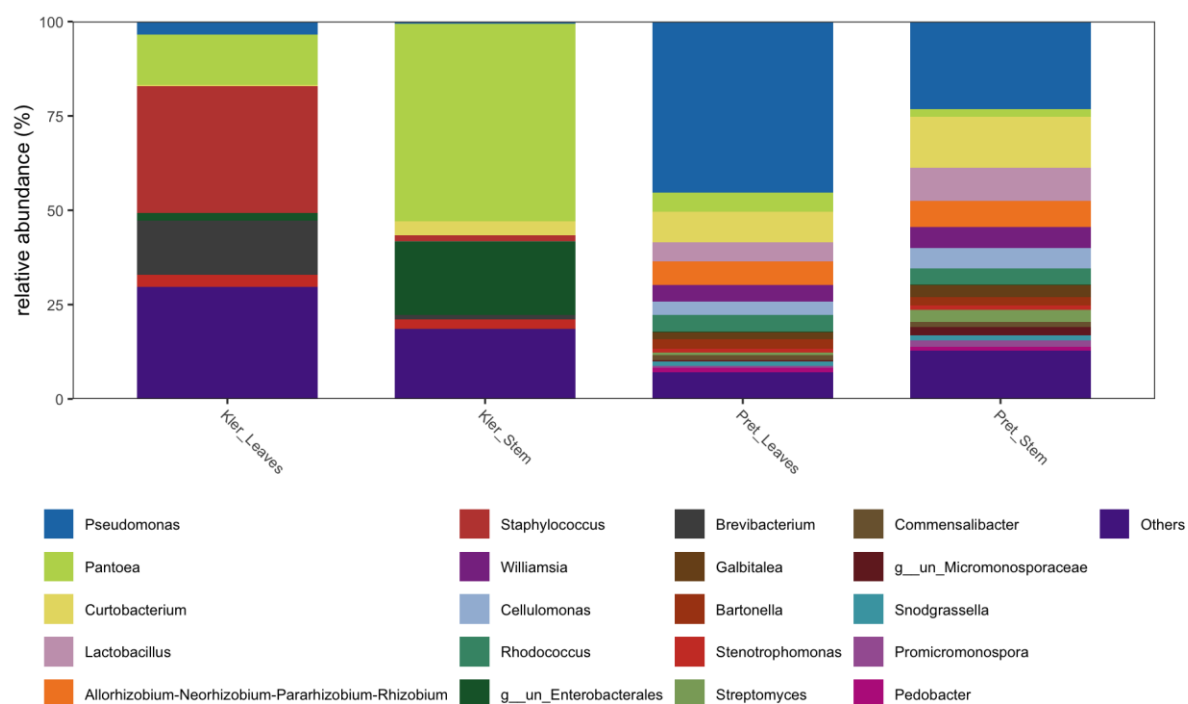


Figure 3.14: The comparative effect of plant parts of *Artemisia afra* relative abundance of bacteria endophytes at genus level for samples collected from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

Figure 3.15 illustrates the effect of location on relative abundance of endophytes at genus level. In samples collected from Klerksdorp, seed cultivation method was utilized while in samples collected Pretoria vegetative cutting cultivation method was utilized. The most dominating endophytes from *A. afra* collected from Pretoria and Klerksdorp were *Pseudomonas*, *Pantoea*, *Curtobacterium*, *Lactobacillus*, *Allorhizobium-neorhizobium-Pararhizobium-Rhizobium*, *Staphylococcus*, *Williamsia*, *Cellulomonas*, *Rhodococcus*. *Pantoea* and *Staphylococcus* were the most dominant endophytes from *A. afra* collected in Klerksdorp, whereas *Pseudomonas* and *Curtobacterium* were the dominant endophytic bacterial taxa from *A. afra* collected in Pretoria.

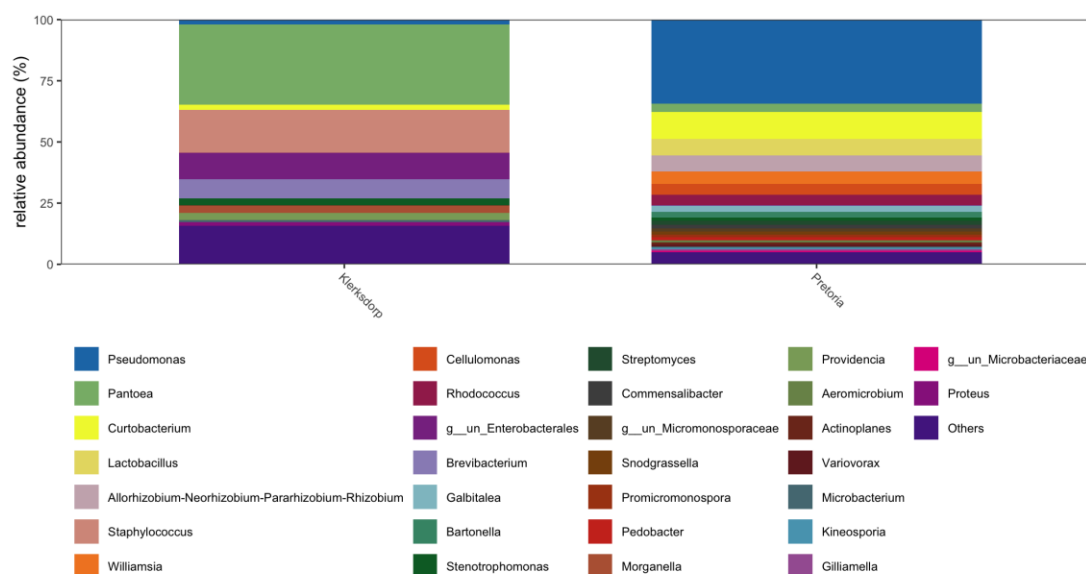


Figure 3.15: The comparative effect of location on relative abundance of *A. afra* bacteria endophytes at genus level for samples collected from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

Figure 3.16 illustrates the relative abundance and phylogenetic clustering of bacterial endophytes from *A. afra*. In samples collected from Klerksdorp, seed cultivation method was utilized while in samples collected Pretoria vegetative cutting cultivation method was utilized.

Most samples obtained from the same sampling location/ cultivation method formed a glade by plant part. Where vegetative cutting cultivation method was utilized, these are the Pretoria-collected samples of *A. afra* grouped in accordance with sample types: *A. afra* stem samples (BMS2 and BMS1; BES2 and BBS2, BES1 and BBS1), *A. afra* leaf samples (BEL2 and BEL1; BBL2 and BBL1; MBL1 and BML3) only (BES3 and BEL3; BBS3 and BBL3) leaf and stem samples grouped together. The diverse clustering of samples from Pretoria could be attributed to their large variability. Where cultivation method was utilized in Klerksdorp, samples exhibited more pronounced clustering, with samples grouping according to their sample plant part. Overall, samples under seed cultivation method in Klerksdorp showed distinct clusters for both the stem including BAS2 BAS1 and BAS3) and leaf (BL3, BAL2 and BAL1).

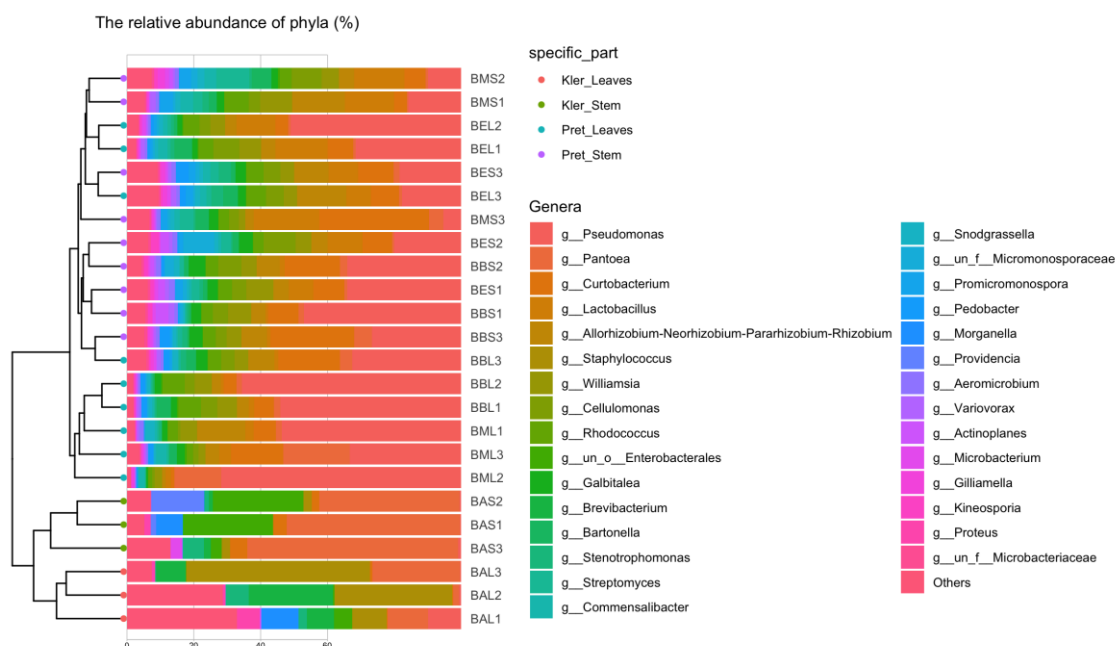


Figure 3.16: Differentially abundant bacteria genera between *A. afra* plant parts for samples collected from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

3.3.7. Correlation between metabolites and endophyte bacterial community

Redundancy analysis (RDA) was performed on relative abundant bacteria genera and abundant metabolites in *A. afra* leaves and stem (Figure 3.17). The lime-coloured dots denote the samples from leaves and the blue dots from stem. Endophytes are in red, while metabolites are blue in colour. Significant correlations were found between bacterial endophytes and metabolites from *A. afra*. Metabolites including proline, xylose, and phosphoglycerate had a positive correlation with bacterial endophytes such as *Paenarthrobacter* and *Xanthomonadaceae*. Metabolites such as Deoxyinosine, Melatonin, Biotin, Sucrose, and Fucose were positively correlated with bacterial endophytes such as *Luteimonas* and *Acinetobacter* present in *A. afra* leaves. This suggests that certain endophytic bacteria influence the metabolite production by the plant.

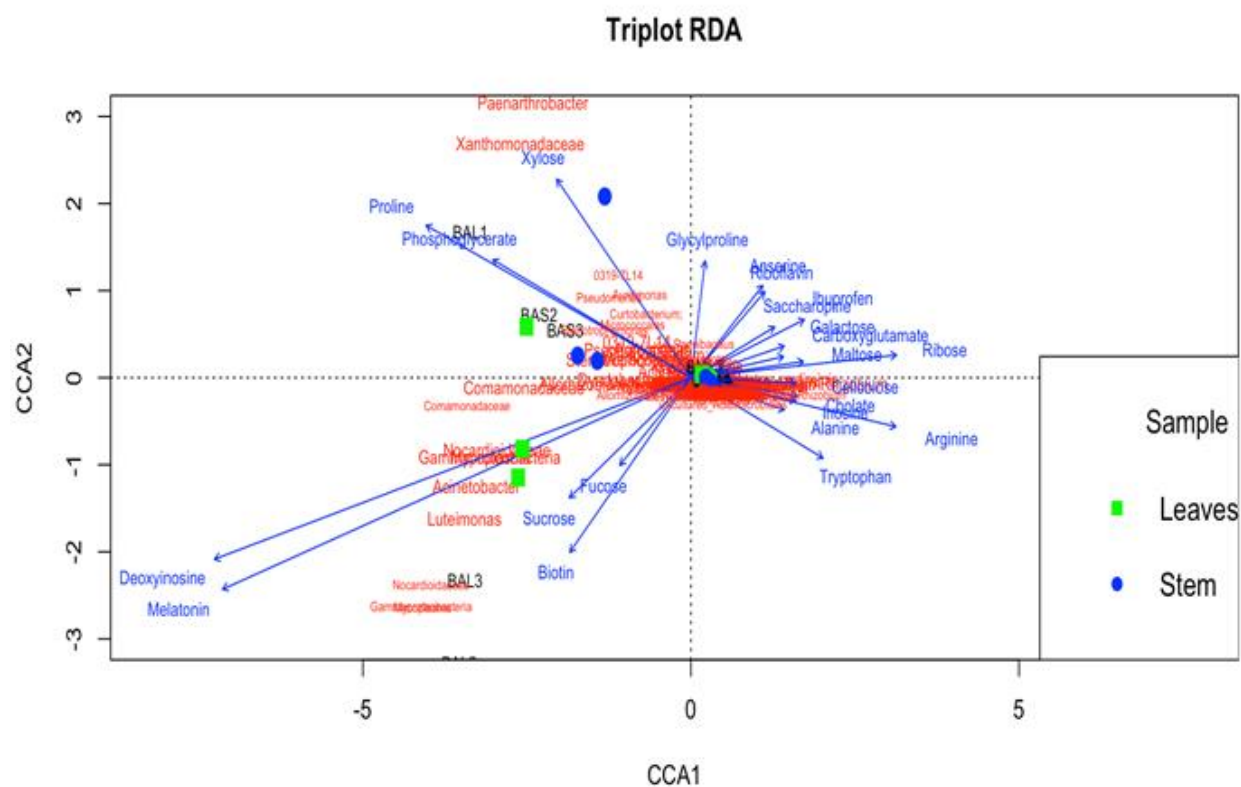


Figure 3.17: Correlation of relative abundant bacteria genera and abundant metabolites in *A. afra* leaves and stem for samples collected from Klerksdorp (whereby seed cultivation method was utilized) and Pretoria (whereby vegetative cutting cultivation method was utilized)

3.4. DISCUSSION

3.4.1. Metabolites variation of *Artemisia afra*

Artemisia afra Jacq. ex Willd. is a medicinal plant that has been used for millennia to cure and control illnesses such as influenza, fever, inflammation, hepatitis, asthma, diabetes, malaria, menstrual abnormalities, loss of appetite, and infectious diseases (Liu et al., 2009; Van Wyk, 2008). Its potential to control or treat a variety of chronic ailments relates to the variety of bioactive chemicals it contains, and results from the present study revealed a number of bioactive compounds produced by *A. afra*. Shown by ¹H NMR spectra (δ 0–8 ppm), resonance signals of sugars (δ 3.0–5.5 ppm) were clearly dominant in the leaves (Figure 3.1 & 3.2) and stem (Figure 3.1 and 3.3) extracts of *A. afra*, followed by amino acids, organic acids. This was in correspondence with Chenomx results, which revealed relatively high levels of sugars such as xylose, fucose, galactose, maltose, sucrose, ribose, UDP-galactose, UDP-glucose and sucrose, which were obtained in the current investigation as one of the constituents of *A. afra*, as demonstrated in Tables 3.1 and 3.2. In addition, alpha amino acids (Pipicolate, Ornithine, and Leucine) and other amino acids (Proline, Trans-4-hydroxy-L-proline, 2-Aminoadipate, Homocysteine, and Tryptophan) were also found. Furthermore, Biotin, Inosine, Cellobiose, Pantothenate and Arabinose were amongst other organic compounds annotated from *A. afra* extracts. *A. afra*, a medicinal plant of South Africa, produces large quantities of xylose (Braünlich et al., 2018). The list of monosaccharides that was obtained by Braünlich et al. (2018), other than xylose, included fucose, glucose, galactose, galacturonic acid, mannose, glucuronic acid and arabinose. In addition, alanine, proline, glucose (α -glucose, β -glucose), sucrose, threonine, and valine were counted amongst metabolites produced by *A. afra* plant samples collected from different geolocations (Liu et al., 2010). From the present study, as seen in Tables 3.1 and 3.2, these compounds were also present in *A. afra* leaf and stem extracts. Vegetative cuttings cultivation samples of *A. afra* leaves from Pretoria had a higher proportion of annotated metabolites than *A. afra* leaves from seed cultivation samples from Klerksdorp. The principal component analysis (PCA) in Figure 3.4 illustrates the variation in bioactive compounds found in *A. afra* leaf samples from these regions. About 96% of the variation in the data was explained by the principal component model, as indicated by its R² X (cum) 0.96. The samples from these locations were also confined to distinctive groups (Figure 3.4B). Sotenjwa et al. (2020) also experienced different variances within *A. Afra* samples collected from different areas were grouped/clustered according to where they were collected, and it was concluded that the

chemistry is conserved within the populations, possibly as a result of similar genetic traits and similar chemistry within each of the groupings. Similar observations were made in *A. afra* stem samples collected from these locations (Figure 3.5). There were intraspecies variations and clustering patterns within the dataset of the *A. afra* stem samples. The PCA score plot (Figure 3.5A) indicates the presence of three loose groups with two main branches (groups) (Figure 3.5B). It is only the OPLS-DA (Figure 3.5C) that had a distinctive grouping of the samples according to the collection site. Other samples (stem both unsupervised and supervised, as well as unsupervised leaf samples) from these provinces are not confined to distinctive individual groups, indicating that they might be containing similar compounds. Sotenjwa et al. (2020) also experienced different variances within *A. Afra* samples collected from different areas were grouped/clustered according to where they were collected. This most typical feature of chemical variation dependent on geological area was also detected in *A. afra* samples from two distinct countries, South Africa and Lesotho (Rafiri et al., 2024). It is also hypothesised that these differences in phytochemical composition result from plants' ability to adapt to shifting macro- and microenvironments (Stevens et al., 2024).

3.4.2. Metabolites profiling of cultivated *Artemisia afra* plants from different environments

Numerous studies on *Artemisia afra* have revealed a high degree of variability in the plant's chemical compounds, which have been ascribed to various combination factors, including the part of the plant, leaves being the most used part, also geographic origin, genetic traits, and biotic and abiotic environmental factors (Dograa et al., 2021; Du Toit & Van der Kooy, 2019; Moyo et al., 2019; Sotenjwa et al., 2020). From the present study, *Artemisia afra* samples were collected from two locations (Pretoria and Klerksdorp) with obviously different environments, and the production of metabolites varied between them as mentioned earlier and shown in Tables 3.1 and 3.2. Different environmental conditions from these locations could be one of the factors that resulted in varied metabolite production by *Artemisia afra*. Because it has been indicated that compounds from various cultivation methods have been found to differ chemically based on their environmental lineages (Albrecht et al., 2012). A region's temporal and geographical variations regulate the production of metabolites. Medicinal plants interact with their surroundings during their growth and development, exposing them to various biotic and abiotic elements that are present in that area or environment (Y. Li et al., 2020). These environmental factors are strong elicitors and have an impact on the metabolomics pathways for metabolite production by plants. The level of stress

tolerance varies among plants; stress can either cause an increase or a decrease in metabolites produced by plants (Mahajan et al., 2020). Furthermore, environmental conditions have distinct effects on plant parts as well as plant molecular, cellular, and morphological responses that influence metabolite production (Gray & Brady, 2016).

3.4.3. Plant part effect on metabolite production by medicinal plants

Research on the phytochemical production of *Artemisia afra* has always concentrated on its leaves (Liu et al., 2009). Liu et al. (2009) evaluated metabolites from the *Artemisia afra* plant parts, and they reported chemical variation across plant parts, with more compounds recovered from the leaves. A similar observation was made in this current study, in Tables 3.1 and 3.2, where more compounds were recovered from leaf than stem samples. Plant leaves play a vital role in the overall life of plants, not only as the primary organs for photosynthesis, but also in the synthesis and storage of plant metabolites (Y. Li et al., 2020). Secretory structures, such as secretory vesicles, secretory trichomes (plant appendages that synthesise and secrete a variety of substances), and resin ducts (viscous substances that help plants defend themselves against pathogens), to name a few, are differentiated in the leaves, such secretory structures being the main site for biosynthesis and accumulation of metabolites (Figueiredo et al., 2008). Therefore, compared to other plant components, leaves are more likely to contain a large abundance of metabolites. Variations in the secretions of these secretory structures can affect the quality and quantity of metabolites that medicinal plants produce (Verma & Shukla, 2015). Plant tissues and parts also influence the expression patterns of genes responsible for metabolite synthesis (Sharma, 2018).

3.4.4. Influence of propagation methods on *Artemisia afra* metabolites production

It has been emphasised that the propagation of medicinal plants is a practical solution to address the challenges associated with the unsustainable collection of these plants from the wild. Cultivation can enhance the productivity of medicinal plants (Alamgir, 2017). The propagation of medicinal plants can involve both asexual techniques, such as cuttings, which ensure uniformity, and sexual techniques, such as seeds, which promote genetic diversity (Kalaskar et al., 2025). Plant life begins with seed germination, and plant propagation from seeds is more vulnerable to environmental conditions such as moisture, temperature, drought, and salinity, all of which can affect the plant's ability to biosynthesise metabolites (Abbasi Khalaki et al., 2021). In the present study, there were differences in the production of metabolites by *Artemisia afra* as affected by different propagation methods (seed propagation

methods and vegetative cutting method), as shown in Tables 3.1 and 3.2, respectively. Plants develop seeds as an outcome of sexual reproduction by cross-pollination, and as a result, seeds are frequently genetically different from the plant parent and amongst themselves (Gantait et al., 2015). Thus, medicinal plants derived from those seeds usually have unique metabolic properties (Gantait et al., 2015). Under the seed propagation method, the seed metabolic activities play a role in the success of the plant's later phases, such as metabolite production and availability of some metabolites in the leaves (Tanga et al., 2018). For example, although L-canavanine is primarily stored in the seed, its availability on the leaves is also considerable, with reported amounts ranging from 0.5 to 14.5 mg g⁻¹ depending on the phases of plant growth and location with varied environmental circumstances (Colling et al., 2010; Van Wyk, 2008). In the present study, vegetative propagation through cuttings yielded more metabolites than seed propagation, demonstrating that cuttings are an ideal propagation method for *Artemisia afra* (Tables 3.1 and 3.2). It has been noted that the production of medicinal plants is accelerated by vegetative propagation using cuttings because plants derived from cuttings are faster and more productive than those propagated from seed germination (Mehalaine & Chenchouni, 2021). Propagation of medicinal plants from the same stalk, as seen in vegetative cuttings, is thought to be unique due to its genetic and biosynthetic capability for metabolite production (Vanisree et al., 2004). In vegetative propagation, genetic recombination does not occur, resulting in a uniform genetic makeup and identical replicas of the parent plant (Mehalaine & Chenchouni, 2021). Nevertheless, this was not the case in the present study. It is important to note that the *Artemisia afra* samples collected through the vegetative cutting propagation method in Pretoria came from three distinct areas of the field: the beginning, middle, and end. As a result, variations in the metabolites synthesised by these plants are expected. These variations were evidenced by the findings of the OPLS-DA model, which revealed unique groupings based on the locations where the plants were sampled in the field (see Figure 3.4D). *In vitro* propagation has been identified as one strategy that can promote the biosynthesis of new genetic variations (Albrecht et al., 2012).

The production of metabolites by propagated medicinal plants is influenced by various factors (Karabulut et al., 2024). Key factors such as temperature, soil pH, humidity, and water availability affect plant growth and development, ultimately impacting the production of plant metabolites (Verstraete et al., 2007). Even when utilising vegetative cutting propagation, environmental variables can hinder metabolite production since optimal

conditions are often unattainable in field settings (Waman et al., 2019). For example, variations in day temperature, relative humidity, and cloud cover can regulate plant physiological activities, which in turn influence metabolite formation, particularly sugar compounds that predominantly accumulate in the leaves of cuttings (Waman et al., 2019). Additionally, the growth, development, and metabolite production of medicinal plants can be regulated by the composition of the soil in which they grow (Porwal et al., 2020). Research has shown that endophytes from the surrounding soil can alter these characteristics, as different endophytes colonize plants in unique ways (Yan et al., 2019). Endophytes can modify the metabolome of plants, and their interactions have been found to enhance the production of metabolites like terpenoids, alkaloids, and flavonoids (Fu et al., 2017). During the colonization of plants by endophytes, shock events occur in response to incoming symbiotic signals, leading to changes in plant material (Escudero-Martinez & Bulgarelli, 2019).

Endophytes inhabit the intercellular spaces of plants and can induce changes in the plant's general characteristics through gene transfer (Kandel et al., 2017). This horizontal gene transfer (HGT) can disrupt the host's reproductive system (Ma et al., 2022; Yue et al., 2012). It has been indicated that genes and enzymes play crucial roles in metabolite synthesis. Endophytes with specialized traits, such as redox response regulatory systems, quorum sensing, and machinery for breaking down plant polymers, are more successful in colonising plants (Vandenkoornhuyse et al., 2015). The interaction between endophytes and medicinal plants can lead to changes in the metabolites produced, although the filtering of endophytes may vary from one plant to another (Pandey et al., 2022). Understanding the direct impact of endophytes on metabolite production in a field environment remains challenging (Pandey et al., 2016). Furthermore, the molecular mechanisms by which endophytes influence the presence of metabolites in plants are not yet fully understood (Mendes et al., 2013). To gain a better understanding of the impact of endophytes on plant metabolite production, it is essential to study the genomes of the key microbiota that influence this process (Kumari et al., 2023). Finally, designing a controlled system, such as a greenhouse, to assess a subset of endophytes and evaluate their complete genomes will be crucial for reconstructing the metabolic networks of both plants and endophytes (Kumari et al., 2023).

3.4.5. Endophytes from *Artemisia afra*

Endophytes are recognised for their significant role in the production of metabolites in medicinal plants. While microbes, including endophytes, have demonstrated promising potential in alleviating the negative effects of environmental factors, their applications and impacts have been inadequately explored. Once endophytes colonise their host plants, a variety of genes regulate their behaviour, leading to changes in both their own genetic traits and those of their host plants..

3.4.6. Plant parts and cultivation methods from different locations as possible drivers of endophytic bacterial community distribution of *Artemisia afra*

It is suggested that plant metabolites are linked to their microbial community structures, which affects their overall productivity (Zhang et al., 2020). Microbial diversity, particularly alpha and beta diversity, is a critical aspect of the microbiome community because it is mostly used to examine the impact of environmental influences on microbes, including endophytes (Willis, 2019). In the present study, alpha diversity was utilised to examine differences between endophytes between plant parts as well as cultivation methods from two different locations (environments), as illustrated in Figures 3.8 and 3.9. Plant parts and cultivation methods by location have an influence on *A. afra* endophytic community structures. *A. afra* stem samples cultivated from seeds in Klerksdorp and *A. afra* leaf samples obtained through vegetative cutting in Pretoria exhibited high species richness, as demonstrated by elevated observed and Chao1 indices. In contrast, *A. afra* leaves from Klerksdorp and stem samples from Pretoria displayed lower species richness, indicated by the lowest observed and Chao1 indices compared to other plant parts from these locations (Figure 3.8). Seed cultivation in Klerksdorp resulted in higher diversity, as evidenced by higher observed OTUs and Chao1 alpha compared to the vegetative cutting cultivation in Pretoria (Figure 3.9). These indices highlight the relative abundance of distinct species within a community (Zhang et al., 2020). It has been reported that microenvironments, such as different parts of plants, create specialisations in the microbiome population. This is due to various colonization patterns adopted by endophytes (Köberl et al., 2013). The present study observed that both the plant part and its location influenced the bacterial structure of *Artemisia* endophytes. While the different plant parts contained a high number of unique operational taxonomic units (OTUs), there were also 412 common OTUs found in both leaf and stem samples (Figures 3.10A and B).

Conversely, samples from different cultivation methods and locations exhibited distinct endophytic communities. This was evident from the number of unique OTUs present in each region, with only 55 OTUs being common between samples from Klerksdorp, which used seed cultivation, and those from Pretoria, which utilised vegetative cutting (Figures 3.10C and D). This demonstrates that different cultivation methods and environments affect the colonisation of endophytes in plants.

The variations observed were further confirmed through Principal Component Analysis (PCA), which revealed that all parameters accounted for 52% of the variation (PCo1) and 11.59% (PCo2). This indicates that the bacterial communities of endophytes are clustered based on plant part and location (see Figure 3.11). A shift in diversity may indicate environmental changes influenced by various factors (Aghdam & Brown, 2021). Research has demonstrated that both abiotic and biotic factors significantly impact the alpha diversity of ecological communities, which in turn affects the overall functionality of the ecosystem (Reese & Dunn, 2018). Diversity serves as an important indicator of ecosystem health, directly relating to its stability, functionality, and productivity (Reese & Dunn, 2018). Furthermore, it has been suggested that through mutualistic interactions with microbes, hosts can select low-diversity, high-fidelity microbial partners for specific functions essential for their survival, thereby limiting the presence of non-beneficial microorganisms, such as pathogens (Reese & Dunn, 2018).

On the other hand, the host plant's innate immune system may alter the bacterial community structure by favouring bacteria that are resistant to the plant's defensive systems (Bodenhausen et al., 2014). Additionally, certain endophytic taxa for colonisation can be selected based on the plant/seed's genetic makeup and colonisation method utilised (Klaedtke et al., 2016). For instance, vertical colonisation occurs when endophytes spread from the seeds or vegetative propagules (with cutting vegetative propagation being the most common method), while horizontal colonisation occurs when endophytes are transmitted from the environment, such as the soil rhizosphere or the phyllosphere (Kumar et al., 2020). Endophytes can utilise both modes of colonisation (Nguyen et al., 2023). Although vertical transfer of bacterial symbionts from parents to offspring is widespread, most bacterial endophytes are most likely to be horizontally transmitted from the surroundings (phyllosphere and rhizosphere) to the host plants (Frank et al., 2017). Seed-based endophytic transmission is less likely to influence plant community structure (Frank et al., 2017). This was validated in a study conducted by Hardoim et al. (2012), in which seeds were utilised to promote

seedlings in sterile conditions, while others were put in a soil environment. Findings demonstrated high diversity, population, and enhanced community structure in plant samples grown in soil in an uncontrolled setting, implying that the majority of endophytes are transmitted horizontally from the environment (Hardoim et al., 2012).

3.4.7. Relative abundance of bacterial endophytes from *Artemisia afra*

The most dominant phyla of bacteria from *Artemisia afra* were Proteobacteria, Actinobacteria, and Firmicutes, their abundance varying according to plant parts and location from which different cultivation methods were used, as shown in Figures 3.12 and 3.13. Microbiomes of medicinal plants remain largely unknown (Singh & Jain, 2022). According to Singh & Jain (2022), Actinobacteria, Bacteroidetes, Proteobacteria, and Firmicutes phyla are commonly found in medicinal plants. The dominance of Proteobacteria, Actinobacteria, Firmicutes was also observed from leaves and stems of *Paris polyphylla var. yunnanensis* and *Stellera chamaejasme* medicinal plants (Jin et al., 2014; Liu et al., 2020). Additionally, high relative abundance of Proteobacteria, Actinobacteria and Firmicutes phyla, as well as *Pantoea*, and *Bacillus* endophytic genera from medicinal plants, has been noted by (Khan Chowdhury et al., 2017).

Pseudomonas, *Pantoea*, *Curtobacterium*, *Lactobacillus*, *Allorhizobium-neorhizobium-Pararhizobium-Rhizobium*, *Staphylococcus*, *Williamsia*, *Cellulomonas*, and *Rhodococcus* were the most abundant genera from *A. afra* (Figures 3.14, 3.15 and 3.16). The dominance of these genus species varied by plant part (Figure 3.14) and location (Figure 3.15), and the majority of samples obtained from the same sampling location clustered together by plant part (Figure 3.16). Only (BES3 and BEL3; BBS3 and BBL3) leaf and stem samples from Pretoria were grouped together. Endophytes primarily migrate from the roots to the leaves via the stem; hence, it is usual for plant parts to contain comparable endophytes. Some genera, including *Bacillus* and *Pseudomonas*, are ubiquitous, can be found throughout the entire plant, and can certainly be drivers of community structure (Köberl et al., 2013). *Bacillus* sp. *Lactobacillus*, which was also abundant in the present, are known as fructophilic lactic acid bacteria (FLAB) together with *Fructobacillus* (Filannino et al., 2019). Due to their great versatility, FLAB have been used in different industry applications. They have been indicated to oxidise NADH and to convert fructose to mannitol, resulting in NAD⁺ cofactor production (Filannino et al., 2019). FLAB microorganisms play a role not only in metabolising sugar but also in combating pathogen growth and development (De Simone et al., 2023). Some of these endophytes that are dominant in the present study, such as *Pantoea*,

Staphylococcus and *Rhodococcus*, are known as opportunistic pathogenic endophytes. It has been indicated that, from a chemical point of view, metabolism allows the plants to communicate with microorganisms such as endophytes by opening some synergistic and antagonistic relationships (Isah et al., 2018). *Pantoea* is found in many environments due to its adaptability. Upon its discovery, it was assumed to be a plant pathogen, but current data suggest it is an opportunistic pathogen that, depending on the environment it is found in, can be either toxic or beneficial (Nadarasah & Stavrinides, 2014). It performs several activities, including bioremediation, pesticide degradation, and plant growth promotion through N-fixation. (Walterson & Stavrinides, 2015). Furthermore, it exhibits antibacterial action, and due to its ubiquity and adaptability, it has lately been proposed as a possible strain of economic importance for the manufacture of medicinal, environmental, and agricultural by-products (Walterson & Stavrinides, 2015). On the other hand, *Rhodococcus*, which falls under Actinobacteria is one of the microbes with high pharmaceutical and agricultural value (Barman & Dkhar, 2020). The capacity of *Rhodococcus* to withstand unfavourable conditions such as high ROS, UV, extreme temperatures, heavy metal toxicity, drought, and extreme pH can best explain the profuseness of this microbe in habitats that include medicinal plants (Ivshina et al., 2022; Pátek et al., 2021). Elucidating the mechanisms used by *Rhodococcus* to cope under harsh conditions can present new opportunities for its extensive application in other facilities, including novel medication synthesis in the medical industry (Ivshina et al., 2022). *Rhodococcus* exhibits antitumor, anticancer, and antiviral, as well as antimicrobial activity with the ability to produce quinolone antibiotics (Ivshina et al., 2022). Because of its ability to endure severe temperatures, *Rhodococcus* is one of the endophytic bacteria whose source of bioactive chemicals has not been completely studied, with a high probability of novel compounds still left undiscovered (Debnath et al., 2016). Consequently, they present a more promising chance to combat the global epidemic of antibiotic resistance issues (Ivshina et al., 2022). Lastly, endophytes infest plants or seeds in the same way that pathogens do; they transmit virulence factors into the plant's cytoplasm, weakening its defensive molecules (Bodenhausen et al., 2014). Therefore, endophytes' capacity to release virulence factors that facilitate plant colonisation may influence their variety and abundance in host plants, whereas their inability to do so would lead to a low population of these species.

3.4.8. Correlation between relative abundant bacterial genera and abundant metabolites from *Artemisia afra*

Medicinal plants have been the source of bioactive compounds for ages. However, emerging research suggests that the plant endophyte interaction may have a significant impact on the metabolites produced by medicinal plants (Singh & Jain, 2022). The plant microbiome is one of the indicators that play a significant impact in plant functionality (Ahmed & Hijri, 2021). In the current investigation, RDA indicated a positive and negative correlation between bacterial endophytes and metabolites from *Artemisia afra* leaves and stem (Figure 3.17). This is not unusual, endophytes and metabolites from *Panax quinquefolius* (L) also had a negative correlation (R. Li et al., 2023). Microbes regulate a number of plant functions, and a minor microbial shift could result in the reduction or enhancement of specific plant processes such as metabolite production (Ahmed & Hijri, 2021). For instance, the great diversity and quantity of opportunistic pathogenic endophytes in medicinal plants may be the cause of the overproduction of proline molecules. Proline was one of the metabolites that *Artemisia afra* generated, as indicated in Tables 3.1 and 3.2 of the current study. Metabolites, including proline, had a positive correlation with bacterial endophytes such as *Paenarthrobacter* and *Xanthomonadaceae* (Figure 3.17). The build-up of proline in cells has been intimately associated with plant response to stressors such as salt, drought, and resistance to pathogens (Anwar et al., 2018). A number of opportunistic endophytes figured in the present study (Figures 3.14, 3.15 and 3.16). However, a direct mechanism by which endophytes influence metabolite production in medicinal plants is still unknown. Nonetheless, it has been hypothesized that endophytes affect medicinal plants metabolites biosynthesis by (i) promoting the development of photosynthetic organs, (ii) transferring genes involved in metabolite production, (iii) upregulating genes involved in metabolite production, which ultimately alters the genetic makeup of plants, and (v) by changing the pathways by which plant metabolites are synthesized (Z. Li et al., 2023; Xu et al., 2021). Endophytes stimulate plant development by improving nutrient acquisition, availability, and uptake, which leads to a high availability of plant material and an increase in photosynthetic materials (Z. Li et al., 2023). Regarding gene regulation, endophytes use transduction pathways to control metabolites production in plant cells (Z. Li et al., 2023). Furthermore, endophytes can also synthesize metabolites that their host plant is unable to produce, which leads to a variety of changes in plant metabolite synthesis and an additional benefit for potential plant development of new metabolites (Z. Li et al., 2023). Also, numerous enzymatic processes

produce metabolites, and it has been noted that endophytes contain several enzymes that their host plants may not have. This has the added advantage of opening up new biosynthesis pathways for the host plants to produce new bioactive compounds (Kumari et al., 2023). Lastly, endophytes can also alter plants' metabolite accumulation and synthesis by microbial degradation of some compounds from one form to another using a range of enzymes (Kumari et al., 2023).

3.5. CONCLUSION

Plant organ type and cultivation method (seed cultivation and vegetative cutting cultivation method) have an influence on metabolite production as well as on the endophytic bacterial community. There were variations in metabolite production from samples of *Artemisia afra* collected in Pretoria, where a vegetative cutting cultivation method was employed, and Klerksdorp, where the seed cultivation method was used. These variations were observed in different plant parts (stems and leaves) and were influenced by the cultivation methods utilised in the two locations. More metabolites were recovered from the leaves compared to the stem samples, with a greater yield noted in Pretoria using the vegetative cutting method than in Klerksdorp, which utilised the seed method. Sugars such as xylose and fucose were produced in high quantities. The variations in metabolite production can be attributed to the different cultivation methods and environmental conditions of the regions from which the plant samples were collected, as well as the leaves' capability to produce metabolites. *Artemisia afra* hosts a variety of beneficial and opportunistic endophytes, which may also contribute to the plant's functionality. Correlation analysis revealed both positive and negative relationships between endophytes and metabolites produced by *A. afra*.

SECTION B: Structure of bacterial endophytic community and chemo-diversity in plant organs of *Lessertia frutescens*

ABSTRACT

Chemo-diversity and endophyte diversity in medicinal plants encompass the vast array of chemical compounds and the microorganisms residing within plant tissues, respectively. Research indicates that the relationship between chemical diversity and endophyte diversity is crucial for enhancing the therapeutic properties of medicinal plants. Endophytes play a significant role in chemical diversity by influencing the biosynthetic pathways of their host plants, which can lead to increased medicinal efficacy and improved stress resistance. This study examined the relationship between chemo-diversity and endophyte diversity in a popular medicinal plant from South Africa, *L. frutescens*. The plant samples were collected from Klerksdorp, South Africa. We compared the metabolite profiles and endophytic bacterial communities in the leaves and stems of *L. frutescens* using nuclear magnetic resonance (NMR) and 16S rDNA metabarcoding, respectively. To investigate how the structure of the bacterial endophytic community influences metabolite production, we used Redundancy Analysis (RDA) to connect the most abundant genera of endophytes with the dominant metabolites found in the leaves and stems of *L. frutescens*. Our findings revealed variability in both the chemical profiles and the endophytic microbial communities across different parts of the plants. Moreover, we discovered a strong correlation between certain bacterial endophytes and specific types of metabolites. In leaf samples, we established positive correlations between several metabolites, including N-acetylglucosamine, and bacterial endophytes such as *Brevibacterium*, *Arthrobacter*, *Xanthomonas*, *Azospirillum*, *Pseudomonas*, and *Kineococcus*. These results underscore the importance of integrated approaches to protect both the plants and their associated endophytes, ensuring the preservation of valuable bioactive compounds for future generations.

3.6. INTRODUCTION

Lessertia frutescens (L.) Goldblatt & J.C. Manning, previously known as *Sutherlandia frutescens* (L.) R.Br., is a medicinal leguminous plant from the Fabaceae family and is native to South Africa (Van Wyk, 2008). This plant is characterised by its small, soft-wooded shrublet and its striking red-orange flowers (Mncwangi & Viljoen, 2012). Its long history can

be traced back to the Nama and Khoisan tribes, who were said to be the first to use it for wound dressing and fever. Due to its wide range of medicinal components, it has also historically been used to prevent and treat internal and prophylactic cancers, which is how its popular name, "cancer bush," originated (Mncwangi et al., 2023). In addition to its biological anticancer properties, it has been shown to have antimicrobial, antidiabetic, immune modulatory, and anti-inflammatory activities (Gibson, 2011; Tobwala et al., 2014). Moreover, *L. frutescens* extracts have been proven to suppress the activity of HIV target enzymes (Wilson et al., 2015). The therapeutic properties of *L. frutescens* are ascribed to its rich phytochemical composition. This plant is known to have a range of metabolites, of which the key ones include sutherlandiosides, sutherlandins, L-canavanine, asparagine, L-arginine, gamma-aminobutyric acid (GABA), with sutherlandiosides and sutherlandins classified as biomarker compounds of this plant (Mncwangi et al., 2023; Shaik et al., 2011a). Leguminous plants, like *L. frutescens* are known to have significant quantities of free protein-bound and non-protein amino acids, particularly in their leaves (Mncwangi et al., 2023). Recent research has shown that the quality and quantity of several compounds in *L. frutescens*, particularly amino acids, have declined (Hlongwane et al., 2023). This decline is concerning because a reduction in certain phytochemical substances may lead to decreased biological activity in the plant. Specifically, the drop in amino acids such as proline has been linked to drought, especially in light of recent drought events in South Africa (Hlongwane et al., 2023). Additionally, some components of *L. frutescens* that possess anti-cancer properties, such as canavanine, have also been reported to have decreased in concentration (Hlongwane et al., 2023). Considering the medicinal properties of *L. frutescens*, it is imperative to search for measures for the preservation of its phytochemical properties. For instance, endophyte diversity is increasingly recognised as a critical factor influencing the growth, development, and stress tolerance of medicinal plants (Kumari et al., 2015; Singh & Jain, 2022). Endophytes, including bacterial endophytes, are microorganisms that reside within plant tissues and play crucial roles in modulating the biosynthesis of secondary metabolites. This process enhances the chemical diversity and overall medicinal properties of plants (Mishra et al., 2021). Like other leguminous plants, *L. frutescens* forms a symbiotic relationship with bacterial endophytes that can promote its growth. Understanding the relationship between chemical diversity and endophyte diversity in *L. frutescens* has important implications for agriculture, particularly in preserving the essential phytochemicals found in this plant. The aim of this study was to investigate the relationship between metabolite production and the community structure of bacterial endophytes in different organs of *L. frutescens*.

3.7. METHODS

3.7.1. *L. frutescens* plant collection

Lessertia frutescens medicinal plants were obtained from Eden nursery in Klerksdorp, North-West Province of South Africa, in April 2021. *Lessertia frutescens* cultivation practices included seed sowing and the use of chemical fertiliser (2:3:2 (N: P: K)) as a source of nutrients. The plants were watered daily or as needed during their growth period. The plant samples were collected and transported on ice to the laboratory at North-West University Potchefstroom campus, where they were surface sterilised. The plants were immediately rinsed with tap water and separated into segments for effective surface sterilisation. Surface sterilisation was carried out as described by (Palanichamy et al., 2018). Plant parts were submerged in 95% ethanol for 30 seconds, and 2.5% sodium hypochlorite solution for 5 mins. The sodium hypochlorite was washed off with 70% ethanol for 1 min. and finally washed three times with double-distilled water to remove the substances. To test the efficiency of surface sterilisation, small portions of each plant part were plated on nutrient agar and incubated at 37 °C for 24-48 h. The absence of microbial growth indicated the success of the sterilisation process. The plant segments (stem and leaves) were air-dried in a sterile environment and later crushed. using a pestle and mortar.

3.7.2. Metabolomics and 16S rDNA metabarcoding

The methods for the evaluation of metabolites from *L. frutescens* (plant extraction and NMR analysis as well as multivariate data analysis) and 16S rDNA metabarcoding (Plant DNA extraction, polymerase chain reaction (PCR) amplification, library preparation and sequencing and 16S rDNA metabarcoding data analysis) were the same already mentioned in section A of this chapter under methods section 3.2.2, 3.2.3, 3.2.4 and 3.2.5.

3.8. RESULTS

3.8.1. *Lessertia frutescens* medicinal plant NMR results

Figure 3.1 b shows the ^1H NMR spectra of several metabolites found in *L. frutescens* and stem samples from Klerksdorp with a zoomed aliphatic region. *Lessertia frutescens* leaf samples had more peaks than stems, suggesting that the leaves may contain more metabolites than the stems. The *Lessertia frutescens* leaf and stem fingerprints' entire ^1H NMR spectra (δ 0–8 ppm) revealed three distinct spectra regions: the sugar region of δ 3.0–5.5 ppm, the region from δ 6.0 to 8 ppm which corresponds to aromatic compound signals, and the region from δ 0.7 to 3.1 ppm which displays resonance signals associated with amino acids and organic acids. The resonance signals of aliphatic compounds were clearly dominant in the leaves and stem extracts of *L. frutescens*, and relatively very few aromatic chemicals. The list of annotated metabolites found in the extracts of *L. frutescens* leaves and stems is presented in Table 3.1b.

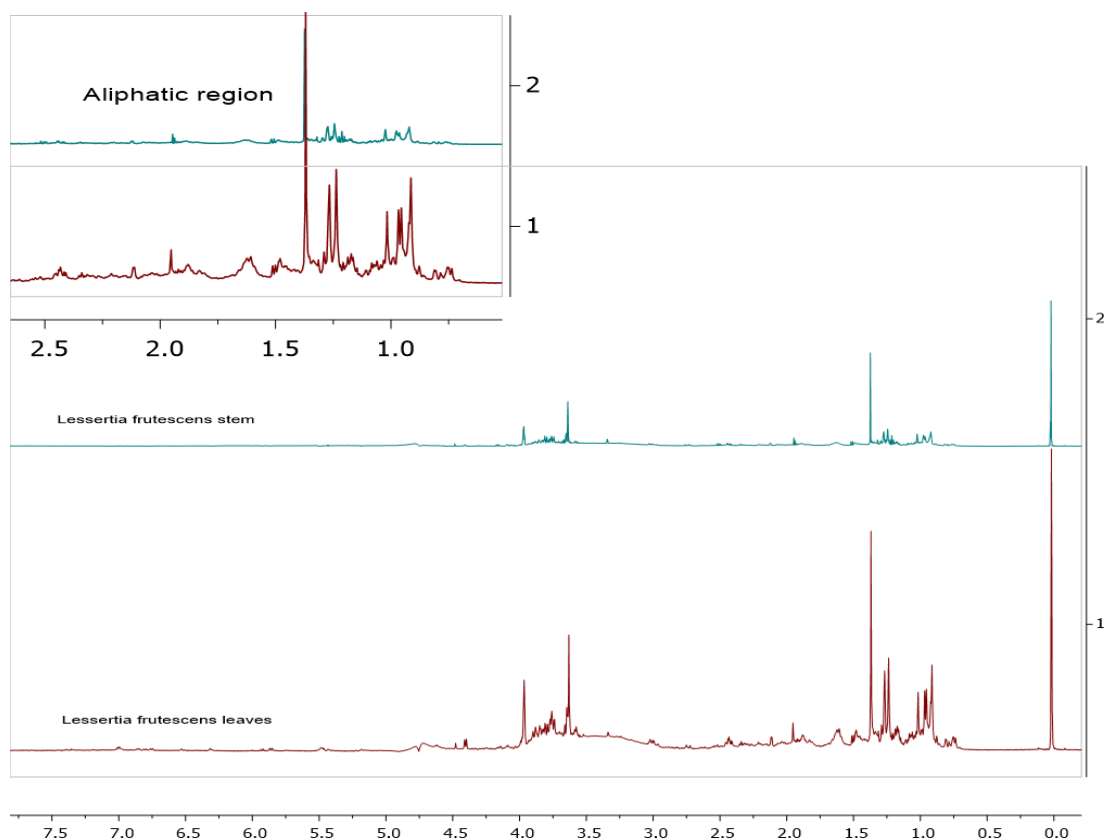


Figure 3.1b: ^1H NMR representative spectra of collective *L. frutescens* leaf vs stem samples from Klerksdorp with zoomed aliphatic region (top left).

Figure 3.2b shows the ^1H NMR spectra of several metabolites found in *L. frutescens* and stem samples from Klerksdorp with zoomed sugar region. *Lessertia frutescens* leaf samples had more peaks than the stem samples, suggesting that the leaves may contain more metabolites than the stem. The resonance signals of the zoomed sugar region (3.0 – 5.5 ppm) was the second dominant represented group after the aliphatic group.

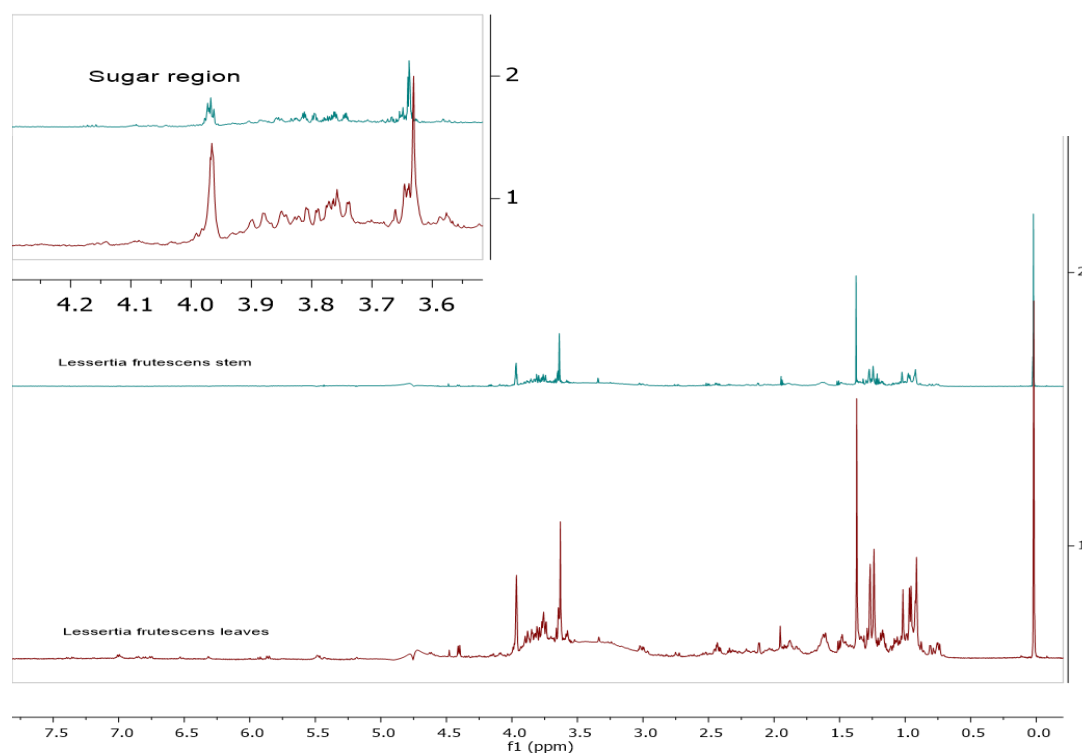


Figure 3.2b: ^1H NMR representative spectra of collective *L. frutescens* leaf vs stem samples from Klerksdorp with zoomed sugar region (top left)

Table 3.1b: Chenomx-assisted annotations metabolites *Lessertia frutescens* medicinal plant parts

<i>Lessertia frutescens</i> plant parts	Metabolites	Concentration (mM)	Chemical shift (ppm)
<i>Lessertia frutescens</i> leaf samples	Ornithine	2.5401	3.78 (m), 1.94 (m), 1.82 (m)
	Arginine	5.4020	3.76 (m), 1.64 (m)
	Pipecolate	2.4155	3.57 (d), 3.40 (m), 1.56 (m)
	Isoleucine	7.1331	1.46 (m), 1.25 (m)
	Proline	32.4240	3.41 (m), 3.33 (m)
	Tryptophan	31.1188	3.29 (m)
	Fucose	48.8112	5.27 (m), 4.55 (d), 4.07 (m), 3.86 (m), 3.63 (d), 3.56 (d)
	Ribose	18.9751	4.86 (d), 3.75 (d)
	Mannose	7.1029	4.89 (s), 4.27 (m), 3.57 (m)
	Threonate	12.1890	3.62 (m)
	Mannitol	2.6806	3.74 (m)
	2-Phosphoglycerate	23.4300	4.46 (s), 3.89 (d), 3.81 (m)

	N-Acetylglucosamine	5.4810	8.08 (s), 4.71 (d), 3.78 (m), 3.74 (m), 3.67 (m), 3.54 (m)
	UDP-N-Acetylglucosamine	1.2238	3.87 (d)
	Biotin	7.0844	4.61 (m), 4.44 (m)
	Ibuprofen	2.5677	3.60 (m)
	Isocaproate	4.3133	1.50 (m)
	Cholate	3.2713	3.50 (m), 2.11 (m), 1.89 (m), 1.150 (m)
	myo-Inositol	3.4012	3.61 (m)
	N-Phenylacetylphenylalanine	2.0587	4.46 (m)
	O-Acetylcarnitine	7.1959	3.59 (d)
	Saccharopine	5.8721	3.75 (m), 1.50 (m), 1.45 (m)
	S-Adenosylhomo	1.4796	4.86 (m)

	cysteine		
	Guanosine	1.6503	4.75 (m), 4.39 (m), 3.81 (d)
	Carnitine	3.9437	4.56 (m)
	Cadaverine	1.4897	1.46 (m)
	Valproate	9.0523	1.25 (m)
	2-Hydroxyvalerate	23.8257	1.33 (m)
	Glucose-1-phosphate	2.9704	3.87 (m), 3.74 (m)
	GTP	1.9869	4.74 (m), 4.55 (m)
<i>Lessertia frutescens</i> stem samples	Lysine	2.8914	1.49 (m), 1.43 (m)
	Isoleucine	3.2600	1.46 (m), 1.24 (m)
	N α -Acetyllysine	9.3474	3.73 (m), 1.66 (dd)
	Leucine	1.6224	3.73 (m), 1.67 (m)
	Proline	42.6234	3.32 (m)

	Fucose	9.1259	4.07 (m), 4.03 (s), 4.01 (m), 3.63 (m), 3.56 (m)
	Glucitol	23.4108	3.62 (m)
	Lactose	25.9664	4.26 (d), 3.95 (m), 3.76 (m), 3.55 (m)
	Lactulose	4.5629	4.56 (d), 4.32 (d), 4.09 (m), 3.81 (m), 3.56 (m)
	Maltose	28.8249	4.65 (d), 3.86 (m), 3.80 (m), 3.63 (s)
	Ribose	17.7943	4.92 (d), 4.45 (d), 3.75 (d)
	Mannose	5.8292	4.89 (d), 3.94 (d), 3.73 (m), 3.56 (s)
	Inosine	19.2491	4.76 (m)
	Erythritol	15.9168	3.62 (m)
	Cellobiose	13.8285	3.32 (m)
	Glucose-6-phosphate	5.9971	4.25 (d)

NB: Peak multiplicity (s = singlet; d = doublet; t = triplet; dd = doublet of doublet; q = quartet; m = multiplet).

3.8.2. Multivariate data analysis for *Lessertia frutescens* samples

To perform unsupervised PCA, Q2 value was 0.73 and R2X value was 0.92 and six components were used to describe the data. To perform a supervised OPLS-DA, the Q2 value was 0.94, the R2X value was 0.98 and four components were used to describe the data. An unsupervised PCA plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *L. frutescens* leaf and stem samples. The principal component model was characterised by R2 X (cum) 0.92, indicating that 92% of the variation in the data was explained by the model and a 73% predictive ability, Q2 (cum) 0.73. The PCA score plot (Figure 3.3bA) shows that there are two groups based on the sample types, with stem samples grouped along the negative. The chemical heterogeneity within the samples was analysed using hierarchical cluster analysis (HCA). The resulting dendrogram from the HCA reveals three distinct groups (Figure 3.3bB). The first group, coloured green, consists of a single stem sample. The second group, depicted in black, includes only leaf samples, while the third group, shown in red, comprises both stem and leaf samples. Notably, the stem and leaf samples are not separated into distinct groups, suggesting that they may have similar chemical compositions. To further explore these groupings, an OPLS-DA model (Figure 3.3bC) was employed to identify the major groupings from the HCA and the specific samples that contribute to these classifications. The chemical variability within the samples was further examined using an OPLS-DA hierarchical cluster analysis. An OPLS-DA model was characterised by 98% variation R2X (cum) 0.98 and a predictive ability of 94% Q2 (cum) 0.94. The dendrogram obtained from the OPLS-DA hierarchical cluster analysis shows two groups (Figure 3.3bD). Group 1 (in green) revealed clustering of leaf samples, and group 2 (in blue) consisted of stem samples.

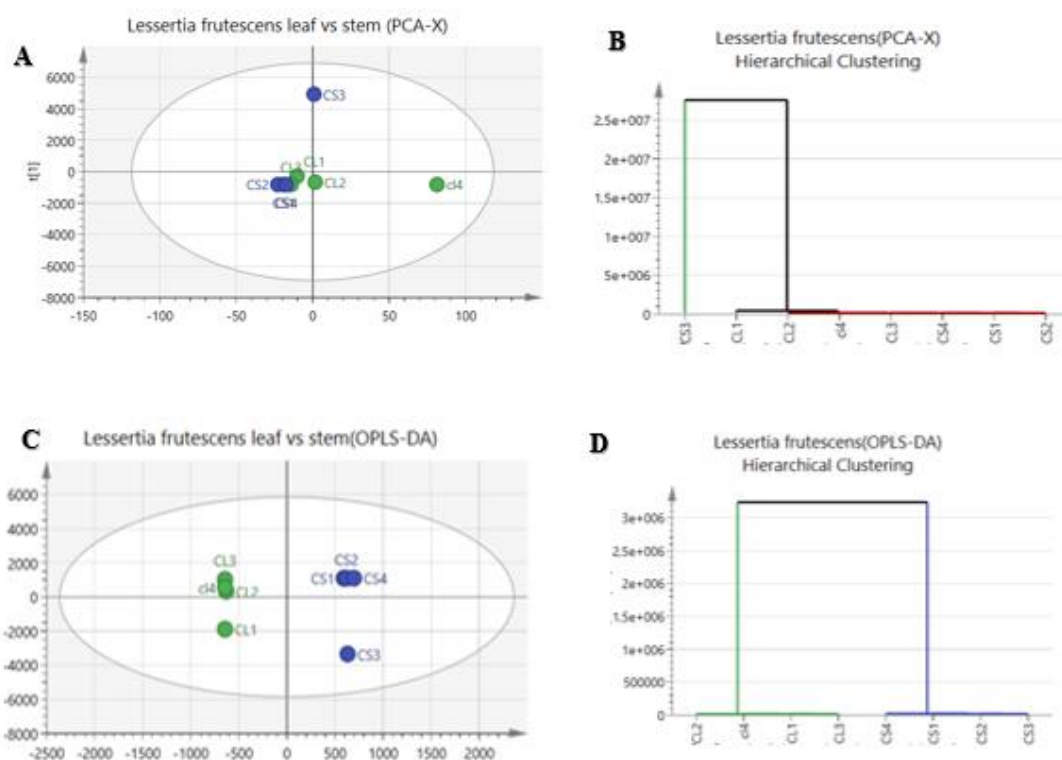


Figure 3.3b: (A) Principal Component Analysis (PCA) scatter plot, (B) Hierarchical Clustering Analysis (HCA) dendrogram indicating inter-and intra- population variation with distinguished groups, (C) Orthogonal partial least squares-discriminant analysis (OPLS-DA) scatter plot, and (D) HCA dendrogram constructed from OPSLS-DA, revealing compounds responsible for clustering of the populations within *L. frutescens* leaf and stem samples

Figure 3.4b shows the comparative contribution plots for stem and leaf samples of *L. frutescens*. In a contribution plot, the upward bars represent the NMR regions associated with active samples, while the downward bars indicate the NMR regions associated with non-active samples. Both stem and leaf samples showed a similar reaction, with more active contributing samples shown by upward bars in Figures 4A and B.

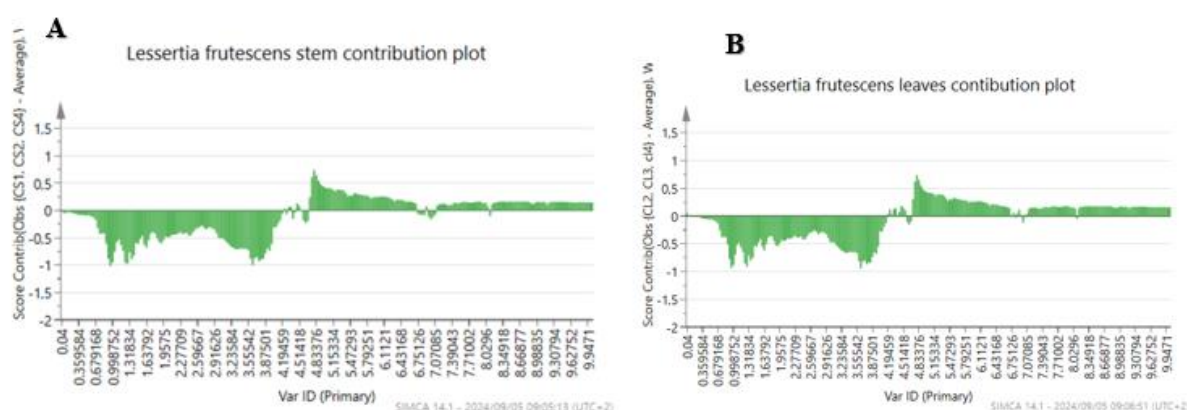


Figure 3.4b (A) Contribution plot (OPLS-DA) from Klerksdorp *L. frutescens* stem samples, (B) Contribution plot (OPLS-DA) from Klerksdorp *L. frutescens* leaf samples

Figure 3.5b illustrates the statistical model validation for OPLS-DA discriminant model. The model's dependability and degree of overfitting were determined by looking at the intercept of the fitting line created by the estimated values R^2X and Q^2 for all samples on the y-coordinate axis. The higher the value of Q^2 , the more predictive the model is, and the higher the value of R^2X , the more robust the explanatory ability becomes. With a Q^2 regression line intercept of -0.129 (Figure 3.5bA), the developed OPLS-DA discriminant model was shown to be well-predictive and not overfit. The predictive residual was cross-validated (p -value < 0.05), and the receiver-operated characteristic (ROC), which computes the area under the curve (AUC), was shown (Figure 3.5bB).

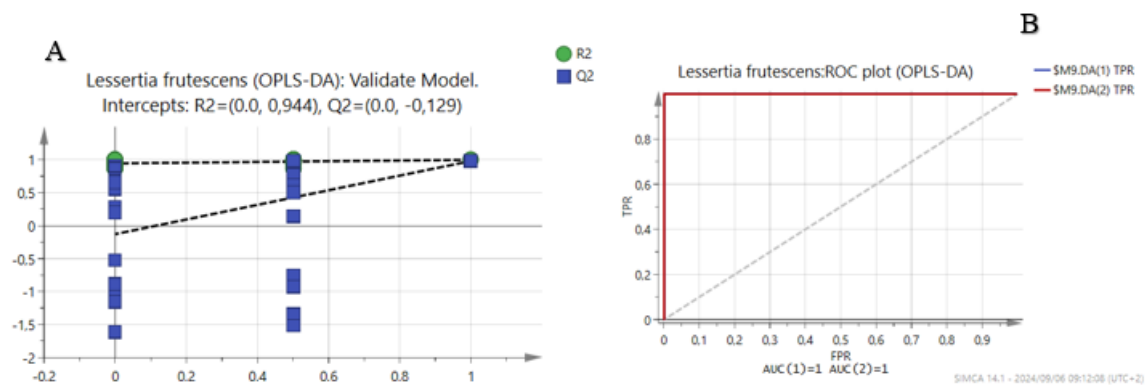


Figure 3.5b: Statistical model validation (A) of the OPLS-DA model and diagnostic performance through ROC (AUC= 1) analysis. The Q2 intercept was -0.129, which is less than 0.05, showing the model's validity

3.8.3. *Lessertia frutescens* microbiome: Plant parts as possible drivers of endophytic bacterial community distribution of *L. frutescens*

Figure 3.6b illustrates Alpha diversity, which summarises the structure of the microbial community with species richness (number of taxonomic groups) indices of endophytes from *L. frutescens* as influenced by plant parts. Alpha diversity indices differed between various taxonomic groups, showing that *L. frutescens* richness is plant part specific. Species with diverse communities have higher species richness. *Lessertia frutescens* leaves followed by roots had a high species richness and diversity, as evidenced by high observed OTUs and Chao1 indices. Stem showed a lower species richness and diversity, as indicated by the lowest observed OTUs and Chao1 indices compared to other plant parts (Figure 3.6b).

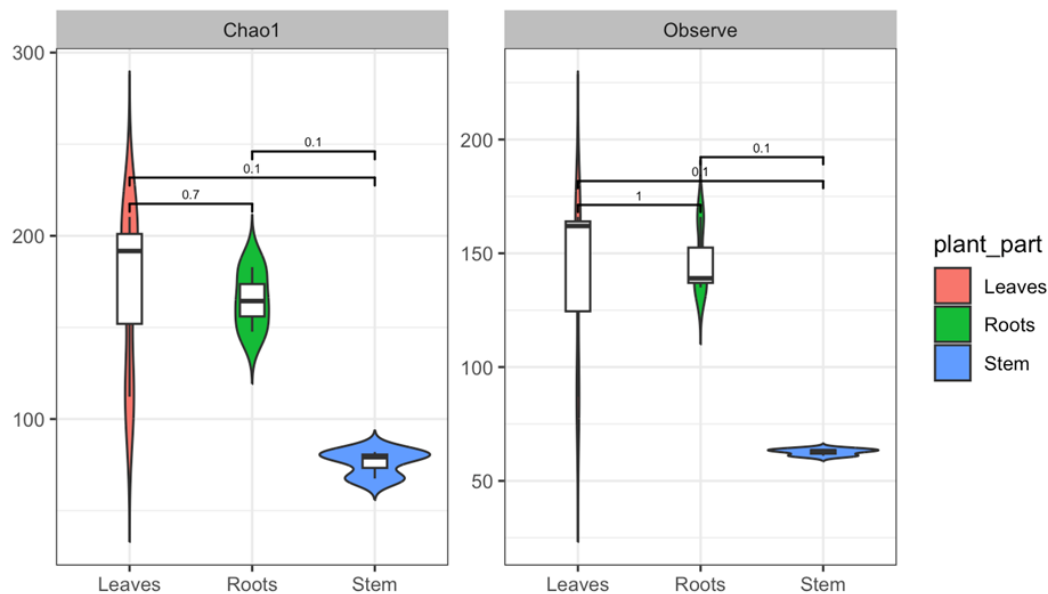


Figure 3.6b: An illustration of Alpha diversity, which summarises the structure of the microbial community with species richness (number of taxonomic groups) indices of endophytes from *L. frutescens* as influenced by plant parts. Box plots show means and quartiles.

Figure 3.7b illustrates the Operational Taxonomic Units (OTU) distribution of *L. frutescens* by plant part. Evaluation of Operational Taxonomic Units (OTU) distribution showed that 300 OTUs were unique in leaves, 174 OTUs were unique for roots and 61 for stem. On the other hand, 13 OTUs were common in leaves, roots and stem samples, ten were common in leaves and roots, eight were common in leaves and stem while 51 were common in roots and stem.

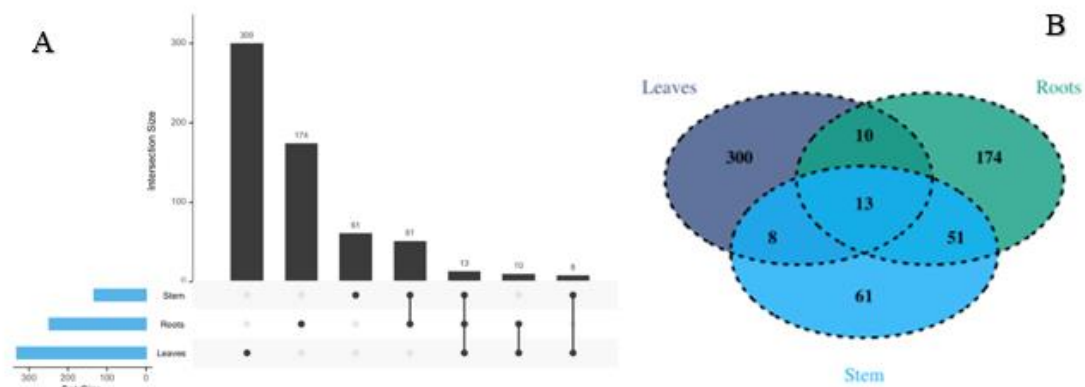


Figure 3.7b: Operational Taxonomic Units (OTU) distribution of *L. frutescens* by plant part (A). The Venn diagram was plotted using the sum of OTUs present in each plant part (B).

Bray-Curtis analysis was utilised to measure the dissimilarities in bacterial endophytic species composition between *L. frutescens* plant parts (Figure 3.8b). Principal Coordinate Analysis (PCA) was used to visualise endophytic bacterial communities associated with *L. frutescens* plant part. The samples varied in all parameters with 46.09% (PCo1) and 22.49% (PCo2) variation as the bacterial community of endophytes clustered by plant parts. There was a distinctive clustering of *L. frutescens* plant samples by plant parts. Leaf samples clustered on the negative side of the PCA, stem samples on the positive side, and root samples on the right side of the PCA (Figure 3.8b).

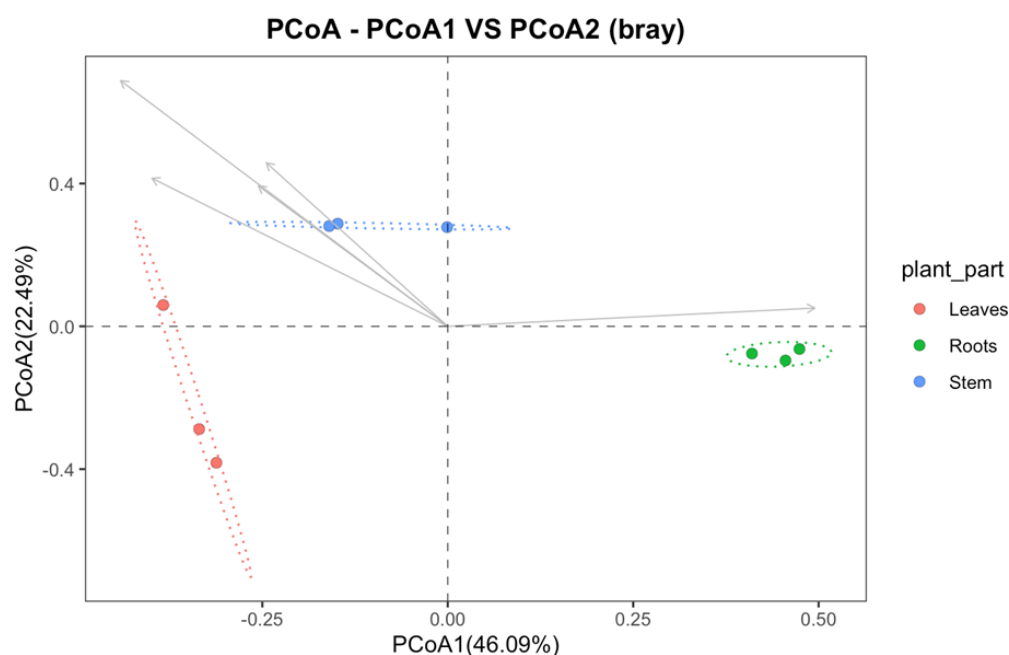


Figure 3.8b: Principal component analysis (Bray-Curtis analysis) illustrating the dissimilarities in bacterial endophytic species composition between plant parts of the *L. frutescens* medicinal plant

3.8.4. Relative abundance of bacterial endophytes from *Lessertia frutescens*

Figure 3.9b represents the relative abundance of top endophytic bacteria genera from *L. frutescens* by plant parts. *Enterobacteriaceae*, *Pseudomonas*, *Microbacteriaceae*, *Proteus*, *Agrobacterium*, *Williamsia*, *Rhodococcus*, *Snodgrassella*, *Rhizobiales*, *Staphylococcus*, *Lactobacillus*, *Actinomycota* and *Streptomyces* were amongst the most abundant bacterial endophytes from all plant parts of *L. frutescens* at the genus level. The most abundant genera in *L. frutescens* leaves were *Enterobacteriaceae*, followed by *Proteus* and *Microbacteriaceae*. The most abundant genera in *L. frutescens* roots were *Pseudomonas*, *Microbacteriaceae* and *Agrobacterium*. In *Lessertia frutescens* stem, *Enterobacteriaceae* were the most abundant.

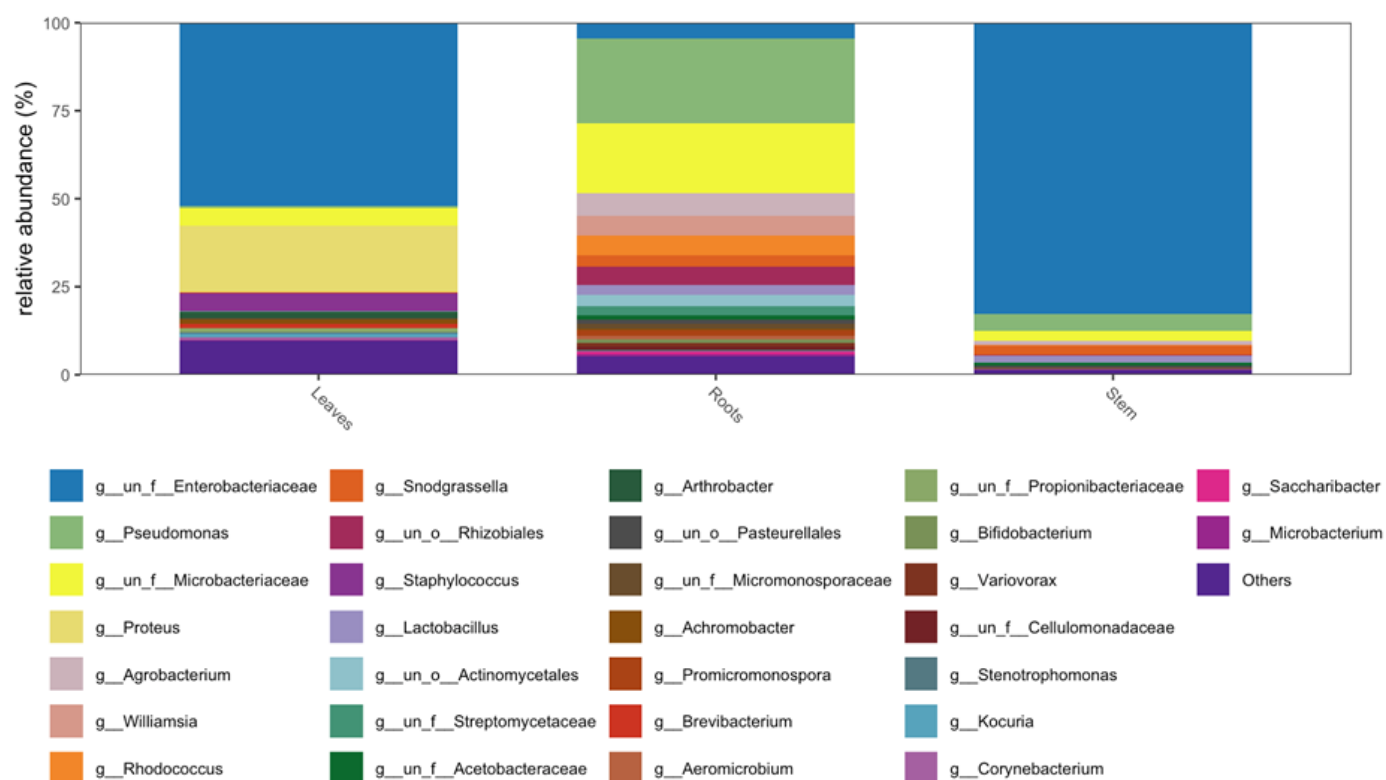


Figure 3.9b: Representation of the relative abundance of top endophytic bacteria from *L. frutescens* by plant parts at the genus level.

Figure 3.10b represents the relative abundance of top endophytic bacteria phyla from *L. frutescens* by plant parts. Overall, Proteobacteria, Actinobacteria, and Firmicutes were the most abundant bacterial endophytes at the phylum level. Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the most abundant in *L. frutescens* leaves. The representation of Proteobacteria and Actinobacteria was similar, and most abundant in *L. frutescens* roots. Firmicutes were the least represented amongst the three. *Lessertia frutescens* stem was predominated by Proteobacteria.

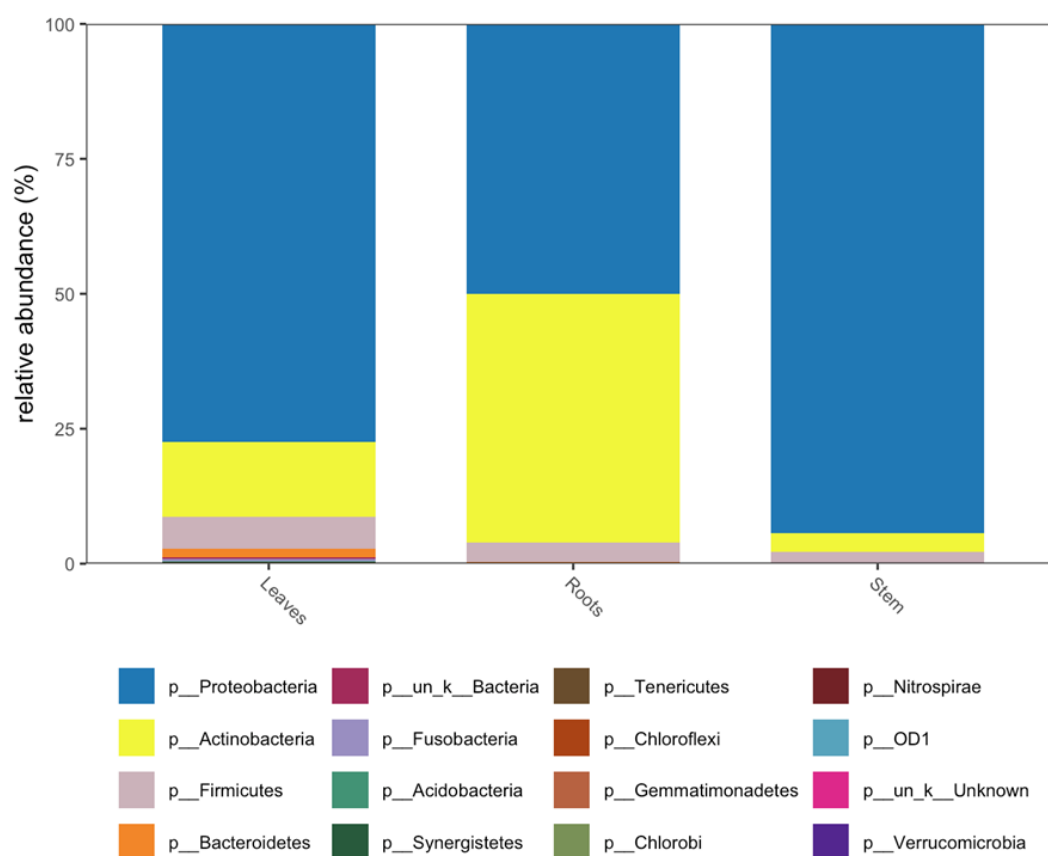


Figure 3.10b: Representation of the relative abundance of top endophytic bacteria from *L. frutescens* by plant parts at phyla level

Redundancy analysis (RDA) was performed on relatively abundant bacteria genera and abundant metabolites in *L. frutescens* leaves and stem (Figure 3.11b). The lime-coloured dots denote the samples from leaves and the blue dots from stem. Endophytes are in red while metabolites are shown in blue. Stem samples correlated negatively with metabolites produced by *L. frutescens*. Significant correlations were found between bacterial endophytes and metabolites from *L. frutescens*. On leaf samples (indicated by line dots), metabolites including ibuprofen and n-acetylglucosamine positively correlated with bacterial endophytes such as *Brevibacterium*, *Arthrobacter*, *Xanthomonas*, *Azospirillum*, *Pseudomonas* and *Kineococcus*.

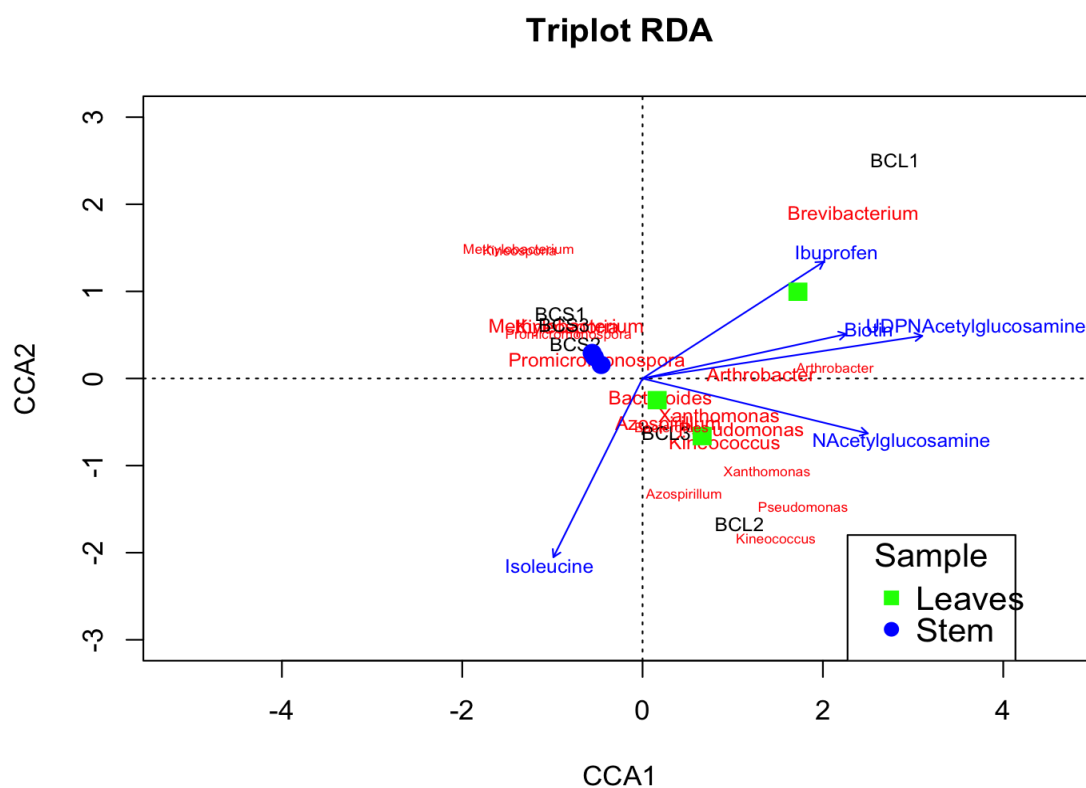


Figure 3.11b: Correlation between relative abundant bacteria genera and of abundant metabolites in *L. frutescens* leaves and stem performed using Redundancy analysis (RDA) on relatively abundant bacteria genera and abundant metabolites in *L. frutescens* leaves and stem.

3.9. DISCUSSION

3.9.1. Effect of plant parts on plant metabolite production

Phytochemical research has revealed high structural diversity in plant metabolite production as influenced by plant parts. Shown by ¹H NMR spectra, the resonance signals of aliphatic compounds were dominant in the leaf and stem extracts of *L. frutescens* (Figures 3.1b and 3.2b). There were chemical variations between leaf and stem samples for *L. frutescens* from this study as shown in Figure 3.3b and Table 3.1b. Campbell, (2012) also reported chemical variations from *L. frutescens* leaf and stem samples. *Lessertia frutescens* medicinal plants are rich in polysaccharides (Zhang et al., 2014). Different compounds were obtained from *L. frutescens* leaves and stems, including proline. Proline, alanine, and l-asparagine were found to be the most abundant amino acids in *L. frutescens*, accounting for more than 60% of the total amino acids obtained (Mncwangi & Viljoen, 2012). The proline and alanine were also present in this study, as shown in Table 3.1b. Proline amino acid from *L. frutescens* over the years had usually ranged in concentration between 0.7-7.5 mg/g, indicating a deterioration in its quality and quantity (Van Wyk & Albrecht, 2008). However, in the present study, proline content from *L. frutescens* was relatively high. Proline concentrations in plant reproductive organs have been reported to be comparatively higher than in other sections of the plant, such as the stem, which could account for the discrepancies in proline content between the stem and leaves (Hlongwane et al., 2023).

The composition and concentration of metabolites have been shown to be plant part-specific (Wink, 2008b). In the present study, as shown in Table 3.1b, stem samples synthesised relatively few but different compounds compared to leaves. Ornithine, arginine, pipecolate and isoleucine were some of the recovered aliphatic amino acids from leaves of *L. frutescens*, as well as proline and tryptophan. Aliphatic compounds recovered from the stem included lysine, isoleucine, α -acetyllysine and leucine. Sugars compounds recovered from leaves included fucose, ribose, mannose, threonate and mannitol. Stem samples synthesised more sugars than leaf samples, including fucose, glucitol, lactose, lactulose, maltose, ribose, and mannose. With more chemicals retained from *Gentianaceae* leaves, (Feng et al., 2018) also observed that metabolite synthesis and concentration are plant-specific.

Variations in bioactive chemicals among *Hypericum* plant parts were also observed by (Çırak et al., 2007). When the entire plant was examined (leaves, stem, and flowers), more metabolites were discovered. However, when plant components were individually analysed,

differences in compounds and their respective concentrations were revealed. For example, *Hypericum perforatum* leaves had hypericin, which was not present in the stem, but the stem contained rutin, which was lacking in the leaves. Both of these chemicals were found chiefly in *Hypericum perforatum* (Çırak et al., 2007). Qi et al. (2017) observed differences in the accumulation of secoiridoid glycosides in the segments of *Gentiana rigescens franch* plants and suggested that distinct plant portions may generate the same chemicals in differing amounts. Even in the present study, leaves synthesised a number of other compounds, including N-acetylglucosamine, UDP-N-acetylglucosamine, guanosine, carnitine, cadaverine, valproate, and biotin, which were not present in the stem. It is proposed that for medication development, all plant parts with medicinal potential should be analysed and integrated for improved biological activity (Wink, 2008a).

Variations in nutrient compositions inside the plant may result in varied plant metabolite production. For example, nitrogen is one of the most significant nutrients that affect the quality and quantity of plant metabolites (Hassan, 2012). An increase in fixed nitrogen in specific plant parts is one of the factors contributing to metabolite variation in leguminous plant parts. Leguminous plants form a symbiotic relationship with bacteria to fix atmospheric N₂. Depending on the legume species, the final product of N₂ fixation is ammonia in the form of ureides or amides (Baral et al., 2016). However, ammonia is transferred from the roots to the leaves as ureides and amides. Once in the leaves, the ureides are catabolised into ammonia, which is then transformed into amino acids and proteins for plant growth (Bosse et al., 2021). The influence of N-fixation in leguminous plants such as *L. frutescens* is thus expected to be higher in the leaves. N-fixation is projected to increase the formation of N-containing metabolites. Lastly, reactive oxygen species (ROS) have been revealed to have a role in legume-rhizobium symbiosis, ROS being well recognised as one of the factors controlling plant metabolite production (Stambulska & Bayliak, 2020).

3.9.2. Plant parts as possible drivers of endophytic bacterial community structure of *Lessertia frutescens*

The interaction between plants and endophytes is well recognised. However, the effect of plant parts on plant endophytic community structure remains unclear in medicinal plants, particularly *L. frutescens*. There is increasing evidence that plant parts have an influence on endophytic community structure. For instance, carrot leaves had a greater diversity of

endophytes than roots, and this was linked to a higher supply of resources in the leaves (Surette et al., 2003). Additionally, *Pseudomonas* was more prevalent in potato stems than roots (Garbeva et al., 2001). The findings of this investigation were consistent with previous observations in which plant parts had an impact on the structure of bacterial endophytic communities. *Lessertia frutescens* plant parts had an influence on OTU richness and endophytic diversity (Figure 3.6b). As indicated by high observed OTUs and Chao1 indices, *L. frutescens* leaves, followed by roots, had a high species richness and diversity, while the stem displayed a lower species richness and diversity (Figure 3.6b). It has also been indicated that a community dominated by one or a few species has a lower evenness index (Zhang et al., 2020). This could be true for the *L. frutescens* stem, which had the lowest indices. Furthermore, OTUs in three different parts in the present study were specific to plant parts. As illustrated in Figure 3.7b, the distribution of OTU revealed that 300 OTUs were unique in leaves, 174 in roots, and 61 in stems. On the other hand, 13 OTUs were common in leaves, roots and stem samples, ten were common in leaves and roots, eight were common in leaves and stem, while 51 were common in roots and stem. After colonising a plant through its roots, endophytes migrate from one location to another, entering the vascular tissues and spreading throughout the plant (Zakria et al., 2007). Therefore, it was not surprising that the leaves, roots, and stems shared OTUs. It was also discovered that each internal plant portion contains a unique microbial structure (Edwards et al., 2015). The discrepancies are brought about by a variety of factors, such as environmental perturbations that cause exposed tissues to have relatively low abundance (Lindow & Brandl, 2003). The presence of different microbiomes within the plant is also caused by variations in resource availability, such as leaves often containing a higher amount of nutrients compared to other plant parts (Fitzpatrick et al., 2018). Having different microbiome community assemblages is also caused by a variety of plant characteristics, including leaf permeability, the production of root exudates, variations in plant parts' immunity to endophytic invasion, and plant parts' capacity to produce antibiotics (Bodenhausen et al., 2013). For instance, leaves are known to produce more bioactive compounds than stems and roots, and thus more influence on microbial variation, diversity and relative abundance could be expected in plant leaves.

Endophyte host plant specificity may also result from varying nutritional demands (Cai et al., 2017). For example, leguminous plants have a symbiotic relationship with nitrogen-fixing microorganisms, and because of their capacity to fix nitrogen and make it available to their host, N-fixing endophytes may be preferred over non-fixing microbes. Thus, endophyte

community structure can be influenced by plant-microbe interaction (Dong et al., 2003). Symbiotic relationships between leguminous plants and bacteria, primarily those in the rhizobiaceae family, that lead to the development of root nodules and ammonia for plant use, are produced from atmospheric nitrogen by bacteria in the nodules (Hnini & Aurag, 2024). However, legumes have been shown to harbour both rhizobial and non-rhizobial endophytic bacteria, the majority of which include *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Agrobacterium* (Hnini & Aurag, 2024). These were abundant in the present study as shown in Figure 9. This abundance in leguminous plants is connected with their plant growth-stimulating activity, employing multiple mechanisms such as biofertilisation, phytostimulation, and biocontrol activity (Hnini & Aurag, 2024).

3.9.3. Relative abundance endophytes from *Lessertia frutescens*

As previously stated, several endophytes, including *Pseudomonas*, were abundant in the current investigation (Figure 3.9b). Their abundance was likewise plant-specific. For instance, the most abundant genera in *L. frutescens* leaves were Enterobacteriaceae, followed by *Proteus* and *Microbacteriaceae*. The most abundant genera in *L. frutescens* roots were *Pseudomonas*, *Microbacteriaceae* and *Agrobacterium*. In *L. frutescens* stem, Enterobacteriaceae genera were the most abundant. Overall, however, *Enterobacteriaceae*, *Pseudomonas*, *Microbacteriaceae*, *Proteus*, *Agrobacterium*, *Williamsia*, *Rhodococcus*, *Rhizobiales*, *Staphylococcus*, *Lactobacillus*, *Actinomycetales* and *Streptomycetaceae* were amongst the most abundant bacterial endophytes from all plant parts of *L. frutescens* at the genus level (Figure 3.9b). Some of these endophytes have high pharmaceutical and agricultural value. For instance, *Streptomycetaceae* have a variety of functions that include antimicrobial activity and plant development (Barman & Dkhar, 2020). On the other hand, the relative abundance of *Rhodococcus* in different environments has been linked to its ability to withstand unfavourable conditions, as high ROS, UV, extreme temperatures, heavy metal toxicity, drought, and extreme pH can best explain the high abundance of this microbe in habitats that include medicinal plants (Ivshina et al., 2022; Pátek et al., 2021). Elucidating the mechanisms used by *Rhodococcus* to cope under harsh conditions can provide new opportunities for its extensive application in other facilities, including novel medication synthesis in the medical industry (Ivshina et al., 2022). *Rhodococcus* exhibits antitumor, anticancer, and antiviral, as well as antimicrobial activity with the ability to produce quinolone antibiotics (Ivshina et al., 2022). Because of its ability to endure severe

temperatures, *Rhodococcus* is one of the endophytic bacteria whose source of bioactive chemicals has not been completely studied, with a high probability of discovering novel compounds (Debnath et al., 2016). Consequently, they present a bigger chance to combat the global epidemic of antibiotic resistance issues. They have also been highlighted as having a potential for biofuel biosynthesis (Ivshina et al., 2022). On the other hand, *Proteus* species, which were also abundant in this study (Figure 3.10b,) are thought to be among the bacteria that can be used in the bioremediation of contaminated environments, guaranteeing environmental safety, because of their capacity to promote plant growth and increase plant tolerance to pollutants (Drzewiecka, 2016).

As one of the dominant endophytic genera in *L. frutescens*' leaves and stems (Figure 3.9b), *Enterobacteriaceae* are extensively distributed bacteria in numerous habitats, including soil, water, and plants (Khalifa, 2020). Several *Enterobacteriaceae* microorganisms have been discovered to have multiple growth-promoting abilities, even though some are opportunistic pathogens. Through a variety of processes, including nutrient acquisition (N-fixation, phosphate solubilisation, hormone and siderophore production), they have been shown to promote plant growth and development. They also function as biocontrol agents by producing antimicrobial compounds (Khalifa, 2020; Ramesh et al., 2014; Taghavi et al., 2010). Among the microorganisms that promote plant growth are *Microbacteriaceae*, which are found in a variety of natural environments, including water, the sea, and plants (Gupta et al., 2022). They were prevalent in the current study, especially in *L. frutescens* leaves and roots (Figure 3.9b). They exhibit a wide range of adaptability under varied conditions and have been demonstrated to be resistant to UV-radiation, heavy metals, drought, and temperature extremes (Corretto et al., 2020). They can break down a range of polysaccharides, including starch (Gupta et al., 2022). They have a high proportion of genes involved in the biosynthesis of important metabolites (Corretto et al., 2020). *Microbacteriaceae* contain a range of bioactive peptides (BAPs), which may contribute to their capacity to tolerate harsh environments (Udenigwe & Aluko, 2012). The ability of endophytes, including the *Microbacteriaceae* genus, to synthesise bioactive peptides (BAPs) demonstrates the medical significance of these microbes. BAPs have a number of activities, including antioxidant, antibacterial, and immunomodulatory effects (Akbarian et al., 2022; Raveschot et al., 2018). *Lactobacillus* is also a well-known producer of bioactive peptides (Stefanovic et al., 2017). *Lactobacillus* was also abundant in the present study and has numerous industrial applications, including fermentation (Raveschot et al., 2018).

Biological nitrogen fixation and rhizobia-legume symbiosis are two economically viable and prominent sustainable farming technologies that promise to assure food security (Laranjo et al., 2014). *Rhizobium* species, such as *Rhizobiales*, have a wide geographic distribution and can nodulate a variety of leguminous plants (Laranjo et al., 2014). *Rhizobiales* spp. were prevalent in the current study (Figure 3.10b), and it is likely that it was one of the endophytes that contributed to the plant's microbial diversity and structure. Yang et al. (2024) also discovered high abundance of Proteobacteria phyla and their corresponding genera, *Rhizobiales*, from *Scutellaria baicalensis* medicinal plant. Through biological nitrogen fixation, *Rhizobiales* play a significant role in promoting plant growth, especially for leguminous plants (Yang et al., 2024). In addition to fixing nitrogen, *Rhizobiales* bacteria also contain methylophs and methanotrophs (Nakai et al., 2020). Furthermore, they can produce phytohormones, and have been demonstrated as able to act as biocontrol agents by protecting plants from diseases (Kim et al., 2014). They have been found to produce a number of compounds that degrade polycyclic aromatic hydrocarbons and are therefore recommended for bioremediation of polluted areas (Staninska-Pięta et al., 2022). *Rhizobia* microorganisms are known to produce N-acyl homoserine lactones, which assist in regulating genes important for quorum sensing, such as genes contributing to the adaptability of these microbes (Sanchez-Contreras et al., 2007).

Lastly, the most dominant bacterial phyla on *L. frutescens* medicinal plants were Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Figure 3.10b). Researchers have reported the abundance of these phyla on medicinal plants. For example, Zhang et al. (2020) observed a significant abundance of Bacteroides from the *Polygonum cuspidatum* medicinal plant. Actinobacteria, Proteobacteria, and Firmicutes were found in high abundance in *Stellera chamaejasme* medicinal plants (Zhang et al., 2024). High abundances of the phyla Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, as well as the genus *Pseudomonas* from medicinal plants were reported by (Dang et al., 2020). Bacteroidetes have been shown to serve a wide range of roles, including promoting plant development and stimulating metabolite production (Zhang et al., 2020). Among Firmicutes phyla, *Bacillus* species are the most prevalent and widely used because of their numerous benefits in a variety of industries, such as agriculture (as a plant growth-promoting and pathogen biocontrol agent), pharmaceuticals (as a metabolite producer), and industry (as a phytoremediation of pollutants) (Hashmi et al., 2020). Proteobacteria are also the most common bacteria in the environment (Miyashita 2015; Yadav et al., 2017). Proteobacteria play a vital role in carbon cycling, due to their ability to degrade cellulose and lignin (Lagos

et al., 2015). They are known to increase root nodulation, promote root growth and suppress soil-borne diseases (Lagos et al., 2015). On the other side, Actinobacteria are natural soil dwellers and can easily penetrate the roots, resulting in their high relative abundance in plants (Golinska et al., 2015). The prevalence of these phyla, such as Actinobacteria from medicinal plants, posits substantial importance, not only in the agricultural sector by enhancing plant growth, but also in the pharmaceutical industry for the production of new drugs (Barman & Dkhar, 2020). For example, it has been shown that antibiotics produced from Actinobacteria endophytes have a significant level of antimicrobial action even at relatively low concentrations, and some of those endophytes include *Streptomyces* (Golinska et al., 2015).

3.9.4. Endophytes' effect on plant metabolite production

While environmental factors influencing the synthesis of metabolites in *L. frutescens* have been demonstrated, there is still a lack of data regarding its response to various factors such as endophytes. Medicinal plants host various endophytes with specific metabolic properties, resulting in a huge and diverse spectrum of plant metabolite richness (Nguyen et al., 2023). The positive correlation between endophytes and metabolites from *L. frutescens* leaves was noted, as illustrated in Figure 3.11b. Because of their higher nutritional importance, leaves have been shown to have a higher relative abundance and diversity of endophytes than stems (Kumari et al., 2023). High diversity and abundance may boost the activity and functioning of these endophytes, including supporting plants in the generation of metabolites. Plants secrete metabolites, which results in the selection of endophytes for colonisation, and in a strong correlation between endophytes and metabolites (Miao et al., 2022). Metabolites are also produced by medicinal plants to combat both biotic and abiotic influences (Köberl et al., 2013). The plant microbiome conveys signals to the plant and activates pathways involved in metabolite biosynthesis (Ahmed & Hijri, 2021). The interior plant tissues can only be successfully colonised and inhabited by endophytes that possess certain characteristics, such as quorum sensing, plant polymer breakdown machinery, and redox response regulatory systems. The interaction between endophytes and plants can then activate plant metabolite synthesis. Prominent endophytes that have been shown to accumulate and produce metabolites in their host plants include *Bacillus subtilis*, *Pseudomonas* sp., and *Stenotrophomonas* (Xu et al., 2021; Ye et al., 2021).

3.10. CONCLUSION

The parts of the *L. frutescens* medicinal plant significantly influence both metabolite production and the composition of its endophytic bacterial community. Multivariate analysis revealed variations in metabolite production between the leaves and stems of *L. frutescens*. Additionally, Chenomx-assisted annotation indicated that leaves contained a greater number of metabolites than stems. Significant correlations were observed between the endophytic bacteria and the metabolites produced by *L. frutescens*. Redundancy analysis (RDA) demonstrated a positive correlation between the bacteria and metabolites in the leaves, while a negative correlation was found for the stem samples.

4. CHAPTER FOUR: PLANT GROWTH-PROMOTING POTENTIAL OF BACTERIAL ENDOPHYTES ISOLATED FROM *LESSERTIA FRUTESCENS*

This chapter forms part of Objective 2 and 3 of the study but only focuses on isolation of endophytes from *Lessertia frutescens* as well as their evaluation for plant growth promotion and enzyme activities. It further evaluated the effect of selected two endophytes of *Lessertia frutescens* on their ability to enhance seedling growth of *Lessertia frutescens* in a pot trial experiment. This chapter was published in South African Journal of Botany. Tsipinana, S., Obi, L., Amoo, S. and Adeleke, R., 2024. Plant growth-promoting potential of bacterial endophytes isolated from *Lessertia frutescens*. *South African Journal of Botany*, 174, pp.768-778. <https://doi.org/10.1016/j.sajb.2024.09.043>.

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Plant growth-promoting potential of bacterial endophytes isolated from *Lessertia frutescens*

Sinawo Tsipinana^a, Linda Obi^a, Stephen Amoo^{a,b}, Rasheed Adeleke^{a,*}

^a Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa
^b Agricultural Research Council – Vegetables, Industrial and Medicinal Plants, Rooiboskloof, Pretoria, South Africa

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Seedling growth

ABSTRACT

Many endophytic bacteria have plant growth promoting attributes that could improve agricultural yield as well as resistance to various types of stress and disease. Detection of such attributes usually involves in vitro screening and the subsequent testing of the isolates on the plants for specific or combined effects on germination, seedling growth and yield. Despite many studies in this field, there is a knowledge gap on the influence of endophytes on early growth of certain economically important plants such as medicinal plants. In the present study, we evaluated the in vitro capabilities of bacterial endophytes isolated from *Lessertia frutescens* (L.) Goldblatt & J.C. Manning (syn. *Sutherlandia frutescens* (L.) W.T. Aiton) for plant growth promotion and their effect on initial seedling growth. Using a culture-dependent approach, plant samples of *Lessertia frutescens* were screened for bacterial endophytes. The isolated bacterial endophytes were subsequently evaluated for their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes. Molecular identification of selected endophytic bacteria based on the 16S rRNA genes of strains that tested negative in the hemolysis test was conducted. The two most promising isolates were evaluated for their ability to promote *Lessertia frutescens* seedling growth in a two-month pot trial study. The obtained results revealed that many of the bacterial endophytes had potential to promote plant growth. Specifically, 86% of the endophytes possessed nitrogen-fixing, phosphate solubilizing, and IAA-producing abilities, while approximately 71% were able to exhibit siderophore-producing capabilities. The endophytes exhibited significant production of essential hydrolytic enzymes, including amylase (86%), gelatinase (86%), protease (29%), lipase (43%), and D-nase (57%). The two best isolates were identified as relatives of *Bacillus* spp. (*Bacillus licheniformis* BaDB06 and *Bacillus velezensis* strain SM-95). Their plant growth-promoting properties such as their ability to enhance plant height and their ability to be used as bio-agent were further confirmed in the pot trial study as they enhanced the growth of *Lessertia frutescens* seedlings compared to the control. This study provides insights into the functional roles of endophytic bacteria of *Lessertia frutescens* in seedling growth and their potential plant growth enhancement, highlighting their potential for sustainable agriculture and ecosystem management.

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ABSTRACT

Many endophytic bacteria have plant growth promoting attributes that could improve agricultural yield as well as resistance to various types of stress and disease. Detection of such attributes usually involves *in vitro* screening and the subsequent testing of the isolates on the plants for specific or combined effects on germination, seedling growth and yield. Despite many studies in this field, there is a knowledge gap on the influence of endophytes on early growth of certain economically important plants such as medicinal plants. In the present study, we evaluated the *in vitro* capabilities of bacterial endophytes isolated from *Lessertia frutescens* (L.) Goldblatt & J.C. Manning (syn. *Sutherlandia frutescens* (L.) W.T.Aiton) for plant growth promotion and their effect on initial seedling growth. Using a culture-dependent approach, plant samples of *Lessertia frutescens* were screened for bacterial endophytes. The isolated bacterial endophytes were subsequently evaluated for their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes. Molecular identification of selected endophytic bacteria based on the 16S rDNA genes of strains that tested negative in the hemolysis test was conducted. The two most promising isolates were evaluated for their ability to promote *Lessertia frutescens* seedling growth in a two-month pot trial study. The obtained results revealed that many of the bacterial endophytes had potential to promote plant growth. Specifically, 86% of the endophytes possessed nitrogen-fixing, phosphate solubilizing, and IAA-producing abilities, while approximately 71% were able to exhibit siderophore-producing capabilities. The endophytes exhibited significant production of essential hydrolytic enzymes, including amylase (86%), gelatinase (86%), protease (29%), lipase (43%), and D-nase (57%). The two best isolates were identified as relatives of *Bacillus* spp (*Bacillus licheniformis* BaDB06 and *Bacillus velezensis* strain SM-95). Their plant growth-promoting properties such as their ability to enhance plant height and their ability to be used as bio-agent were further confirmed in the pot trial study as they enhanced the growth of *Lessertia frutescens* seedlings compared to the control. This study provides insights into the functional roles of endophytic bacteria of *Lessertia frutescens* in seedling growth and their potential plant growth enhancement, highlighting their potential for sustainable agriculture and ecosystem management.

Keywords: *Lessertia frutescens*, endophytes, indole acetic acid, plant growth promoting, seedling growth

4.1. INTRODUCTION

Exploring interactions between plants and microbes presents significant potential for sustainable agriculture, providing innovative approaches to enhance crop yield while minimizing dependence on chemical interventions (Sudheer et al., 2020). Among these microorganisms, endophytes inhabiting plant tissues have emerged as key contributors to enhancing plant growth and bolstering resilience against environmental pressures (Anukool Vaishnav et al., 2019). Endophytes have gained a massive commercial and economic interest due to their significant role in improving plant health and development (Cardoso et al., 2019). Endophytes and their host plants have no competition for space and essential substances such as nutrients (Ghasemnezhad et al., 2021). Instead, endophytes play a vital role in plant health through various processes, such as phyto-stimulation, biological nitrogen fixation, phosphate solubilisation, siderophores production as well as protection of host plants against stress and diseases. Endophytes from several crops, including maize, soybean, wheat, and some vegetables, have been exploited for their potential to promote plant growth (Rana et al., 2020). However, research interests on endophytes from medicinal plants as microbial bio-stimulants are currently low. Endophytes from medicinal plants have been reported to exhibit antifungal, antiviral and antibacterial and other biological properties, similar to those of their host plants (Palanichamy et al., 2018). The use of endophytes from medicinal plants with antimicrobial traits could play a big role in protecting plants from biotic stress while promoting plant growth.

Leguminous medicinal plants such as *Lessertia frutescens* L. R. Br. (= *Sutherlandia frutescens* L.) is a shrub belonging to the Fabaceae family and is native to the Southern Africa: Namibia, Lesotho, Botswana, Zimbabwe, and South Africa (Aboyade et al., 2014; Zonyane et al., 2019). Even though it is available in the coastal and inland parts of South Africa, *Lessertia frutescens* is highly distributed in the Western Cape and Karoo regions of South Africa (Street & Prinsloo, 2013). *Lessertia frutescens* holds significant medicinal and ecological importance. Identified bioactive compounds within the *Lessertia frutescens* exhibit pharmacological activities including anti-inflammatory, antioxidant, immunomodulatory, and anticancer properties, among others. Extracts and formulations from *Lessertia frutescens* are currently being investigated for their potential therapeutic benefits in conditions such as diabetes, HIV/AIDS, cancer and immune –related disorders (Mudau et al., 2022). The ability of *Lessertia frutescens* to thrive in different habitats including disturbed habitats helps to

stabilize ecosystems and aid in rehabilitation of degraded environments (Zonyane et al., 2020). Understanding and harnessing the dual significance of *Lessertia frutescens* can pave the way for holistic approaches to health and environmental sustainability. *Lessertia frutescens* harbours a variety of endophytic microbes with different essential functions. Endophytes associated with *Lessertia frutescens* have been implicated in various applications, including antimicrobial abilities (Sishuba, 2022) and the production of bioactive compounds (King, 2021), among others. However, the full extent and significance of their contributions to plant growth and development have largely remained unexplored. Exploring endophytes from *Lessertia frutescens* can be helpful in promoting the cultivation of this medicinal plant, which is highly valued in the pharmaceutical industry. Understanding the diversity and dynamics of the endophytic bacterial community within *Lessertia frutescens* holds significant potential for exploration and application in agriculture and medicine. It is critical to ascertain the degree to which essential microbes can influence the growth of their host plant and to track their impacts separately from those of other endophytes. Thus, this study aims to evaluate the ability of plant-growth promoting endophytes isolated from *Lessertia frutescens* medicinal plant as potential gateways to sustainable agriculture by evaluating their plant growth-promoting attributes along with their ability to produce hydrolytic enzymes and their ability to enhance *Lessertia frutescens* seedling growth.

4.2 METHODS

4.2.1 Isolation of bacterial isolates from *Lessertia frutescens*

Lessertia frutescens medicinal plants (18 plants) were obtained from Eden nursery in Klerksdorp, North-West Province of South Africa in April 2021. *Lessertia frutescens* cultivation practices included seed sowing and the use of chemical fertilizer (fertilizer 2:3:2 (N: P: K)) as a source of nutrients. The plants were watered daily or as needed during their growth period. The plant samples were collected and transported on ice to the laboratory at North-West University Potchefstroom campus, where they were surface sterilized. The plants were immediately rinsed with tap water and separated into segments for effective surface sterilization. Surface sterilization was carried out as described by (Palanichamy et al., 2018). Plant parts were submerged in 95% ethanol for 30 seconds, 2.5% sodium hypochlorite solution for 5 mins, sodium hypochlorite was washed off by 70% ethanol for 1 min and finally washed three times with double distilled water to wash the substances off. To test the efficiency of surface sterilization, small portions of each plant part was plated on nutrient agar and incubated at 37 °C for 24-48 h. The absence of microbial growth indicated the

success of the sterilization process. The plant segments (roots, stem and leaves) were air-dried in a sterile environment and later crushed using a pestle and mortar. For each sample, 1 g of crushed plant material was mixed with 9 ml of sterile distilled water and shaken. The suspension was serially diluted (10^0 - 10^{-6}) and 100 μ L aliquots of 10^{-6} dilution were plated in nutrient agar and incubated at 37 °C for 24 h for bacterial endophytes isolation. The endophytes were then sub-cultured until pure cultures were obtained.

4.2.2 Test for pathogenicity of endophytes: haemolytic test

All the pure culture isolates of the bacterial endophytes were subjected to hemolysis test as described by (Amaria et al., 2023). Bacterial endophytes were spot inoculated on sheep blood agar. The agar plates were then incubated for 48 h at 30 °C. The change in colony color and/or appearance of clear zone around the colony of the bacterial endophytes on the blood agar indicated positive hemolytic reaction of the strain. A change in the colour of the strain to dark or greenish grey indicated partial lysis of the blood cells, which is known as alpha hemolysis, while the appearance of a clear zone around the strain indicates a complete lysis of the blood cells and the reaction is known as beta hemolysis. The absence of color change or a clear zone around the colony indicates a negative hemolytic reaction of blood cells to the bacterial endophytes and the reaction is known as the gamma hemolysis. The endophytes that tested negative for hemolysis were identified molecularly and then tested for their plant growth promoting properties by testing their ability to fix biological nitrogen, solubilize insoluble phosphate, and produce indole acetic acid and siderophore as well as their ability to produce hydrolytic enzymes.

4.2.3 Molecular identification of bacterial endophytes from *Lessertia frutescens* plants

Endophytes that tested negative for haemolytic test were identified using SANGER sequencing as described by (Palanichamy et al., 2018) with modifications. Bacterial genomic DNA from each pure endophytic strain was extracted using Fungal/Bacterial DNA miniPrep™ kit (Zymo Research Corporation, USA) following manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify the V3-V4 region of bacteria's 16S rDNA gene using universal forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). To achieve the final volume of 25 μ L, the PCR reaction included 12.5 μ L of Taq 2x Master Mix with Standard Buffer (New England, Biolabs Inc.), 1 μ L of each primer (10 μ M), 5 μ L of bacterial DNA, and nuclease-free water to bring to 25 μ L. Following that, the mixture was amplified for 35 cycles at the following settings: 95°C for 5 min for initial denaturation, 95°C for 30 seconds

for denaturation, 52°C for 30 seconds for annealing, 72°C for 1 minute for extension, and 72°C for 1 minute for final extension in a T100 (BioRad) thermal cycler. The size of the amplicons were confirmed by electrophoresis on a 1% agarose gel in TBE buffer, and the gel was visualized on a Gel DOC (BioRad Laboratories) after 45 minutes of running at $\geq 80V$. The amplicons were sequenced using SANGER sequencing at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) and the sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database by the Basic Local Alignment Search Tool (BLAST) program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLAST was used to assign unknown sequences by comparing them to known sequences to deduce their function based on the function of the strains' closest matches. BLAST was only used as a baseline for identification to better understand the evolutionary links between these endophytes. The sequences were submitted to the Genbank NCBI database to authenticate their identities. Additionally, whole genome sequencing data further validated the identification of several endophytes, primarily those used in the greenhouse experiment. For whole genome sequencing, the genomic DNA libraries were prepared, sequenced on an Illumina NovaSeq platform and 150 bp paired-end reads were generated.

Preparation of the genome assembly and annotation

The total sequenced reads generated from *B. paralicheniformis* and *B. velezensis* were filtered to remove adapter sequence and low-quality fragments using Trimmomatic v0.36 (Bolger et al., 2014) with a minimum quality score of 15 and a minimum sequence length of 70. The adapter regions were clipped using a mismatch value of 2, a palindrome clip threshold of 30, and a simple clip threshold of 10. The assembly was performed with the quality reads using SPAdes v3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020). Completeness of the assembled genome and presence of undesired sequence fragments were assessed using CheckM v1.0.18. The genome assembly was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v6.7 (Li et al., 2021) and Rapid Annotations using Subsystems Technology (RAST) with SEED viewer v. 2.0 (Overbeek et al., 2014). Mapping of the entire circular genome was visualised using Proksee (Grant et al., 2023) while the CRISPR array and Cas clusters were determined by CRISPR/Cas finder, and CARD resistance genes Identifier was used to identify antimicrobial resistance genes within the genome.

4.2.4 Determination of plant growth promoting attributes of endophytic bacteria

(a) Dynamics of nitrogen fixation by endophytic bacteria

Two approaches were utilized to determine the nitrogen-fixing capacity of bacterial endophytes isolated from *Lessertia frutescens*. The ability of bacterial endophytes to fix biological nitrogen was determined as described by (Hamza et al., 2017) on a nitrogen-free media (Burk's media) containing magnesium sulphate (0.2 g), dipotassium phosphate (0.8 g), monopotassium phosphate (0.2 g), calcium sulphate (0.13 g), ferric chloride (0.00145 g), sodium molybdate (0.000253 g), sucrose (20 g) and bacteriological agar (15 g) in 1 L. The endophytes that tested negative for hemolysis were grown in this N-free medium and incubated at 30 °C for 7 days. Bacterial endophytes developed into strong, elevated globular-like structures, demonstrating the dynamic nature of nitrogen-fixing activity on nitrogen-free media. The *NifH* gene, which regulates the microbial biological nitrogen fixation, was identified molecularly, providing additional evidence of those endophytes' capacity to fix nitrogen. The *NifH* gene profiles from the endophytes that demonstrated nitrogen fixation were identified as described by (Rusmana & Lestari, 2015) using PoL-F GC (5'-TGCGAYCCSAARGCBGACTC-3') and PoL-R (5'-ATSGCCATCATYTCRCCGGA- 3') primer set as found by (Poly et al., 2001). To achieve the final volume of 25 µL, the PCR reaction included 12.5 µL of Taq 2x Master Mix with Standard Buffer (New England, Biolabs Inc.), 1 µL of each primer (10 µM), 5 µL of bacterial DNA, and nuclease-free water to bring to 25 µL. Following that, the mixture was amplified for 30 cycles at the following settings: 95°C for 5 min for initial denaturation, 95°C for 1 min for denaturation, 52°C for 1 min for annealing, 72°C for 1 minute for extension, and 72°C for 5 minute for final extension in a T100 (BioRad) thermal cycler. The size of the amplicons were confirmed by electrophoresis on a 1% agarose gel in TBE buffer, and the gel was visualized on a Gel DOC (BioRad Laboratories) after 45 minutes of running at $\geq 80V$.

(b) Phosphate solubilisation

P-solubilization of bacterial endophytes were evaluated qualitatively on Pikovskaya agar medium (PVK) enriched with 0.5% of insoluble tricalcium phosphate (TCP) $Ca_3(PO_4)_2$ as indicated by (Pikovskaya, 1948). Pure isolates were inoculated on nutrient broth and incubated at 37 °C for 24 h. Three wells (with equal distance from between each and close to the centre) were then made on Pikovskaya's agar plates and 100 µl overnight cultures were transferred to each hole, and the plates were incubated at 30 °C for 7 days. The diameter of

the well and halo were measured. The same bacterial isolate was added to each of the three wells, and this was performed twice (two plates) to provide six replicates, with each well representing one replicate. The agar plates were examined for formation of a clear zone around the colonies for their capacity to solubilize TCP and their solubilizing indices (SI) were calculated as follows:

$$SI = \frac{\text{Solubilisation diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

The average SI from the wells were then computed, and the results were utilized to generate a pie chart that compared each bacteria's phosphate solubilizing capacity to others.

(c) Siderophore production

To determine the ability of the endophytes to produce siderophores, the Chrome Azural S (CAS) agar plate method was used, as described by (Mukherjee & Dutta, 2019). Blue agar solution was prepared by dissolving 60.5 mg of CAS in 50 ml of double distilled water and was mixed with 10 ml of iron (III) solution containing 1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 10mM HCl. This solution was autoclaved after adding 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) diluted in 40 mL of double distilled water. In a separate 1 L bottle, a mixture of 15 g of bacteriological agar, 100 ml (10 mM9 salts), 30.24 g of piperazine (PIPES), 12.0 g of a 50% (w/w) NaOH solution to raise the pH of PIPES (6.8), and 25% NaCl, all dissolved in 750 ml of double distilled water, was autoclaved. When the agar and blue solution reached 50 °C, the two solutions were mixed with stirring, and the agar was poured in agar plates and allowed to solidify before use. The endophytic microbes were then spot inoculated in blue agar and incubated for 7 days at 30 °C and this was replicated 6 times for each endophyte. The presence of a clear zone around the strain demonstrated the endophyte's ability to produce siderophores.

(d) Indole acetic acid (IAA) production

Indole acetic acid production by endophytes was determined as described by (Putrie et al., 2017). In 6 replicates, pure isolates were incubated in 10 mL nutrient broth supplemented with 1% of L-tryptophan for 48 h at 30 °C on a shaker at 200 rpm. Two ml of the microbial suspension was centrifuged at 10,000-rpm speed for 10 min at 4 °C. Following the transfer of the supernatant into a test tube, 2 ml of Salkowski's reagent (150 mL concentrated sulfuric acid (H_2SO_4), 250 mL distilled water, and 7.5 mL of 0.5 M FeCl_3) was added. The mixture was then incubated for 30 min at room temperature in the dark. The absorbance was

measured using a spectrophotometer at a wavelength of 540 nm with V-1100 D Spectrophotometer (Labex (pty), LTD). The standard IAA calibration curve was set up by determining the prepared different concentrations of IAA solution (0, 5, 10, 20, 50 and 100 µg/mL) as described by (Sarker & Al-Rashid, 2013). Two ml of each standard prepared was transferred to separate tubes, and 2 ml of Salkowski's Reagent was added to each standard. They were incubated at room temperature in the dark for 30 min, and IAA concentrations were measured at 540 nm. The results were plotted, and the equation from the standard curve was used to calculate the concentration of IAA for the samples.

4.2.5 Determination of the hydrolytic activity of extracellular enzymes

The plate method was used to detect enzymatic activity qualitatively, in which solid agar media was supplied with the basic components and appropriate substrates for the enzymes examined in this study. Proteolytic activity of bacterial endophytes was measured using the method described by (Olajuyigbe & Ajele, 2005) and (Khan et al., 2017) on skim milk agar containing 3% (w/v) skim milk (Oxoid, UK) and 35 g/L Mueller-Hinton agar (Merck, Germany). After incubation, colonies with a clear zone of skimmed milk hydrolysis suggested the endophyte's protease activity. Amylase activity was detected on Tryptone Soya Agar (Merck, Germany) supplemented with 1% w/v soluble starch, and 3 g of beef extract. Following incubation, the plates were flooded with 1% lugol solution, and the presence of a clear zone indicated endophyte amylase activity (Khan et al., 2017). Gelatinase activity was determined on agar plates containing Tryptone Soya Agar (Merck, Germany), supplemented with 3% gelatine powder (Merck, Germany). The endophytic isolates were spot inoculated, and the development of a turbid or clear zone around the bacteria following incubation indicated endophytic gelatinase activity. Lipase activity of endophytes was determined on agar plates containing Tryptone Soya Agar (Merck, Germany), supplemented with 1% of tween 80 (Sigma Aldrich, German). The endophytic isolates were also spot inoculated, and the development of a turbid or clear zone around the bacteria following incubation indicated endophytic lipase activity. The Dnase activity of the endophytes was determined using Dnase agar (Oxoid, UK) following the manufacturer's instructions. After incubation, the plates were flooded with 1 M hydrochloric acid (HCl) and the appearance of clear zones around the colony indicated the endophytes' Dnase activity. All the plates for enzyme determination were incubated at 37 °C for 24-72 h.

4.2.6 Pot experiment

The pot seedling trial was conducted in a growth chamber using two strains (*Bacillus licheniformis* BaDB06 and *Bacillus velezensis* SM-95) that showed all the plant growth promotion capacity and highest IAA production. The best IAA producing strains were chosen because IAA is known to stimulate seedling growth. *Lessertia frutescens* seeds were obtained from Seed for Africa Company (Cape Town, South Africa). The experiment consisted of 4 treatments replicated 6 times. The treatments were Treat-C (control=0 inoculum), Treat-A (A = *B. licheniformis* BaDB06 inoculum), Treat-B (B = *B. velezensis* SM-95 inoculum) and Treat-AB (AB = *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum). Six seeds for each replicate were incubated overnight in a nutrient broth containing the following: (1) nutrient broth only for the control (Treat-C), (2) *B. licheniformis* BaDB06 for Treat-A, (3) *B. velezensis* SM-95 for Treat-B, and (4) *B. licheniformis* BaDB06 + *B. velezensis* SM-95 for Treat-AB. The seeds were then planted in pots of the same size (20 cm in diameter) containing sterile coconut peat and they were placed in a growth chamber for the duration of the experiment. Temperatures at the growth chamber were kept at 30 °C during the day and 18 °C at night with constant 70% humidity. All treatments were watered every other day with 100 ml of double distilled water and fertilized with 100 ml of Hoagland solution on a weekly basis for nutritional supplementation right after emergence. The seedling growth was recorded after two months.

4.2.7 Statistical analysis

The influence of *Bacillus licheniformis* BaDB06 and *Bacillus velezensis* SM-95 on *Lessertia frutescens* seedling growth was determined statistically. Obtained data were subjected to one-way analysis of variance (ANOVA) to determine the differences among different treatment groups. The normality assumption was checked based on the Shapiro-Wilk Test ($\alpha=0.05$). Tests were set at 5% significance level ($P < 0.05$). Post hoc tests, Fisher's Least significant difference (LSD) and Tukey Honest Significance Difference (HSD) were used for pair-wise comparisons.

4.3 RESULTS

4.3.1 Hemolytic test results

A total of 25 bacterial endophytes were isolated from *Lessertia frutescens* plant parts, all together. Only seven endophytes tested negative for hemolysis, 10 of the isolates had a beta hemolytic reaction on blood cells and eight had an alpha haemolytic reaction on blood cells.

4.3.2 Plant growth promotion attributes

Table 4.1 summarizes plant growth-promoting activities of the *Lessertia frutescens* endophytic bacteria that tested negative for hemolysis. These endophytes from *Lessertia frutescens* exhibited a variety of activities, with each demonstrating at least one plant growth-promoting attribute. In total, 86% fixed biological nitrogen, 86% solubilized insoluble phosphate, 86% produced IAA, and 71% produced siderophores.

Table 4.1: Plant growth-promoting activities of some isolated *Lessertia frutescens* endophytes

Sample ID	Plant Part	Phylogenetic affiliation with similarity (16S rDNA sequencing)	Genebank Accession number	Similarity (%)	BNF	PS	SD	IAA
CBL1	Leaf	<i>Bacillus subtilis</i> LCDB-BP2	OP703608.1	97	+	+	-	+
CBL7	Leaf	<i>Bacillus licheniformis</i> BaDB06	JX237842.1	99	+	+	+	+
CBR4	Root	<i>Bacillus licheniformis</i> QT445	MT065669.1	97	-	+	-	+
CBR6	Root	<i>Kosakonia</i> sp. MBWS2.(8)	OP990041.1	99	+	+	+	+
CBR7	Root	<i>Bacillus velezensis</i> SM-95	MT377909.1	100	+	+	+	+
CBR11	Root	<i>Bacillus paralicheniformis</i> AJVR1	MT459810.1	97	+	+	+	+
CBR16	Root	<i>Bacillus subtilis</i> ZIM3	MT539995.1	99	+	-	+	-
Total					6/7 (86%)	6/7 (86%)	5/7 (71%)	6/7 (86%)

BNF=Biological Nitrogen Fixation, PS=Phosphate Solubilisation, SD=Siderophore and IAA=Indole Acetic Acid, AM=Amylase, PT=Protease, GT=Gelatinase, LP=Lipase and DN=D-nase

Figure 4.1 demonstrates bacterial endophytes that amplified for *NifH* gene

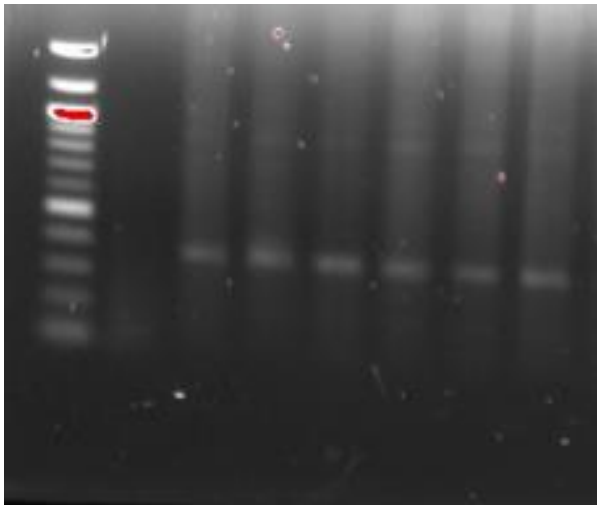


Figure 2.1: The *NifH* gene for endophytic bacteria isolated from *Lessertia frutescens* medicinal plant

Figure 4.2 exhibits the ability of each bacteria to solubilize phosphate in comparison to others. Each strain's percentage contribution to other strains has also been noted, with strains with high solubilizing indices having a higher percentage contribution. Six of the endophytes were capable of solubilizing insoluble tricalcium phosphate on agar plate with solubilizing indices between 1.29 and 1.36. In terms of the percentage contribution to the solubilization of insoluble phosphate, *Bacillus licheniformis* BaDB06, *Bacillus subtilis* LCDB-BP2, *Bacillus paralicheniformis* AJVR1, and *Kosakonia* sp. MBWS.8 were the equal phosphate solubilizers.

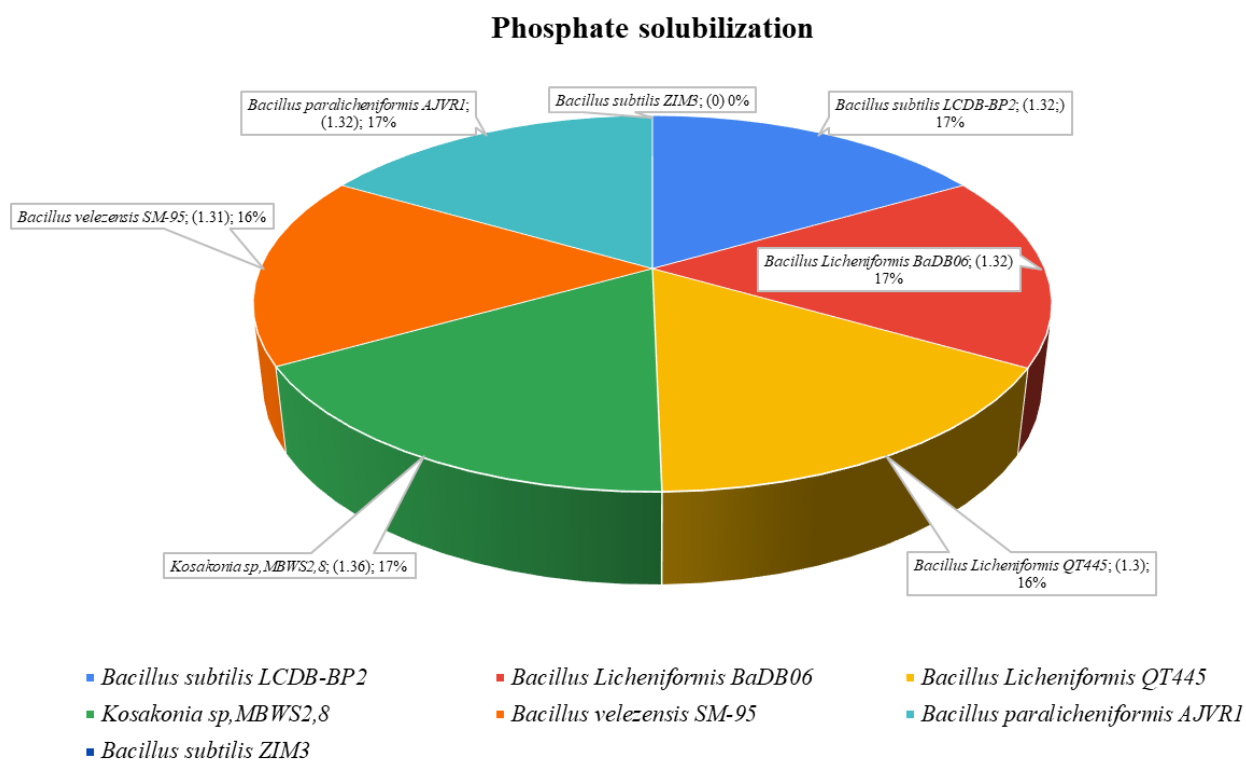


Figure 4.2: Solubilization of tri-calcium phosphate by bacterial endophytes of *Lessertia frutescens*

Figure 4.3 exhibits the ability of each bacterium to produce siderophore in comparison to others.

The percentage contribution of each strain against other strains has also been noted, with the strains that had significant siderophore production having a high% contribution. Five of the endophytes were capable of producing siderophore with average siderophore production with halo zones ranging from 2.5-5.33 mm. *Bacillus subtilis* ZIM3 was the highest producer with 26% contribution followed by *Bacillus licheniformis* BaDB06 at 24 % and the least producer was *Kosakonia sp.* MBWS2,8 at 12% contribution.

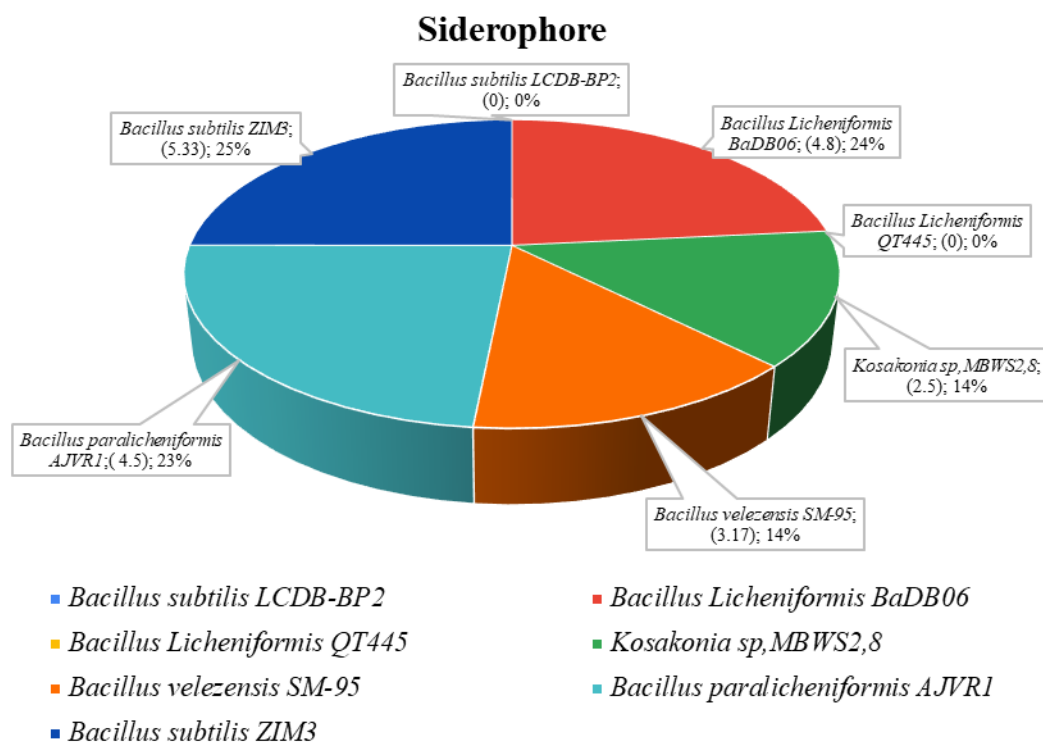


Figure 4.3: Siderophore production by endophytes isolated from *Lessertia frutescens*

Figure 4.4 exhibits the ability of each bacterium to produce indole acetic acid in comparison to others. The percentage contribution of each strain against other strains has also been noted, with strains with high indole acetic acid synthesis having a high% contribution. Six of the endophytes demonstrated their ability to produce IAA with concentrations ranging from 0.15-1.60 $\mu\text{g/mL}$. *Bacillus velezensis* SM-95 was the highest IAA producer (1.60 $\mu\text{g/mL}$) with 49% total contribution and followed by *Bacillus licheniformis* BaDB06 (0.64 $\mu\text{g/mL}$) with 20% total contribution while *Kosakonia* sp. MBWS.8 was the least IAA producer (0.15 $\mu\text{g/mL}$) with only 5% contribution.

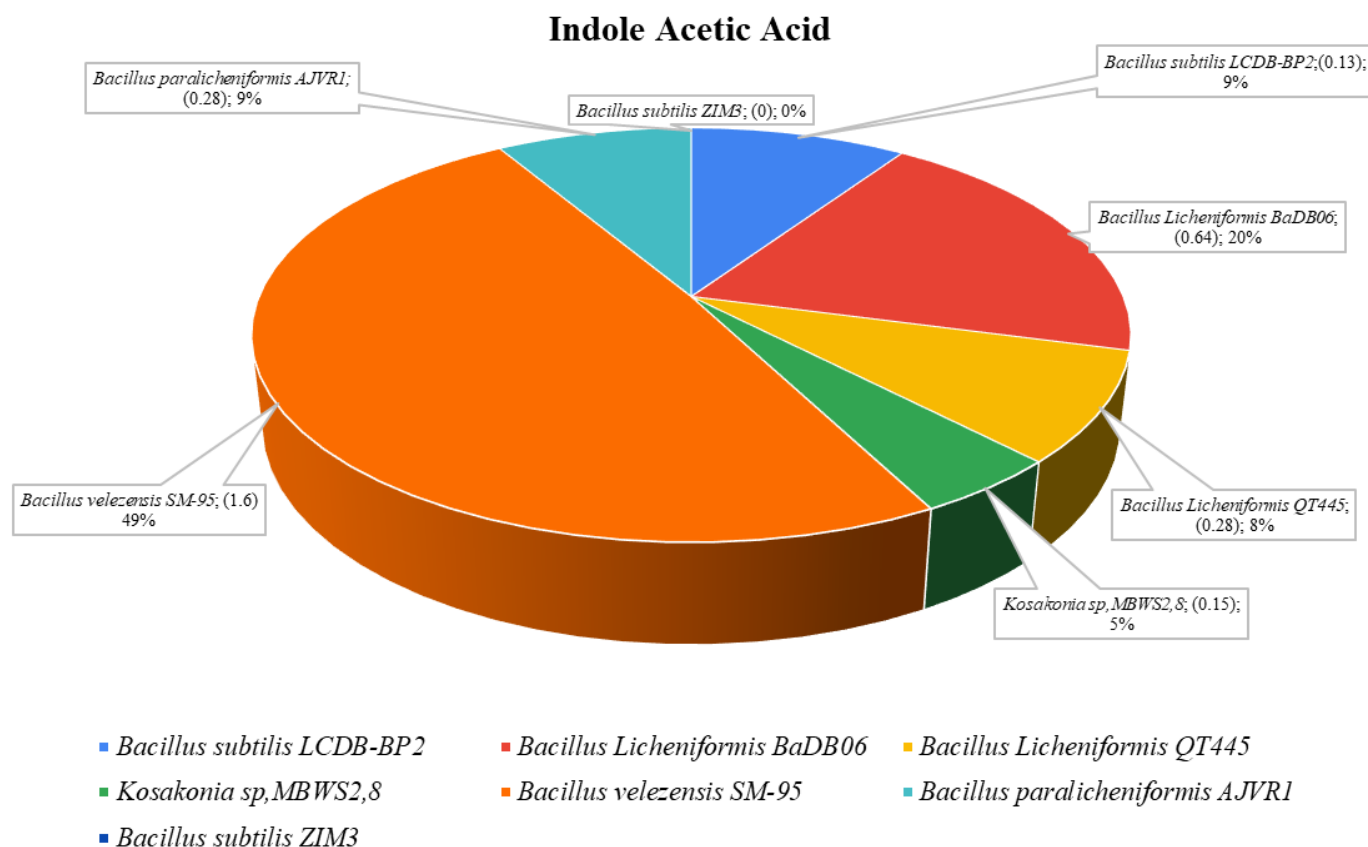


Figure 4.4: Indole acetic acid production by isolated endophytes from *Lessertia frutescens*

4.3.3 Hydrolytic enzyme activity

Table 4.2 illustrates hydrolytic enzyme activities of some *Lessertia frutescens* endophytic bacteria. Endophytes from *Lessertia frutescens* demonstrated ability to produce essential hydrolytic enzymes (amylase (86%), gelatinase (86%), protease (29%), lipase (43%), and D-nase (57%). CBL7, CBR7, CBR4, and CBR11 were the only strains that showed favourable results for all of the plant growth-promoting properties investigated (IAA, siderophore, phosphate solubilization, and N-fixation). CBL1 (amylase, protease, gelatinase, and lipase), CBR4 (amylase, gelatinase, lipase, and D-nase), and CBR16 (amylase, protease, gelatinase, and D-nase) were the dominant strains in hydrolytic enzyme activity, producing four of the five enzymes tested. CBL7 (amylase and gelatinase) and CBR11 (lipase and gelatinase) were the least enzyme-producing strains, producing only two out of five enzymes.

Table 4.2: Hydrolytic enzyme activities of *Lessertia frutescens* endophytes

Sample ID	Plant Part	Phylogenetic affiliation with similarity (16S rDNA sequencing)	AM	PT	GT	LP	DN
CBL1	Leaf	<i>Bacillus subtilis</i> LCDB-BP2	+	+	+	+	-
CBL7	Leaf	<i>Bacillus licheniformis</i> BaDB06	+	-	+	-	-
CBR4	Root	<i>Bacillus licheniformis</i> strain QT445	+	-	+	+	+
CBR6	Root	<i>Kosakonia</i> sp. strain MBWS2.(8)	+	-	-	-	+
CBR7	Root	<i>Bacillus velezensis</i> strain SM-95	+	-	+	-	+
CBR11	Root	<i>Bacillus paralicheniformis</i> strain AJVR1	-	-	+	+	-
CBR16	Root	<i>Bacillus subtilis</i> strain ZIM3	+	+	+	-	+
Total			6/7(86%)	2/7 (29%)	6/7 (86%)	3/7(43%)	4/7(57%)

Figure 4.5 illustrates the hydrolytic enzyme activity of endophyte isolated from *Lessertia frutescens*. With a zone of hydrolytic activity ranging from 2 to 15 mm, gelatinase was the most abundantly produced enzyme. Six of the endophytes also produced amylase, which had a hydrolytic activity ranging from 1 to 7 mm. Protease and lipase were the least produced enzymes. CBR16 was a notable producer of these enzymes with the capacity of producing,

gelatinase, amylase, protease and D-nase, followed by CBL1, which produced gelatinase, amylase, protease, lipase, and CBR4 which gelatinase, lipase, D-nase and amylase. The strains that produced the fewest enzymes were CR11, which could only create protease and gelatinase, while CBR6 could only make gelatinase and D-nase as well as CBL7 that produced gelatinase and amylase but had a better hydrolytic activity.

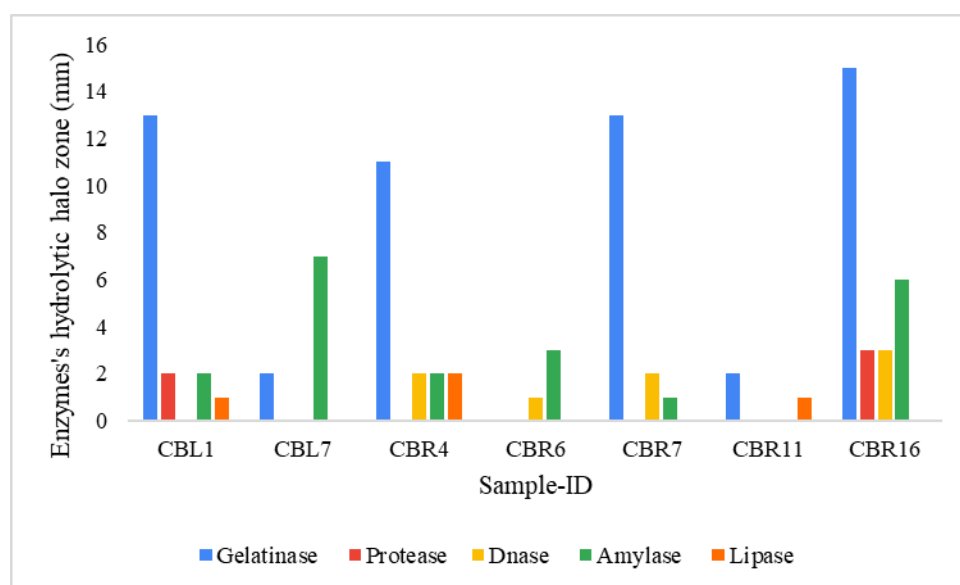


Figure 4.5: Hydrolytic enzyme activity of endophytes from *Lessertia frutescens*

4.3.4 *Lessertia frutescens* seedling growth

Endophytic bacteria significantly affected *Lessertia frutescens* seedling growth after two months (Figures 4.6, 4.7). Compared to the control (without inoculum) with the lowest seedling height, observable leaf loss and yellowing, the combined treatment (Treat-AB) resulted in the highest seedling growth (Figure 4.6). Similarly, the individual treatment with *B. licheniformis* BaDB06 and *B. velezensis* SM-95 significantly promoted seedling height.

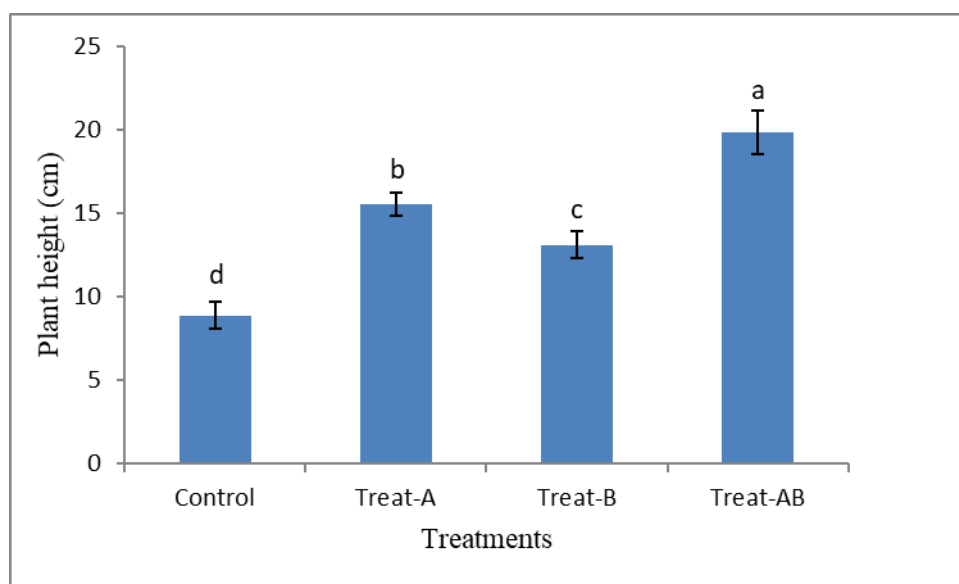


Figure 4.6: Effect of bacterial endophytes on *Lessertia frutescens* seedling growth after 2 months. Error bars represent standard deviation (n=6). Different letters on the upper portion of each bar indicates significant differences between treatments after 2 months ($P < 0.05$). A = *B. licheniformis* BaDB06, B = *B. velezensis* SM-95 and AB = *B. licheniformis* BaDB06 + *B. velezensis* SM-95.

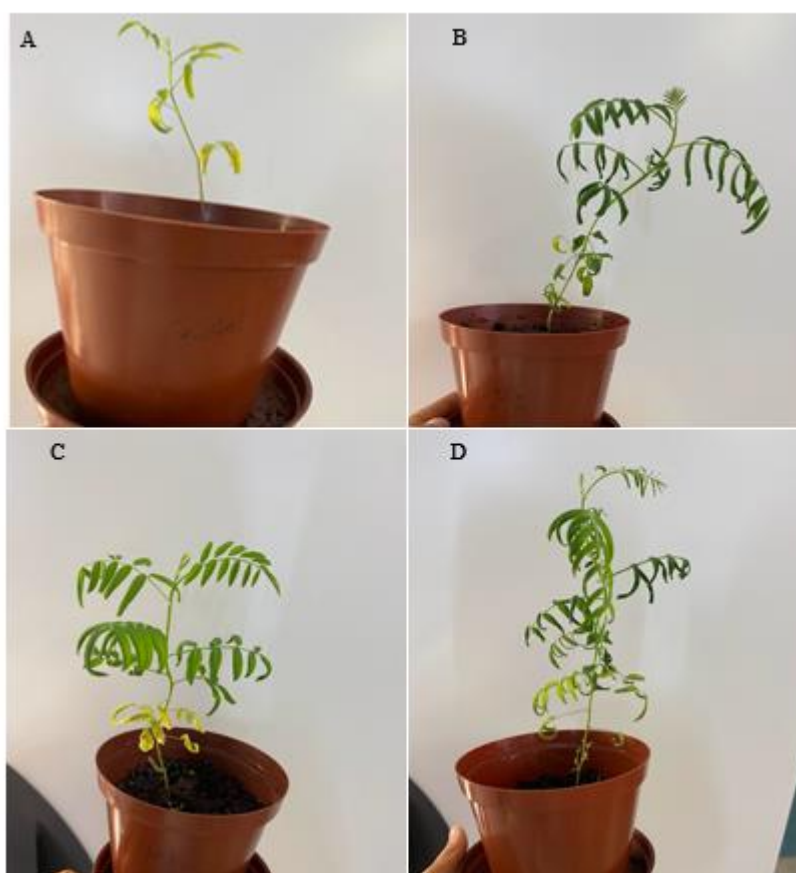


Figure 4.7: Seedling growth of *Lessertia frutescens* in a pot experiment after two months, (A) Control (treatment without an inoculum), (B) Treat- A (treatment with *B. licheniformis* BaDB06 inoculum), (C) Treat- B (treatment with *B. velezensis* SM-95 inoculum) and (D) Treat- AB (treatment with *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum).

4.4 DISCUSSION

Besides offering raw materials and diverse bioactive compounds which are vital in agriculture and pharmaceuticals, medicinal plants host a variety of bacterial endophytes with diverse potentials including plant growth promotion (Guerriero et al., 2018). The current investigation thus sought to isolate endophytes from the medicinal leguminous plant *Lessertia frutescens* and evaluate their capacity to promote plant growth. As indicated in section 4.3.1, only seven endophytes showed no harm to blood cells and the majority of them belonged to *Bacillus*. This presents a significant advantage in agriculture as such organisms are suggested to be non-pathogenic. From the present study, as indicated in Table 4.1, a greater number of isolates were obtained from the roots compared to other plant components such as leaves, while none of the isolates from stems exhibited negative hemolysis. The higher abundance of endophytes in roots relative to other plant parts can be attributed to their natural affinity for soil habitats, with colonization predominantly occurring through the root system (Golinska et al., 2015). Limited bacterial entry into the xylem (Afzal et al., 2019; Arora & Ramawat, 2017) results in fewer bacterial endophytes being isolated from non-root tissues compared to roots. The study predominantly identified *Bacillus* species, with only one strain being non-*Bacillus*. *Bacillus* species are potential bio-fertilizer candidates because their spores allow for simple colonisation of either a seed or roots and they have a good survival rate in various soil types and conditions (Lopes et al., 2018).

Nutrients such as nitrogen and phosphorus, are primary macronutrients with high demand from plants, significantly impacting crop outcomes by determining success or failure. Understanding different avenues utilized by endophytes to stimulate plant growth is critical, especially when selecting the optimal strains for bio-fertilizer production. Endophytes play a crucial role in N acquisition and exchange. For instance, free-living and symbiotic endophytes can fix biological nitrogen by converting atmospheric nitrogen into soluble form, which is accessible to plants (Ek-Ramos et al., 2019; Hassan, 2017). It is believed that endophytes use carbon assimilates produced by plants as an energy source to transform insoluble nitrogen into soluble ammonia which plants can utilise (de Vilhena Araújo et al., 2020). It is not surprising that more than 80% of endophytes isolated from *Lessertia frutescens* were able to fix nitrogen as *Lessertia frutescens* is a leguminous plant. Symbiotic nitrogen-fixing bacteria, known as alpha and beta rhizobia, perform BNF with legume species, enhancing ecosystem sustainability (Bomfeti et al., 2011). There is a tight relationship between *Nif* gene notation and nitrogen-fixing activity (Hurek et al., 2002). As shown in Figure 4.1, all six endophytic bacteria that tested positive for nitrogen fixation

possessed the *NifH* gene. *NifH* gene fragments have been effectively used to identify diazotrophic bacteria (n-fixing microorganisms) in the environment using the polymerase chain reaction (PCR) technique and the *NifH* gene's presence aids in clarifying the relationship between the associated n-fixing endophytes and their host (Terakado-Tonooka et al., 2013). *NifH* genes play a vital role in plant nitrogen regulation. For example, biological N-fixation uses the nitrogenase enzyme to catalytically convert atmospheric nitrogen to ammonia (Terakado-Tonooka et al., 2013). Whereas, *NifH* encodes the Fe protein (dinitrogenase reductase) in nitrogen-fixing bacteria and controls the production of nitrogenase synthesis (Thaweenut et al., 2011). Plant development is mostly restricted by N, which is usually supplemented with chemical fertilizers, which are hazardous to the environment (Defez et al., 2017). Endophytes from *Lessertia frutescens* have the potential to be a better supply of nitrogen, reducing reliance on chemical fertilizers. Additionally, maximizing high crop yield without damaging the environment while conserving biodiversity and ecosystems is extremely important (Defez et al., 2017). The plant-endophyte interaction has been shown to be one of the most sustainable strategies for improving crop production (Sher et al., 2024).

Bacillus species, especially *B. subtilis*, not only fix nitrogen but also promote nodulation by other bacteria, facilitating symbiotic microbe colonization (Blake et al., 2021). Endophytes also employ processes like acidification and chelation to aid in P exchange by releasing immobile insoluble phosphate and make this available for plant use (Hassan, 2017; Jayakumar et al., 2020). About 86% of the endophytes were found to solubilize insoluble tricalcium phosphate (TCP) on agar plate with solubilizing indices ranging from 1.29 to 1.36 as shown in Figure 4.2. The disappearance of TCP indicates the high potential of those endophytes to solubilize inorganic bound phosphate (TCP) (Bekkar & Zaim, 2023). In addition, formation of halo zones around the colonies are in response to decrease in pH due to organic acid production, exchange reaction and ion chelation (Bekkar & Zaim, 2023). Endophytes have been suggested to solubilize different forms of insoluble phosphate differently, for instance, they solubilise inorganic phosphate through lowering the pH of the soil, by competing with P for absorption sites in the soil and through chelation reaction of cations bound to P (Ingle & Padole, 2017; Sharma et al., 2013). The hydroxyl and carboxyl group of acids form chelates with cations (Al, Fe and Ca) (Khan et al., 2009). The PSM results in chelation reactions, and this leads to the release of bound P in the soil (Ingle & Padole, 2017). Endophytic microbes produce certain enzymes such as phytase and

phosphatase to mineralise the P-bound making it available for plant usage (Ghosh et al., 2021; Ingle & Padole, 2017).

Endophytes can also improve plant growth through production of siderophores. As shown in Table 4.1, 71% of endophytes isolated from *Lessertia frutescens* were able to produce siderophores. Even though iron is only needed in minute amounts, it is essential for plant growth and it plays a part in enzymatic activities that are necessary for physiological processes (Eid et al., 2021; Ghosh et al., 2021). Iron exists in two oxidation states in the environment: ferric (Fe^{3+}), which is insoluble, and soluble ferrous (Fe^{2+}), which plants can only use (Ghosh et al., 2020). The insoluble ferric (Fe^{3+}) form exists as either oxides, hydroxides, carbonates and phosphates and it is not available for plant use (Eid et al., 2021). Iron deficiency in plants leads to leaf chlorosis and reduced metabolic activities (Chowdappa et al., 2020). Additionally, siderophore-producing endophytes can also act as biocontrol of phytopathogens by chelating iron and limiting its availability for uptake by pathogens (Burrage & Jeon, 2021; Ek-Ramos et al., 2019). For instance, when the hydroxamate iron binding is utilized, two oxygen molecules from the hydroxamate group operate as chelating agents, creating hexadentate octahedral with Fe^{3+} and rendering iron inaccessible to pathogens, leading to their ultimate death (Chowdappa et al., 2020). Siderophores degrade pathogen cell walls, which aids in the prevention of sporulation (Mohamad et al., 2022). Thus, siderophores offer dual benefits to plants as they shield plants from pathogens and also help them with iron acquisition and absorption (Iqbal et al., 2021). An investigation by (Myo et al., 2019) reported that bacterial endophytes, *B. velezensis* produced siderophores and IAA significantly improved tomato growth.

Endophytes also promote plant development through biosynthesis and regulation of phytohormones (Mercado-Blanco and Lugtenberg, 2014). As shown in Table 4.1, about 86% of endophytes were able to produce IAA with concentrations ranging from 0.15-1.60 $\mu\text{g/mL}$. *Bacillus velezensis* SM-95 as the highest IAA producer (1.60 $\mu\text{g/mL}$) has also been triggering systemic resistance in plants besides production of auxins (J. Wang et al., 2022). The second best performing isolate, *Bacillus Licheniformis* BaDB06 (0.64 $\mu\text{g/mL}$) has been associated with the ability to produce IAA and improve plant yield (Kwon et al., 2021). It is not surprising that most of the endophytes were able to produce IAA, in the present study L-tryptophan was used as an indicator and IAA is known to be produced through the help of amino acid, tryptophan which is a product of L-tryptophan metabolism (Ghosh et al., 2021). Auxins like indole acetic acid (IAA), helps in plant tissue differentiation, cell division and elongation, apical dominance, and plant pathogen protection (Mukherjee et al., 2022).

Not only does a lack of essential nutrients cause stagnation and lower crop yield, but it also makes plants vulnerable to pathogenic attack due to inadequate immunity (Ghosh et al., 2021; Ghosh et al., 2020). Endophytes not only provide essential nutrients such as N and P to crops, but they also indirectly stimulate plant growth by antagonizing pathogens and producing lysis enzymes (Ghosh et al., 2021). It has been established that more than 8% of the *Bacillus* genome is dedicated to producing inhibitory components like lytic enzymes (Márquez et al., 2020). For example, *B. velezensis*, which was present in this study (Table 4.1), forms stable relationships with plants, which encourages the promotion of plant growth and the inhibition of soil-borne plant diseases. It does so through formation of biofilms, production of quorum-quenching enzymes such as phytase production which plays a role in phosphorus solubilisation (Iqbal et al., 2021; Rabbee et al., 2019). Screening new potential endophytes that produce enzymes is essential to understand the role of endophytes in plant defense (Saran et al., 2007). Among these enzymes are hydrolytic enzymes such as gelatinase, lipase, protease, and amylase (Naik et al., 2019) and these enzymes were evaluated in the present study. Findings from the current study demonstrated that different endophytes from *Lessertia frutescens* have varying capacities for producing these enzymes as shown in Table 4.2. Mohamad et al. (2022) also screened bacteria endophytes for hydrolytic enzymes and found that a number of those bacteria could produce enzymes such as protease, lipase. The ability of endophytic bacteria to produce amylase, lipase, cellulase, and protease enzymes was also confirmed by (Aydi Ben Abdallah et al., 2019). Microbial enzymes indirectly enhance plant growth by assisting in the mobilization of certain activities, such as the production of pathogen-protecting chemicals and the catabolism of complex organic molecules that promote plant growth (Yan et al., 2018). The fact that the majority of these endophytes from the present study produced several enzymes is remarkable.

It has been indicated that having a single microorganism that can synthesis both lipase and protease is one of the rare cases (Suryia Prabha et al., 2015) and this was the case for *Bacillus subtilis* LCDB-BP2 isolated from the leaves as shown in Table 4.2. Furthermore, these microbial enzymes enable the colonization of plants by endophytes and the transformation of inaccessible forms of nutrients into plant-usable ones (Maggini et al., 2018). In a real experiment *B. paralicheniformis* isolated from *Enicostema axillare* improved the production of the tomato's defensive enzymes and thus the tomato plant was resistant to *F. oxysporum* infection and such defence was attributed to the ability of the strain to produce hydrolytic enzymes (Jinal et al., 2020). Although medicinal plants like *Lessertia frutescens* are commercial plants, proper commercialization, which includes greater sustainable returns and

consistency of generated raw materials, can only be achieved through the application of sustainable propagation cropping systems (Kleynhans et al., 2016). *Lessertia frutescens* is known to have multiple curative properties including, anti-cancer, anti-virus, anti-microbial, anti-stress, and anti-diabetic qualities and consequently its demand has surged dramatically (Kleynhans et al., 2016). Standard medicinal plant production can lead to increased plant biomass yield (Isah et al., 2018). Thus, the need for medicinal plants propagation cannot be emphasised any less as the supply sometimes becomes less than the demand adding to issues associated with inconsistent supply. As earlier mentioned, the present study evaluated the effect of native bacillus endophytes of *Lessertia frutescens* on its seedling growth. As shown in Figure 4.6 and 4.7, endophytes significantly improved *Lessertia frutescens* seedling growth after two months compared to control with the combined treatment (= *B. licheniformis* BaDB06 + *B. velezensis* SM-95 inoculum) showing the highest seedling growth. The mode of action of endophytes' influence on plant growth commences in seed where the receptors in the plasma membrane send a signal to the seed (Shasmita et al., 2018). Both the seed and microbial cellular activities are the determinant for cell responsiveness, survival and adaptability in changes in environment (Shasmita et al., 2018). It is only after endophytes have been established inside the seed's systems that they can alter the host's metabolic and growth pathways. They can then trigger seed germination, seedling development, and other plant growth stages while giving fitness and an improved growth rate (Finch-Savage & Bassel, 2016).

On the other hand, endophytes' capacity to enhance seedling growth has been attributed to their ability to produce plant-growth promoting attributes. IAA, for example, has been suggested to help in seed germination and the production of adventitious and lateral roots, as well as in improving shoot length (Jayakumar et al., 2020). It is not surprising that both of the endophytic strains employed in the present investigation showed a significant difference in seedling growth compared to control as shown in Figure 6, during the seed germination trial test because they both produced IAA. Furthermore, IAA endophytic bacteria helps in improving nutrition absorption and exchange by plants due as they trigger root length growth and photosynthetic plant components (Fadiji & Babalola, 2020). This appears to be the first study that reports on the influence of *Bacillus licheniformis* and *Bacillus velezensis* on *L. frutescens* plant growth. However, the effect of these strains (*Bacillus licheniformis* and *Bacillus velezensis*) and their consortia on medicinal plant *T. vulgaris* growth and oil content at 65 and 140 days was investigated by (Abdel-Hamid et al., 2021). The control heights at 65 and 140 cm were 10 and 12.7 cm and *Bacillus licheniformis* inoculation resulted in the

maximum *T. vulgaris* plant height of 42.3 and 55.9 cm at 65 and 140 days, respectively. This was followed by the combined treatment (*Bacillus licheniformis* and *Bacillus velezensis*) with 38.9 and 55.7 cm in the same order of days. These strains' capacity to improve plant development was linked to their ability to boost nutrient availability to plants through this plant growth promoting traits, IAA and siderophore synthesis, phosphate solubilization, and N-fixation. Due to the potential of microorganisms, particularly endophytes, to influence plant development and biomass accumulation, they have been specified as one of the factors of plant health and growth (Klaedtke et al., 2016). Lastly, endophytes are a sustainable source of nutrients, and this study revealed that native *Lessertia frutescens* can be used as a better alternative for nutrient nourishment to promote plant growth. This can ensure better supply of *Lessertia frutescens* as it has been indicated that the monthly demand of *Lessertia frutescens* in South Africa is roughly 20 tons for production of commercial herbal dietary supplements and supplying this demand is becoming increasingly challenging as the population grows (Shaik et al., 2011a). Furthermore, the market and economic value of medicinal plants in South Africa cannot be overlooked, as they constitute a significant contribution with annual turnovers of over US\$ 60 million from over 20 000 tons of medicinal plant trades (Van Wyk, 2011).

4.5 CONCLUSION AND RECOMMENDATIONS

Endophytes are a sustainable source of nutrients, and this study revealed that native *Lessertia frutescens* endophytes can be used as a better alternative for nutrient nourishment to promote plant growth. The capacity of most of the strains to possess a wide variety of hydrolytic activities as well as plant growth promoting abilities indicates their long-term benefits in agriculture. *Bacillus licheniformis* BaDB06 and *Bacillus velezensis* SM-95, promoted *Lessertia frutescens* seedling growth. This has demonstrated that *Bacillus* species are the ideal candidates for bio-fertilization and biocontrol since they can promote plant growth and produce bio-control agents like siderophores and hydrolytic enzymes.

5. CHAPTER FIVE: PLANT GROWTH-PROMOTING BACTERIAL ENDOPHYTES FROM *ARTEMISIA AFRA*: CHARACTERIZATION AND POTENTIAL FOR SUSTAINABLE AGRICULTURE

This chapter forms part of Objective 2 and Objective 3 of the study but focuses on isolation of endophytes from *Artemisia afra*, as well as their evaluation for plant growth-promotion and enzyme activities.

ABSTRACT

Bacterial endophytes are well-known inoculants and have been used to promote plant growth and enhance plant resistance against abiotic and biotic factors. While endophytes from medicinal plants have been identified and employed for diverse bioactivities in the medicinal and pharmaceutical industries, there has been little research done on their ability to promote plant development. This chapter sought to investigate plant growth-promoting bacterial endophytes from *Artemisia afra* sourced from two different environments. Bacterial endophytes were isolated from *A. afra* and tested for pathogenicity using sheep blood agar. A molecular phylogenetic analysis was then performed on the strains that tested negative for pathogenicity. These endophytes were also assessed for their ability to fix biological nitrogen, solubilise insoluble tricalcium phosphate, and produce indole acetic acid and siderophores. The endophytes' ability to produce extracellular enzymes like amylase, protease, gelatinase, lipase, and d-nase was also evaluated. There were only 14 endophytes that did not exhibit pathogenicity, and these were: *Bacillus paralicheniformis* KW1, *Bacillus subtilis* KSW6, *Bacillus simplex* HS, *Bacillus subtilis* cf18B, *Bacillus subtilis* 118, *Sporosarcina pasteurii*, *Lysinibacillus fusiformis*, *Bacillus subtilis* H1, *Bacillus australimaris*, *Bacillus drentensis*, *Enterobacter* sp. MLB03, *Enterobacter* sp., *Bacillus licheniformis* AP, and *Bacillus clausii*. Data obtained in this study revealed that 64% of the endophytes fixed biological N, 79% solubilised insoluble tricalcium phosphate, 86% produced IAA, and 79% produced siderophores. The endophytes also demonstrated a substantial ability to produce essential extracellular enzymes (protease (57%), gelatinase (64%), d-nase (43%), amylase (57%) and

lipase (36%). Because these endophytes can promote plant growth, they present a viable substitute for chemical fertilisers and pesticides in the context of sustainable crop production.

5.1. INTRODUCTION

In this day and age, producing adequate food in a sustainable manner for an ever-increasing population remains a challenge (McCarthy et al., 2018). Global population is expected to reach 9 billion by 2050 (Kumar, 2013). Relying on conventional agronomic approaches for agricultural production remains a significant challenge, requiring additional agricultural land to meet the demand for sufficient food. In addition, agricultural land is prone to degradation as a result of changing climatic conditions, or the prolonged use of chemical fertilisers, both of which have long been recognised to have an impact on soil fertility (Roy et al., 2023). The forever changing climatic conditions have also exacerbated incidences of phytopathogenic attack with negative impact on crop production (Munaweera et al., 2022). Thus, there is a rising demand for sustainable agricultural intensification strategies that can be used to ensure food security (Ajibade et al., 2023). Consequently, the end of the era of synthetic agrochemicals awaits the effective discovery of biological agricultural products. However, upon developing sustainable agriculture, environmental safety should be kept in mind to avoid creating more problems (Munaweera et al., 2022). Under these circumstances, it is preferable to use naturally occurring resources, like endophytes, which stimulate plant growth. Endophytes coexist with plants and their mutual relationship is one of the best attributes that can make them a suitable solution as they already have established an interactive relationship that includes diverse chemical communication through gene transfers and certain bioactive compounds (Aswani, Vipina Vinod, et al., 2020; Golinska et al., 2015). The endophyte's ability to transfer specific genes can be useful in boosting the plant's genomic ability while promoting plant growth. Endophytes use a variety of methods to stimulate plant growth, the most prominent of which include (1) nutrient cycling through fixation of biological nitrogen and solubilisation of insoluble phosphate, as well as the production of siderophore, which ensures Fe availability to plants, (2) biosynthesis of phytohormones such as IAA, (3) protection against pathogenic attacks, and (4) inducing plant systemic resistance (Dilshad et al., 2023). For example, nitrogen is one of the vital and limiting elements in plant growth and development. It represents about 2% of the total plant

dry matter that enters the food chain. The atmosphere contains around 80% of nitrogen in inert form, but plants cannot directly access dinitrogen gas. However, microbes, including endophytes, fix biological nitrogen and make it available for plant usage. Consequently, in order to increase agricultural productivity, endophytes have been employed to promote plant growth (Martinez-Alvarado et al., 2022). Endophytes with medicinal potential are thought to be underutilised in terms of their applicability in the preservation of environmental biodiversity and plant growth promotion. Several studies have proven that endophytes associated with medicinal plants can promote plant health and growth using the well-known mechanisms of biofertilisation, phytostimulation, and biocontrol (Golinska et al., 2015; Palanichamy et al., 2018). In addition, endophytes can execute activities similar to those of their host plants. Examining endophytes from therapeutic plants that can flourish in a variety of conditions can be advantageous. Plants in the Asteraceae family are widespread and tolerant of a wide range of climatic conditions (Pasta et al., 2022). *Artemisia afra*, a medicinal plant, is a member of the Asteraceae family (Liu et al., 2009; Pasta et al., 2022). Thus, this chapter sought to investigate plant growth-promoting bacterial endophytes from *Artemisia afra* sourced from two different environments.

5.2. METHODS

Healthy *Artemisia afra* plants were collected from Agricultural Research Council Roodeplaat - Vegetable, Industrial and Medicinal plants (ARC-VIMP) located in Gauteng Province of South Africa and from Eden nursery in Klerksdorp, North-West Province of South Africa. Surface sterilisation, isolation of endophytes, pathogenicity tests, molecular identification of endophytes, screening for plant growth-promoting attributes, and enzyme activity assays were conducted as described in the methodology section of Chapter 4 (Section 2).

5.3. RESULTS

5.3.1. Pathogenicity: Hemolysis Patterns of endophytes

A total of 30 bacterial endophytes were isolated from *Artemisia afra* plant. Only 14 isolated endophytes tested negative for haemolysis, whereas seven of the isolates had a beta haemolytic reaction on blood cells, and nine had an alpha haemolytic reaction on blood cells.

5.3.2. Plant growth promotion

Table 5.1 illustrates plant growth-promoting activities of endophytic bacteria from *Artemisia afra*. Endophytes from *A. afra* exhibited a variety of plant growth activities, each demonstrating at least one plant growth-promoting ability. In total, 64% of the isolates fixed biological nitrogen, 79% solubilised insoluble phosphate, 86% produced IAA, and 79% produced siderophores. Isolates AS9 (*Bacillus licheniformis* AP), AR1 (*Bacillus paralicheniformis* KSW1), EL2 (*Bacillus subtilis* KSW6), BL2 (*Bacillus subtilis* cf18B), ML2 (*Bacillus subtilis* 118), MS3 (*Bacillus subtilis* H1) were the only endophytes that tested positive for all the evaluated plant growth-promoting attributes.

Table 5.1: Identity and plant growth-promoting activities of endophytic bacteria isolated from *Artemisia afra*

Sample ID	Location	Plant part	Identity	Accession number	% similarity	BNF	PSB	IAA	SD
AL4	Klerksdorp	Leaves	<i>Bacillus drentensis</i>	MT525288.1	97	+	+	-	-
AS2	Klerksdorp	Stem	<i>Enterobacter</i> sp. ML	JQ765414.1	97	-	+	-	-
AS8	Klerksdorp	Stem	<i>Enterobacter</i> sp.	HE663080.1	97	+	-	+	-
AS9	Klerksdorp	Stem	<i>Bacillus licheniformis</i> AP	MG705593.1	99	+	+	+	+
AR1	Klerksdorp	Roots	<i>Bacillus paralicheniformis</i> KSW1	MN133894.1	99	+	+	+	+
AR12	Klerksdorp	Roots	<i>Bacillus clausii</i>	MT110681.1	98	-	-	+	+
EL1	Pretoria	Leaves	<i>Bacillus simplex</i> HS	MG905633.1	99	-	+	+	+
EL2	Pretoria	Leaves	<i>Bacillus subtilis</i> KSW6	PP338071.1	99	+	+	+	+
BL2	Pretoria	Leaves	<i>Bacillus subtilis</i> cf18B	ON 71512.1	99	+	+	+	+
ML2	Pretoria	Leaves	<i>Bacillus subtilis</i> 118	KX828383.1	99	+	+	+	+
ML6	Pretoria	Leaves	<i>Sporosarcina pasteurii</i>	MN330023.1	99	-	+	+	-
MS2	Pretoria	Stem	<i>Lysinibacillus fusiformis</i>	MH714737.1	99	-	+	+	+
MS3	Pretoria	Stem	<i>Bacillus subtilis</i> H1	CP026662.1	99	+	+	+	+
MS4	Pretoria	Stem	<i>Bacillus australimaris</i>	MT510169.1	99	+	-	+	+
Total						9/14(64%)	11/14(79%)	12/14(86%)	11/14 (79%)

NB: BNF=Biological nitrogen fixation, PSB=Phosphate solubilisation, IAA = Indole Acetic Acid and SD = Siderophore
AL4: A = *Artemisia*; L = Leaves; 4 = strain number 4; **AS2, 8, 9:** A = *Artemisia*; S = Stem; 2, 8 and 9 = strain numbers; **AR1, 12:** A = *Artemisia*; R=Roots; 1 and 12 = strain numbers; **EL1, 2:** E (collected at the end of the field); L = Leaves; 1 and 2 = strain numbers; **BL2:** B (collected from the beginning of the field); L = Leaves; 2 = strain number and **MS2, 3, and 4:** M (collected from the middle of the field); S = Stem; 2, 3, and 4 = strain numbers

Figure 5.1 demonstrates the ability of bacterial endophytes to solubilise insoluble phosphate; eleven of the endophytes were capable of solubilising insoluble tricalcium phosphate on an agar plate with solubilising indices ranging between 1.34 and 2.15. In terms of the percentage contribution to the solubilisation of insoluble phosphate, *Bacillus paralicheniformis* KSW1 (AR1) was the highest solubilizer of tricalcium phosphate with a solubilisation index (SI) of 2.15 with 13% contribution, followed by *Bacillus subtilis* KSW6 with SI of 1.90 with 11% contribution. On the other side, *Bacillus simplex* HS was the lowest solubilizer of tricalcium phosphate with 8% contribution.

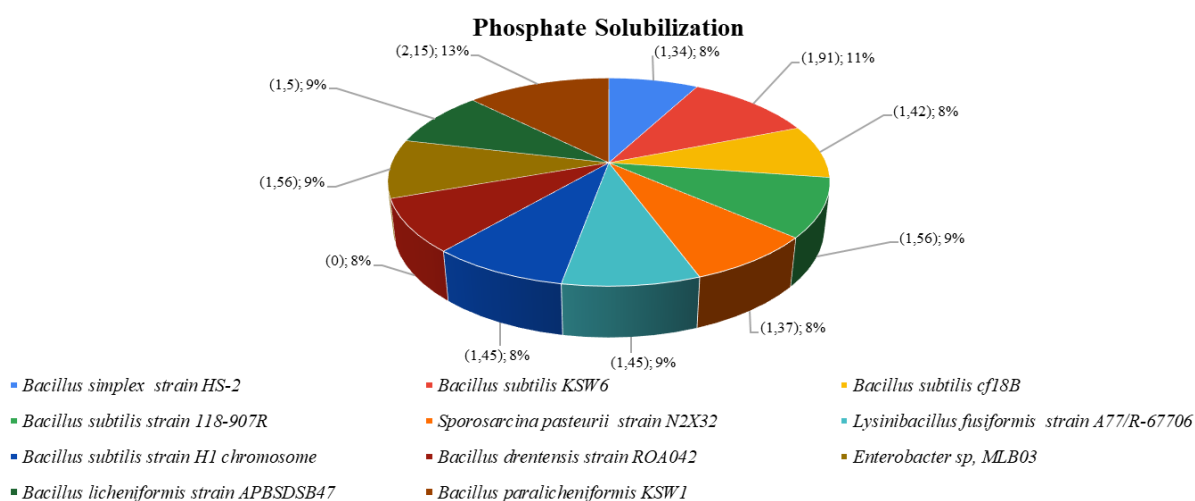


Figure 5.1: Percentage distribution of phosphate-solubilising bacterial endophytes isolated from *Artemisia afra*. The numbers in parenthesis signify the solubilizing index (SI) of each of the isolates

Figure 5.2 demonstrates indole acetic acid production by bacterial endophytes isolated from *Artemisia afra* and 12 isolates demonstrated their ability to produce IAA. The endophytes IAA production concentrations ($\mu\text{g/mL}$) were as follows, ranging from highest to lowest: *Bacillus paralicheniformis* KSW1 (3.499), *Bacillus subtilis* KSW6 (2.153), *Bacillus subtilis* 118 (1,37), *Bacillus clausii* (0.688), *Enterobacter* sp, LPPA 1490 (0.688), *Lysinibacillus fusiformis* A77/R-67706 (0.501), *Bacillus licheniformis* AP7 (0.489), *Bacillus simplex* HS-2 (0.41), *Sporosarcina pasteurii* N2X32 (0.344), *Bacillus subtilis* cf18B (0.206), *Bacillus australimaris* (0.144) and *Bacillus subtilis* H1 (0.034).

Indole Acetic Acid production

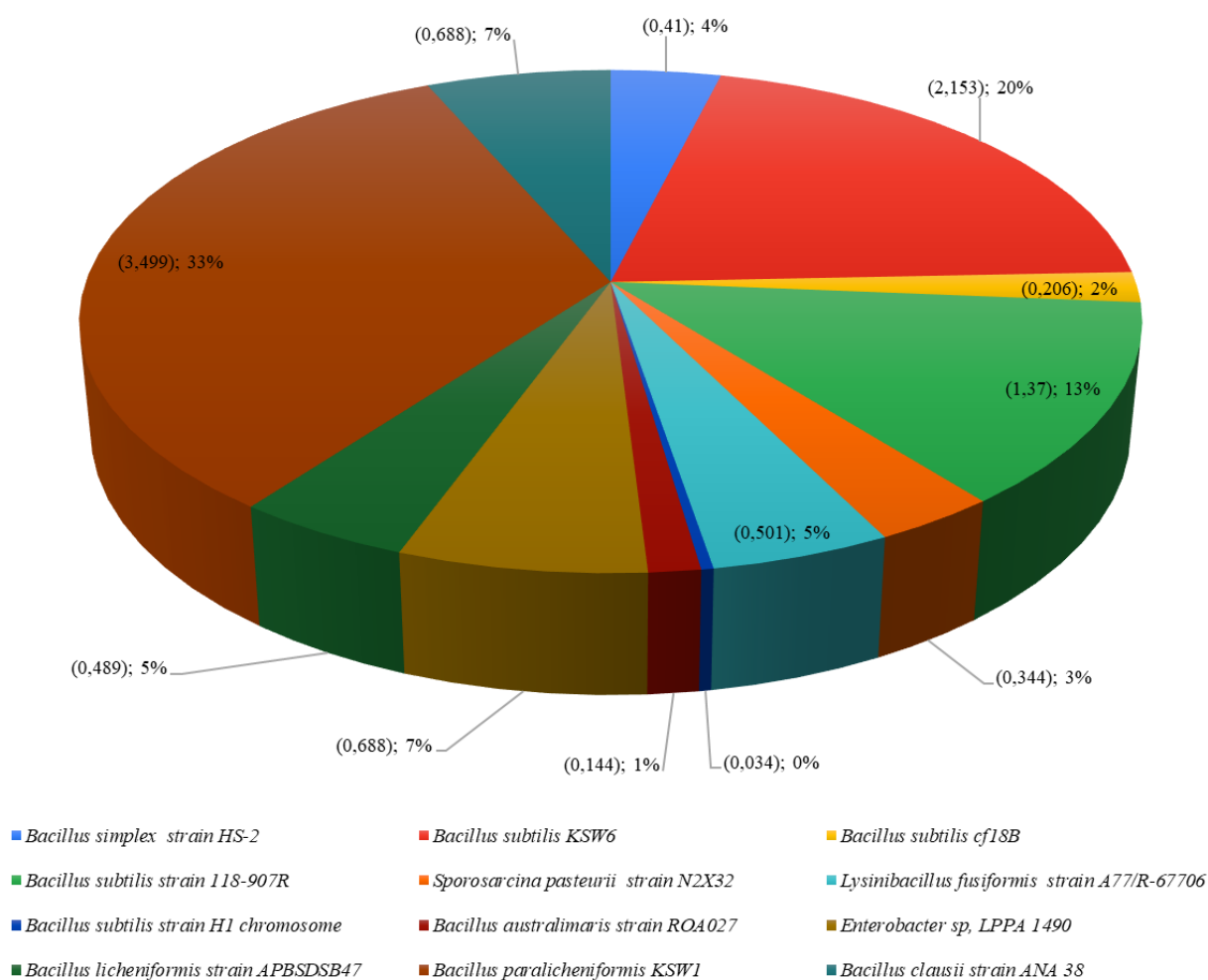


Figure 5.2: Percentage distribution of Indole Acetic Acid producing bacterial endophytes isolated from *Artemisia afra*. The numbers in parentheses signify IAA production capacity of each of the isolates in µg/mL

Figure 5.3 demonstrates the ability of bacterial endophytes to produce siderophore; 10 of the endophytes could produce siderophore with siderophore production halo zone ranging from 2-11 mm. The endophyte's siderophore productions were as follows, ranging from highest to lowest: *Bacillus paralicheniformis* KSW1 (11mm), *Bacillus subtilis* KSW6 (9 mm), *Bacillus australimaris* (8 mm), *Bacillus subtilis* 118 (6 mm), *Bacillus licheniformis* AP (6 mm), *Bacillus subtilis* H1 (5 mm), *Lysinibacillus fusiformis* A77/R-67706 (3 mm), *Bacillus clausii* (3 mm), *Bacillus subtilis* cf18B (2 mm) and *Bacillus simplex* HS-2 (2 mm).

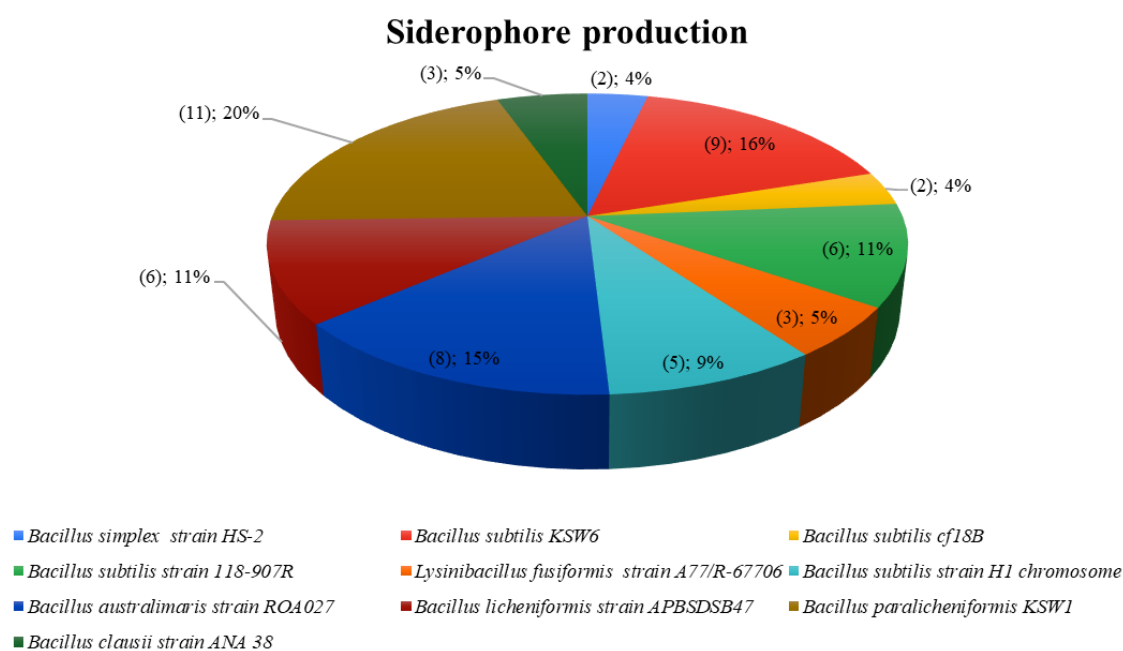


Figure 5.3: Percentage distribution of siderophore production by bacterial endophytes isolated from *Artemisia afra*.

5.3.3. Enzyme activity

Table 5.2 illustrates hydrolytic enzyme activities of *A. afra* endophytic bacteria. Endophytes from *A. afra* demonstrated a substantial ability to produce essential hydrolytic enzymes: protease (57%), gelatinase (64%), d-nase (43%), amylase (57%) and lipase (36%). AR1 (*Bacillus paralicheniformis* KSW1), and EL2 (*Bacillus subtilis* KSW6) were the major producers of these hydrolytic enzymes as they produced all the enzymes evaluated, followed by MS2 (*Lysinibacillus fusiformis*) that produced 4/5 of the tested enzymes. AS2 (*Enterobacter* sp. ML) and BL2 (*Bacillus subtilis* cf18B) were the least enzyme-producing strains, producing only one out of five enzymes.

Table 5.2: An illustration of the hydrolytic enzyme activities of identified endophytic bacteria from *A. afra*

Sample ID	Identity	Protease	Gelatinase	D-nase	Amylase	Lipase
AL4	<i>Bacillus drentensis</i>	+	+	-	-	-
AS2	<i>Enterobacter</i> sp. ML	-	-	+	-	-
AS8	<i>Enterobacter</i> sp.	-	+	-	+	-
AS9	<i>Bacillus licheniformis</i> AP	-	+	-	+	-
AR1	<i>Bacillus paralicheniformis</i> KSW1	+	+	+	+	+
AR12	<i>Bacillus clausii</i>	+	+	-	-	-
EL1	<i>Bacillus simplex</i> HS	+	-	-	+	-
EL2	<i>Bacillus subtilis</i> KSW6	+	+	+	+	+
BL2	<i>Bacillus subtilis</i> cf18B	-	+	-	-	-
ML2	<i>Bacillus subtilis</i> 118	-	+	+	+	-
ML6	<i>Sporosarcina pasteurii</i>	-	-	+	+	+
MS2	<i>Lysinibacillus fusiformis</i>	+	-	+	+	+
MS3	<i>Bacillus subtilis</i> H1	+	+	-	-	-
MS4	<i>Bacillus australimaris</i>	+	-	-	-	+
Total	14	8/14 (57%)	9/14 (64%)	6/14 (43%)	8/14 (57%)	5/14 (36%)

Figure 5.4. demonstrates gelatinase enzymatic activity of endophytes isolated from *Artemisia afra*. With 9 out of 14 endophytes able to produce gelatinase and a hydrolytic zone that ranges from 2 to 22 mm, gelatinase was the most prevalent and highly produced enzyme among the endophytes, with EL2 and AR1 being the most notable producers. In terms of the percentage contribution to the gelatinase enzymatic activities, *Bacillus subtilis* KSW6 was the highest producer with an enzymatic activity of 22 mm halo zone with 19 % contribution. Followed by *Bacillus paralicheniformis* KSW1 (AR1) at 21 mm enzyme activity with 18% contribution

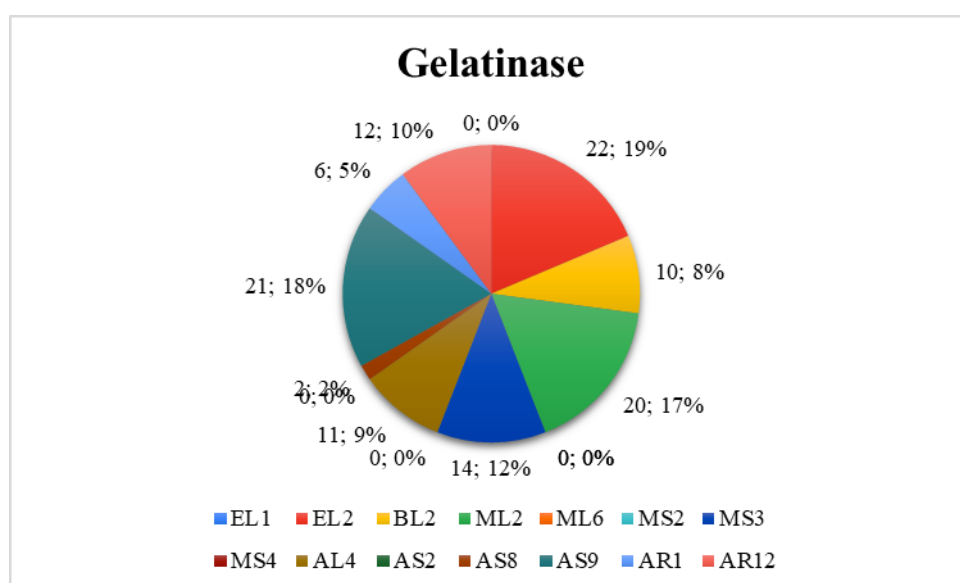


Figure 5.4: Gelatinase enzymatic activity of endophytes isolated from *Artemisia afra*

Figure 5.5 demonstrates amylase enzymatic activity of endophytes isolated from *Artemisia afra*. Eight endophytes with hydrolytic activity ranging from 1 to 11 mm produced amylase, with EL2 and AS8 being the most prominent producers. In terms of the percentage contribution to the amylase enzymatic activities, *Bacillus subtilis* KSW6 was the highest producer with an enzymatic activity of 11 mm halo zone with 24 % contribution. Followed by *Enterobacter* sp. (AS8) at 8 mm amylase enzyme activity with 17% contribution

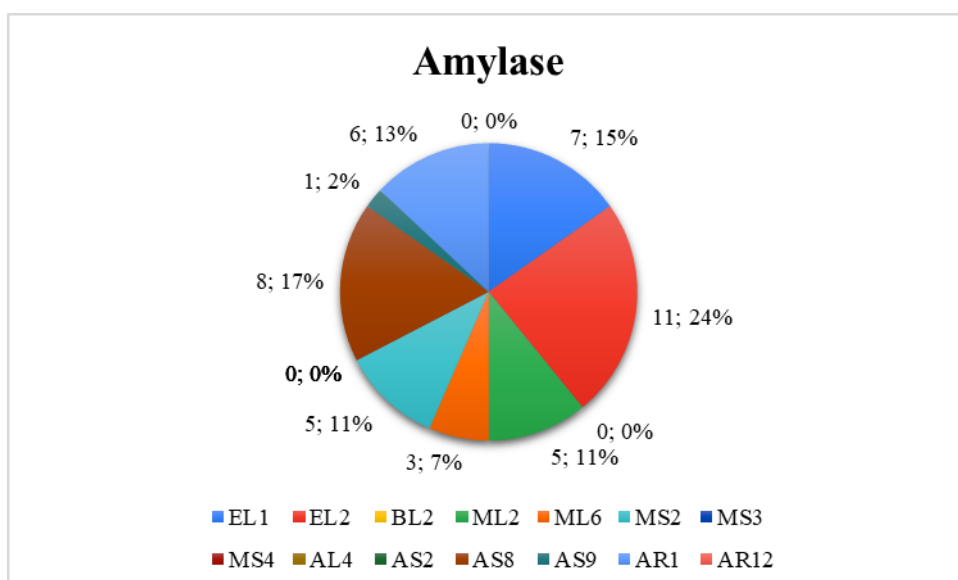


Figure 5.5: Amylase enzymatic activity of endophytes isolated from *Artemisia afra*

Figure 5.6 demonstrates protease enzymatic activity of endophytes isolated from *Artemisia afra*. Protease was the third most generated enzyme in this study, with eight endophytes capable of producing it with hydrolytic activity ranging from 2 to 10 mm hydrolysis zone. MS2 and EL1 were the leading producers of Protease. In terms of the percentage contribution to the protease enzymatic activities, *Lysinibacillus fusiformis* (MS2) was the highest producer with an enzymatic activity of 10 mm halo zone with 28 % contribution. Followed by *Bacillus simplex* HS (EL1) at 7 mm enzyme activity with 19% contribution.

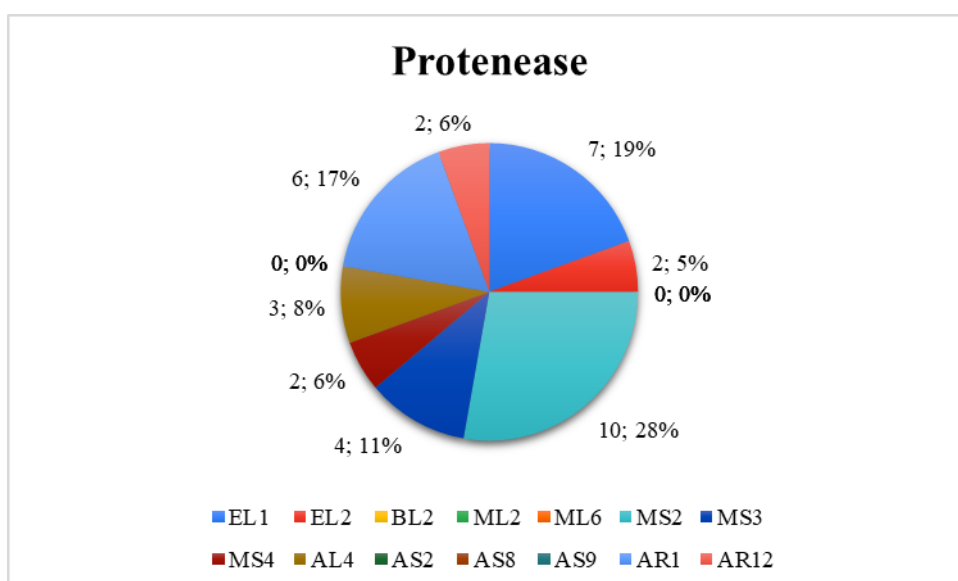


Figure 5.6: Protease enzymatic activity of endophytes isolated from *Artemisia afra*

Figure 5.7 demonstrates lipase enzymatic activities of endophytes isolated from *Artemisia afra*. Lipase as one of the least produced enzymes with five endophytes producing lipase with a hydrolytic zone of 2 to 7 mm. *Bacillus clausii* (AR12) was the major producer of lipase at 7 mm enzyme activity with 35% contribution, followed by *Bacillus paralicheniformis* KSW1 (AR1) at 4 mm halo zone enzyme activity with 20% contribution.

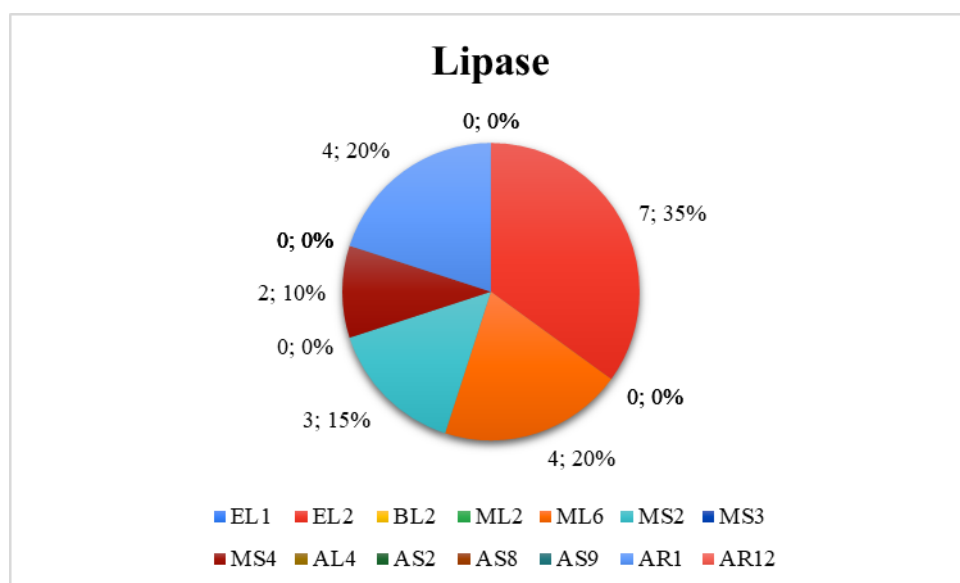


Figure 5.7: Lipase enzymatic activity of endophytes isolated from *Artemisia afra*

Figure 5.8 demonstrates D-nase enzymatic activity of endophytes isolated from *Artemisia afra*. D-nase another least produced enzymes with only 6 out of 14 producing d-nase with a hydrolytic zone of 1 to 3 mm. *Bacillus clausii* (AR12) was the major producer of D-nase at 3 mm enzyme activity with 22% contribution, followed by *Bacillus paralicheniformis* KSW1 (AR1) at 3 mm halo zone enzyme activity with 21% contribution.

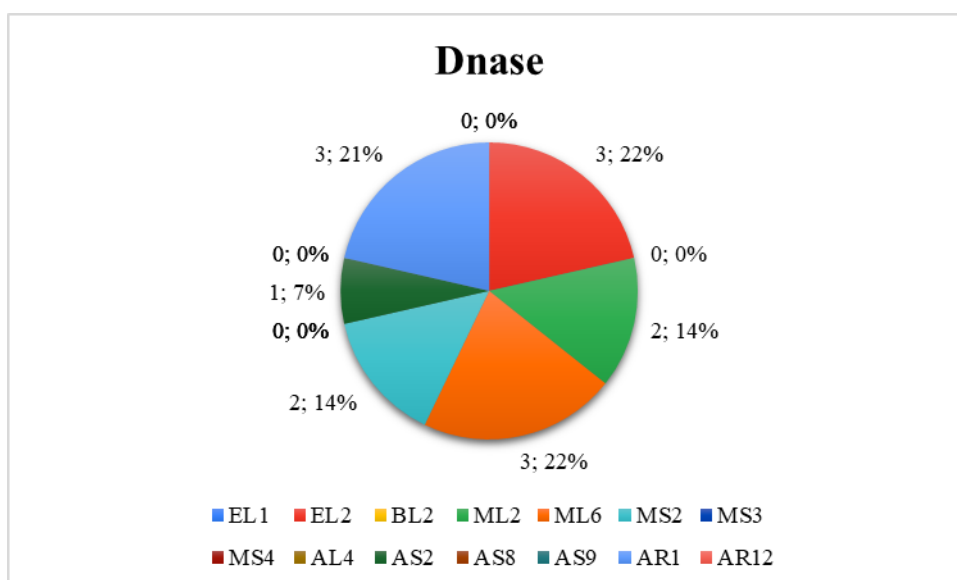


Figure 5.8: D-nase enzymatic activity of endophytes isolated from *Artemisia afra*

5.4. DISCUSSION

5.4.1. Endophytes from *Artemisia afra* and their plant growth-promoting abilities

The primary limiting factor in plant growth and development as well as agricultural productivity is low retention and storage of nutrients by plants. Plant growth-promoting microbes, including endophytes, have been increasingly utilised in agriculture to enhance crop production, especially in the event of changing climates (Senger et al., 2022). Even though endophytes are present in all plant sections, their distribution and community structure vary depending on the plant tissue and growing conditions. As shown in Table 5.1, *Bacillus* species were dominating in *A. afra* from both locations (Pretoria and Klerksdorp). Plant anatomical traits play a vital role in generating a distinct ecological niche of the endophyte. Furthermore, diverse root exudates, for example, provide a distinct, strong rhizospheric effect, allowing them to attract a specific set of bacterial communities (Maggini et al., 2018). The environment can also have a stronger impact on the distribution of bacterial endophytes within plants, causing various plants from the same or different environment to host strains belonging to the same bacterial genus (Maggini et al., 2018). This could also be because some *Bacillus* species, such as *Bacillus subtilis* biofilms, can have a destructive and antagonistic relationship with other associate microorganisms, resulting in significant changes in bacterial community assemblages (Rosenberg et al., 2016). *Bacillus* species are therefore beneficial for the development of biofertilizers due to their high survival rate (Lopes et al., 2018). In addition, their capacity to stimulate plant growth and generate

biocontrol agents makes them the ideal candidates for biocontrol and biofertilization (Hashem et al., 2019). Endophytes from *A. afra* exhibited a variety of plant growth activities, with each demonstrating at least one plant growth-promoting ability as shown in Table 5.1.

Although it is more usual for leguminous plants to contain nitrogen-fixing microorganisms through nodule development, a number of non-leguminous plant endophytes can also fix nitrogen (Eid et al., 2021). This was true for the present study as 64% endophytes isolated from the non-leguminous plant, *A. afra*, showed ability to fix biological nitrogen in an in vitro experiment. Plants demand N in relatively higher levels, and the available N in the soil has been found to be insufficient for maximum production in terms of crop yield. The capacity of N-fixing bacteria is of considerable help in boosting nitrogen availability for plants and have a good influence on crop yield. The second key element that plants need in large quantities is phosphorus. Phosphorus has low mobility, which further reduces its availability in the soil; yet it plays a crucial role in plant growth and development since it is engaged in several processes such as cell division. On the other hand, constant application of chemical P-based fertiliser only increases P in the fixed pool since the majority of it is fixed into insoluble complexes that are not available to plants. However, phosphate-solubilising microbes help to solubilise fixed P, making it available for plant usage (Sarmah & Sarma, 2023). As shown in Table 1, 79 % of total endophytes isolated from both locations were able to solubilise the insoluble tricalcium phosphate. Their solubilising indices ranged from 1.34 to 2.15 as indicated in Figure 5.1.

Additionally, endophytes stimulate plant growth by synthesising phytohormones. Plants produce phytohormones, which control a wide variety of signalling pathways that allow plants to adapt to various environmental conditions. Thus, inoculation with microorganisms that produce phytohormones can often be critical to plants' survival in difficult environments. Their influence is more significant on plant root growth and development (L. Zhu et al., 2021). Endophytes isolated from *A. afra* exhibited a great ability to produce IAA as shown in Figure 5.2, with AR1 (*Bacillus paralicheniformis* KSW1) (3.499 $\mu\text{g/mL}$) being the highest producer followed by EL2 (*Bacillus subtilis* KSW6) (2.153 $\mu\text{g/mL}$) and MS3 (*Bacillus subtilis* HI) was the least producer with 0.034 ($\mu\text{g/mL}$) IAA concentration.

The majority of iron in soil is found in an insoluble form (Fe^{3+}), which is unavailable to plants. Various endophytes have been proven to chelate Fe through siderophore synthesis, making it available for plant utilisation. As illustrated in Table 5.1 and Figure 5.3, this current study mirrored that as 79% of the endophytes isolated from *A. afra* exhibited a great ability to produce siderophore, with siderophore activity ranging from 2 - 11 mm of halo zone. It has been noted that due to their strong affinity for Fe^{3+} , siderophores can chelate iron, and form soluble

complexes such as Fe-phytosiderophore complex. This process eventually depletes phytogetic bacteria of Fe and releases it for use by plants (Luo et al., 2022). Siderophore production by endophytes enable them to outcompete their competitors through iron sequestration from the same niche, thus proving to be advantageous in colonisation (Maggini et al., 2018).

The following strains, *Bacillus paralicheniformis*, *Bacillus clausii*, *Bacillus subtilis*, *Bacillus simplex*, *Sporosarcina pasteurii*, *Lysinibacillus fusiformis*, *Bacillus australimaris*, *Bacillus drentensis*, *Enterobacter* sp., and *Bacillus licheniformis* were isolated from *A. afra* from both the locations as shown in Table 5.1 and their application of different sectors, particularly their plant growth promotion in the agricultural sector, has been discussed below.

(a) *Bacillus paralicheniformis* (AR1)

As shown in Table 1, *Bacillus paralicheniformis* tested positive for all evaluated plant growth-promoting attributes namely P-solubilisation, N-fixation, IAA and siderophore production. Among the other endophytes examined in this study, *Bacillus paralicheniformis* had the highest production capacity on every plant growth-promoting characteristic examined. *Bacillus paralicheniformis* solubilised insoluble tricalcium phosphate with a SI of 2.15, produced IAA at a concentration of 3.499 µg/mL, and siderophore with 11 mm halo zone (Figures 5.1, 5.2 and 5.3). *Bacillus paralicheniformis* is widely used in a variety of fields, such as agriculture, medicine, and pharmaceuticals (Muras et al., 2021). For instance, in a greenhouse experiment, *Bacillus licheniformis* significantly increased the growth and development of peppers and tomatoes (García et al., 2004). The utilisation of *B. licheniformis* for industrial production of amylase dates back to the 1900s (Muras et al., 2021).

(b) *Bacillus subtilis* and *B. simplex*

Bacillus subtilis is one of the well-known plant growth-promoting *Bacillus* species (Schwartz et al., 2013). This was evident in the present study in which four *B. subtilis* endophytes were obtained with varying capacity in potential plant growth promotion. All these strains were able to fix biological N as demonstrated in Table 5.1. *Bacillus subtilis* is a good competitor, and as previously stated, it has a damaging and antagonistic interaction with other associated microorganisms (Rosenberg et al., 2016). All the strains were also able to solubilise insoluble tricalcium phosphate, *Bacillus subtilis* KSW6 solubilised insoluble tricalcium phosphate with SI of 1.91 followed by *B. subtilis* 118-907R with SI of 1.56, *B. subtilis* H1 chromosome at 1.45 and

B. subtilis cf18B having SI of 1.42 (Figure 5.1). Organic molecules in soil, such as phytate, contain up to 50 - 80% phosphate, which cannot be hydrolysed (Liu et al., 2022). However, phytase can dissolve phytate, releasing the P bound in it, and making it available for plant use. Several *Bacillus* strains have the *phyC* gene, which is responsible for the synthesis of phytase (Neubauer et al., 2012). Moreover, all the strains were able to produce IAA, *Bacillus subtilis* KSW6 produced IAA at a concentration of 2.153 µg/mL that made it the second highest IAA-producing strain in this study, *B. subtilis* cf18B produced 0.206 µg/mL, *B. subtilis* 118-907R at 1.37 µg/mL IAA concentration, and *B. subtilis* H1 chromosome, which was, however, the least producer of IAA (0.034 µg/mL) even amongst other strains (Figure 5.2).

The strains showed different capacity in siderophore production as well, with *Bacillus subtilis* KSW6, producing siderophore with 9 mm halo zone, *B. subtilis* 118-907R with 6 mm halo zone of siderophore production, followed by *B. subtilis* H1 chromosome at 5 mm and *B. subtilis* cf18B at 2 mm halo zone of production (Figure 5.3). *Bacillus subtilis* can also act as a biocontrol agent because it produces a wide spectrum of cell wall-disintegrating enzymes and biocontrol agents like siderophores, which were observed from this study as shown in Table 5.1 and Table 5.2. All the examined enzymes, including protease, gelatinase, d-nase, amylase, and lipase, with halo zones of 2 mm, 22 mm, 3 mm, 11 mm, and 7 mm, respectively, were produced by *Bacillus subtilis* KSW6 as indicated in Figures 5.4 – 5.8. *Bacillus subtilis* cf18B only produced one enzyme (gelatinase with 10 mm halo zone), while *B. subtilis* 118-907R produced three enzymes, namely, gelatinase, d-nase, and amylase with enzymatic activities of 20 mm, 2 mm and 5 mm respectively. On the other side, *B. subtilis* H1 chromosome produced only two enzymes, protease at 4 mm activity and gelatinase at 14 mm halo zone activity (Figures 5.4, 5.5, 5.6, 5.7 and 5.8). In an in vitro experiment that explored the interspecies interaction between biofilms of *B. subtilis* and *B. simplex*, *B. subtilis* overcame *B. simplex* (Rosenberg et al., 2016). The ability of *B. subtilis* to conquer *B. simplex* was connected with its capacity to produce cannibalism and surfactin toxins at quantities inert to *B. subtilis* but inhibitory to *B. simplex*. Even though *B. simplex* could not outcompete *B. subtilis*, it has good properties, including plant growth stimulation, biocontrol, and even bioremediation. In the present study, *Bacillus simplex* HS demonstrated its ability to solubilise insoluble tricalcium phosphate at 1.34 solubilizing index, producing at a concentration of 0.41 µg/mL, and siderophore with 2 mm halo zone (Figures 5.1, 5.2, 5.3). It tested negative for biological N fixation (Table 5.1). Upon examining the potential of *B. simplex* for nitrogen fixation in an N free media, there was no growth (Schwartz et al., 2013). Nevertheless, when inoculated in a pea plant, *B. simplex* induced changes in its root architecture

by enhancing growth of lateral roots. In the same study, co-inoculation of *B. simplex* and *B. subtilis* resulted in larger pea nodules, and it was therefore concluded that co-inoculation might be employed as one of the techniques to promote better N-fixation by bacteria. Both of these strains were said to have shown the ability to produce siderophores and solubilise insoluble phosphate. *Bacillus simplex* was examined for its capacity to improve maize and soybean growth and development, and the results revealed that inoculation with *B. simplex* considerably boosted maize and soybean production (Senger et al., 2022). *Bacillus simplex* was evaluated to act as biocontrol on soybean, findings indicating that seeds coated with *B. simplex*, strain sneb545, induced resistance of soybean to *Heterodera glycines* (Xing et al., 2020).

Additionally, *Bacillus* spp. are widely used in several sectors. Certain species, owing to their capacity to generate a variety of enzymes, have been employed in the biodegradation of contaminants. In the present study, *Bacillus simplex* HS only produced two enzymes, namely protease and amylase with enzymatic activities of 7- and 7-mm halo zones (Figures 5.5 and 5.6). *Bacillus simplex* was assessed for its capacity to biodegrade phenol, one of the main pollutants having a serious influence on the environment (Magharbeh et al., 2021). According to the results, the strain's ability to produce degrading enzymes that enable the bioconversion of the pollutants, enabled it to degrade about 90% (or 700 mg/L) of the phenol in approximately 96 hours of incubation.

(c) *Bacillus clausii*

Bacillus clausii is a spore-forming, rod-shaped, aerobic, and non-pathogenic Gram-positive bacterium (Ianiro et al., 2018). Some of its physiological features include resilience to heat and acidity, making it suited for a wide range of conditions (Ianiro et al., 2018). There are indications that it can both prevent and treat acute diarrhoea (Ianiro et al., 2018). Although *Bacillus clausii* has gained popularity in the pharmaceutical and medical industries for its probiotic properties, little is known about its capacity to stimulate plant growth. In the present study, *Bacillus clausii*, produced IAA at a concentration of 0.688 µg/mL concentration and it produced siderophore with 3 mm halo zone (Figures 5.2 and 5.3). It is well known that several *Bacillus* species can inhibit the growth of plant diseases by producing a range of antibiotic compounds. The ability of *Bacillus clausii* to act as a biostimulant was tested by (Oulebsir-Mohandkaci et al., 2021). The number of lateral roots, root length, number of leaves, and plant height of rapeseed were all increased by *Bacillus clausii* inoculation. Furthermore, phytases and phosphatases, which are important for solubilising insoluble inorganic phosphate, are among the plant growth metabolites

that the strain produced. In the same study, the strain was able to produce siderophore and IAA (Senesi et al., 2001). *Bacillus clausii* in this study showed a noticeable ability to produce two enzymes namely gelatinase and protease with halo zones of 12 mm and 2 mm respectively as shown in Figures 5.4 and 5.6.

(d) *Sporosarcina pasteurii*

The *Sporosarcina pasteurii* is a spore-forming gram-positive bacterium that is primarily found in environments high in urea and ammonium (Y. Wu et al., 2021). This work appears to be the first to report on the isolation of *S. pasteurii* from *A. afra* and investigating its potential plant growth promotion capability. *S. pasteurii* N2X32 tested negative for biological N fixation and siderophore production (Table 5.1). *S. pasteurii* N2X32 solubilised insoluble tricalcium phosphate with SI of 1.37 and produced IAA at concentration of 0.344 µg/mL (Figures 5.1 and 5.2). *S. pasteurii* is one of the most prominent strains largely employed in biomineralization, ureolytic one to be particular, and this is due to its high urease activity, as it is claimed to constitute roughly 1% of urease in its cells (J. Zhu et al., 2021). Consequently, this strain has gained massive attention due to its possible biotechnological applications in, for instance, the environmental, construction and medical sectors. (Garcia-Arroyo et al., 2018). It has been employed in bioremediation of heavy metals through the microbial-induced carbonate precipitation process (Fang et al., 2021; Jalilvand et al., 2020). It does this by producing the urease enzyme, which hydrolyses urea into ammonium and carbonate ions (Kumari et al., 2016). The generated carbonate ions react with the heavy metal ions to form stable carbonate crystals, which results in heavy metal stabilisation (Krajewska, 2018). *Sporosarcina pasteurii* (ML6) produced the three enzymes, namely d-nase, amylase and lipase at 3-, 3- and 4-mm halo zone activity, respectively (Figures 5.8, 5.5 and 5.7). The enzyme activity of the strain could be useful in indicating additional applicability of the strain in different industrial sectors.

(e) *Lysinibacillus fusiformis*

Lysinibacillus fusiformis is an endospore-producing gram-positive bacterium. It is well known for its capacity to produce inactive endospores that are resistant to UV radiation, temperature fluctuations, and chemicals (Khadka et al., 2020). *Lysinibacillus fusiformis* is also a well-known antagonising bacteria with tolerance to heavy metal toxicity (Passera et al., 2021). Regardless of its qualities, this strain has been hardly explored for its probable agricultural application, and its plant growth-promoting mechanisms are therefore poorly understood. The strain has been found to be capable of solubilising insoluble phosphate, producing siderophore, and auxins, particularly

IAA (Passera et al., 2021). This was observed in the present study where *L. fusiformis* A77/R-67706 solubilised insoluble tricalcium phosphate with a solubilising index of 1.45, produced IAA at a concentration of 0.501 µg/mL, and siderophore with 3 mm halo zone (Figures 5.1, 5.2 and 5.3). The strain tested negative for biological N fixation (Table 5.1). In a greenhouse experiment, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *L. fusiformis* were amongst the bacterial strains that promoted black pepper growth with single bacterium increasing leaf number by 50% to 70%, and enhanced wet and root biomass by two folds compared to the control (Nguyen et al., 2019).

They also inhibited fungal pathogenic attack through production of antifungal compounds. The ability of these strains to enhance growth and root development was associated with their IAA production and P-solubilisation capacity. The IAA production capability of *L. fusiformis* and its potential to bioremediate heavy metals were tested on amelioration of Cd toxicity on a tomato plant by (L. Zhu et al., 2021). The findings revealed that *L. fusiformis* stimulated JA signalling pathways in tomato roots and inhibited Cd entrance. In an in vitro experiment, *L. fusiformis* was also assessed for its potential to biosorb certain heavy metals such as Ni, Cd, Cr, and Pb (Jibrin et al., 2020). After 35 days of incubation on an agar plate containing these heavy metals and the strain, *L. fusiformis* managed to biosorb 99.33% of 15 ppm Ni, 99.94% of Cd, 99.97% of Cr, and 99.96% of Pd at a 10-ppm concentration. *Lysinibacillus fusiformis* has lately received interest in biotechnological fields due to its capacity to produce a diverse spectrum of enzymes with several industrial applications (Passera et al., 2021). On the other hand, *L. fusiformis* A77/R-67706 produced the following enzymes: protease, d-nase, amylase and lipase with activities of 10 mm, 2 mm, 5 mm and 3 mm halo zones, respectively (Figures 5.5, 5.6 and 5.7). It has been reported to produce thermostable protease at extreme temperatures (Khadka et al., 2020).

(f) *Bacillus australimaris*

Bacillus australimaris is a gram-positive aerobic bacterium. When *B. australimaris* was evaluated for its capacity to improve *Z. mays* and *T. aestivum* seedling growth, it raised the number of leaves on these crops by more than 130 and 160 percent, respectively (Dilshad et al., 2023). The potential of *B. australimaris* ROA027 for plant growth promotion was investigated in this study. *B. australimaris* ROA027 produced 0.144 µg/mL of IAA and an 8 mm halo zone of siderophore (Figures 5.2 and 5.3). The strain tested positive for biological N-fixation but was unable to solubilise insoluble tricalcium phosphate, as illustrated in Table 5.1.

The efficacy of *B. australimaris*, which tested positive for IAA and siderophore, to promote *Barleria lupulina* Lindl seedling germination in conjunction with *Enterobacter kobei* was investigated in an experiment by (Kumar & Dubey, 2022). Co-inoculation of these strains resulted in a considerable increase in shoot length, wet and dry biomass, and more than 90% germination of the seeds. On the other hand, in the present study, *B. australimaris* ROA027I could only produce two enzymes, protease and lipase, which had 2 mm and 2 mm halo zone activity respectively, as shown in Figures 5.6 and 5.7. *Bacillus australimaris* showed greater enzymatic activity for lipase, amylase, and protease in a study by (Kumar & Dubey, 2022). It also solubilised zinc and phosphate. Positive gelatinase activity results for *B. australimaris* were also validated by (Y. Liu et al., 2016).

5.4.2. Production of hydrolytic enzymes by endophytes isolated from *Artemisia afra*

Endophytes also indirectly promote plant growth by protecting the plant from pathogenic attacks. They achieve this by producing extracellular enzymes that break down the cell wall of the pathogen. Microbial enzymes aid in the mobilisation of specific activities, such as the biosynthesis of pathogen-protecting compounds, and the catabolisation of complex organic molecules that promote plant growth (Yan et al., 2018). Among these enzymes are extracellular enzymes including gelatinase, lipase, protease, and amylase (Naik et al., 2019). Additionally, these microbial enzymes support the colonisation of endophytes by plants and the transformation of inaccessible forms of nutrients into forms that plants can use (Maggini et al., 2018). To gain a deeper knowledge of the endophyte's function in plant defence, it is imperative to screen new potential endophytes that generate enzymes (Saran et al., 2007). *Bacillus* spp. which commonly occurred in this present study, as shown in Table 5.1, have been shown to produce qualitative and quantitative stable extracellular enzymes in a variety of conditions (Ghelardi et al., 2022). The present study assessed different endophytes' extracellular enzyme activity, as indicated in Table 5.2, and found that these endophytes had varying capacities to produce the evaluated enzymes. Endophytes from *A. afra* demonstrated a substantial ability to produce essential hydrolytic enzymes: protease (57%), gelatinase (64%), d-nase (43%), amylase (57%) and lipase (36%). Bacterial endophytes from *A. afra* have demonstrated their remarkable ability to produce extracellular enzymes with hydrolytic activity varying from 1 to 22 mm halo zones (Figures 5.4 – 5.8). Microbial enzymes are more desirable since they are environmentally friendly, and simpler to produce and improve, microbes being able to be genetically engineered to generate the

required quantity and quality (Khadka et al., 2020). Protease enzymes, which were produced by 57% of the endophytes from this study, are among the most substantial groups of enzymes, valued at around \$4 billion, and are used in a variety of processes, including the processing of food, bioremediation, and pharmaceuticals (Khadka et al., 2020). The ability of protease to hydrolyse proteins is widely known. Protease readily hydrolyses lipase, and having a single microbe that can synthesise both lipase and protease, as has been reported, is one of the rare cases (Suryia Prabha et al., 2015). In the present study, as illustrated in Table 5.2 and Figure 5.6, AR1 (*Bacillus paralicheniformis* KSW1), EL2 (*Bacillus subtilis* KSW6) and MS4 (*Bacillus australimaris*) there were some of the endophytes that were able to produce both these enzymes. *Bacillus* spp. are also notable for aflatoxins (highly carcinogenic mycotoxin compounds) detoxifiers (Khadka et al., 2020). These toxins can enter human systems through contaminated food. Some *Bacillus* species can also inhibit microbial pollutants and detoxify aflatoxins by producing enzymes such as chitinase, protease, and glucanases (Khadka et al., 2020).

5.5. CONCLUSION AND RECOMMENDATIONS

Artemisia afra harbours endophytic bacteria with varying plant growth-promoting abilities. The endophytes evaluated in this study demonstrated considerable phosphate solubilising activity, as evidenced by their phosphate-solubilising indices ranging from 1.34 to 2.15, with 79% of these able to solubilise insoluble tricalcium phosphate. Approximately 86% of *A. afra* isolates produced IAA, with AR1 (*Bacillus paralicheniformis* KSW1) producing the most at 3,499 (µg/mL) and MS3 (*Bacillus subtilis* H1) producing the least at 0.034 (µg/mL). On the other hand, 64% of endophytes were able to fix biological nitrogen in a N-free medium, while 79% formed siderophores with a halo zone spanning from 2 to 11mm. Furthermore, endophytic bacteria from *Artemisia afra* exhibited variable extracellular enzyme activity; with hydrolytic activity ranging from 1 to 22mm. Gelatinase (64%), protease (57%), and amylase (57%) were the most produced enzymes. AR1 (*Bacillus paralicheniformis* KSW1), EL2 (*Bacillus subtilis* KSW6) were the only endophytes that could generate all of the examined enzymes. The AS2 (*Enterobacter* sp. ML) and BL2 (*Bacillus subtilis* cf18B) were the least producers of these enzymes, having produced only one of the different enzymes that were evaluated. The capacity of these endophytes to have both enzymatic and plant growth-promoting properties indicates that they may have a broad use in both the pharmaceutical and agricultural sectors. The use of these endophytes in a

greenhouse experiment may give validation to these preliminary findings. For instance, *Bacillus* spp. are good contenders for the production of biofertilizers as they can promote growth and produce biocontrol agents with their additional ability to sporulate under extreme environments. They have a longer shelf-lifespan, being able to form endospores, a possible added benefit for production of long-lasting biofertilizers.

6. CHAPTER SIX: GENOMIC AND METABOLOMIC ANALYSES OF ENDOPHYTIC *BACILLUS* SPECIES REVEALS ENHANCED GROWTH AND METABOLITE PRODUCTION IN *LESSERTIA FRUTESCENS* (*SUTHERLANDIA FRUTESCENS*)

ABSTRACT

The concept of coevolution elucidates how endophytes and their host plants have developed biochemical interactions over time. This relationship shapes the biosynthetic pathways of metabolites produced by both endophytes and their host plants. One primary method involves the endophytes' ability to generate similar signaling pathways as their hosts. On the other hand, inoculation of endophytes with host plants has been shown to stimulate the expression of silent biosynthetic genes, leading to the production of novel metabolites. The present study investigated the effect of endophytic *Bacillus paralicheniformis* and *Bacillus velezensis* bacteria on *Lessertia frutescens* (L.) Goldblatt & J.C.Manning (syn. *Sutherlandia frutescens* (L.) W.T. Aiton) growth and metabolite production in a pot trial experiment. The experiment was conducted for 3 months. Four treatments were replicated six times in a completely randomised pattern. Nuclear magnetic resonance (NMR) analysis was used to assess the metabolites from pure bacterial endophytes and *L. frutescens* leaves after harvesting. Whole genome sequencing was utilised to look at genes that promote plant growth in *B. paralicheniformis* and *B. velezensis*, as well as genes linked to their metabolites production capacity. *Bacillus paralicheniformis*, *B. velezensis* and their combination significantly increased *L. frutescens* growth as evidenced by increased plant height, wet and dry total biomass, wet and dry shoot and root biomass. Additionally, the endophytes increased the macronutrient content of *L. frutescens* leaves. Treat-CL increased the accumulation of macronutrients (nitrogen, phosphorus, magnesium, calcium and sulfur and iron). In comparison to the control (without inoculum), *L. frutescens* produced more metabolites when treated with inoculum of *B. paralicheniformis*, *B. velezensis* and their combination. However, inoculated *L. frutescens* plants and pure bacterial endophytes produced more comparable metabolites. *Bacillus paralicheniformis* and *Bacillus velezensis* as well as their co-culture also produced more comparable metabolites. co-culturing increased the quantity of metabolites, not the variation. The whole genome sequencing results revealed that *B. paralicheniformis* and *B. velezensis* contained genes (*NtrC*, *NarT*, *NasA*, and *NifU*) that are involved in the biological conversion of nitrogen from one form to another.

Additionally, *B. paralicheniformis* and *B. velezensis* carried genes (*Fur*, *Feu A*, *FeuB*, *FeuC*, *FeoA*, *FeoB*, and *FhuA*, *FhuB*, *FhuC*, *FhuG*) that are involved in the production and transportation of siderophores, as well as genes (*PstA*, *PstB*, *PstC*, *PstS*, *PhoR*) that are involved in phosphate solubilisation, phosphate uptake, and transportation. Furthermore, the metabolite profiles derived from NMR data were consistent with the genetic functions derived from whole genome data. Bacterial endophytes were shown to be the most effective source of metabolites.

6.1. INTRODUCTION

In South Africa, *Lessertia frutescens* is a highly sought-after medicinal plant that holds significant economic value (Van Wyk & Albrecht, 2008). *Lessertia frutescens* is in high demand due to its several medicinal benefits, which include anticancer, antiviral, antimicrobial, anti-stress, and antidiabetic effects (Gibson, 2011). Collecting medicinal plants from the wild remains the most common practice in Africa, including South Africa. Although medicinal plants such as *L. frutescens* are commercial plants in South Africa, proper commercialisation, which includes greater sustainable returns and consistency of produced raw materials, can only be accomplished through the application of sustainable propagation and cropping systems (Kleynhans *et al.*, 2016). Commercialisation and cultivation of *L. frutescens* started in the 1990s and early 2000s (Van Wyk, 2011), with literature claiming that it began as far back as in 2000. Large-scale cultivation of *L. frutescens* only started when South Africa Company, Phyto Nova (Pty) Ltd, followed with the production of tablets from dry powdered leaves and stems (van der Walt *et al.*, 2016). About 20 tons of dried leaves of *L. frutescens* are required to offer herbal nutritional supplements for just one million people in South Africa, the demand constantly rising to the extent that the available raw plant materials are sometimes insufficient to meet the demand (Shaik *et al.*, 2010). The increased demand for plant material for the production of low-cost phytopharmaceutical medications is conducive to overharvesting and a decline in the genetic diversity of species such as *L. frutescens* (Shaik *et al.*, 2010). Conservation methods are therefore necessary, the ultimate goal being to increase the production of these metabolites from medicinal plants for the purpose of producing high-quality drugs (Isah *et al.*, 2018). This can be accomplished by utilising plant development studies that enable the amplification of these crucial bioactive compounds (Isah *et al.*, 2018). For instance, given the mutualistic interaction between endophytes and plants, incorporating native endophytes into the plants' sustainable agriculture techniques can have a stronger impact on ensuring plant health, growth and enhancing their capacity to synthesise novel metabolites. Inoculation of medicinal plants, notably leguminous ones, such as *L. frutescens* with beneficial microorganisms is an emerging

scientific endeavour aimed at enhancing medicinal plant bioactive component synthesis in a sustainable manner (Masenya et al., 2022). Certain endophytes produce metabolites that are chemically similar to those synthesized by their host plants, thereby revealing a complex interplay between these organisms. This phenomenon underscores the ecological and biochemical relationships that exist in plant microbiomes and has significant implications for agriculture and medicine, particularly in the realms of sustainable practices and drug discovery (Singh et al., 2022). The ability of endophytes to mimic host plant metabolites can enhance the production of bioactive compounds, which play critical roles in plant defence and resilience against environmental stressors. Research has shown that these microorganisms can induce the expression of plant secondary metabolites, such as phenolics and alkaloids, thereby contributing to the host's growth, resistance to pathogens, and overall health (X. Liu et al., 2016). This mutualism not only benefits the plants by enhancing their defenses and stress tolerance but also presents opportunities for bioprospecting novel compounds from both endophytes and their host plants, that could be harnessed for pharmaceutical applications (Singh et al., 2022; Wang et al., 2023). The present study investigated the influence of two endophytic bacteria, *B. paralicheniformis* and *B. velezensis*, on plant growth and metabolite production of *L. frutescens* in a pot trial experiment. The two isolates were previously obtained from the same plant. Furthermore, quality and quantity of metabolites production by both *Bacillus* species were also evaluated.

6.2. METHODS

6.2.1. Selection of bacterial endophytes for inoculum production for use in the greenhouse experiment

Eighteen *Lessertia frutescens* medicinal plants were obtained from Eden nursery in Klerksdorp, North- West Province of South Africa in April 2021. The plants were surface sterilised using 95% ethanol and 2.5% sodium hypochlorite solution, and rinsed with distilled water as described (Palanichamy et al., 2018). To test the efficiency of surface sterilisation, small portions of each plant part were plated on nutrient agar and incubated at 37°C for 24-48 h. The absence of microbial growth indicated the success of the sterilisation process. The plant segments (roots, stem and leaves) were air-dried in a sterile environment and later crushed using a pestle and mortar. For each sample, 1g of crushed plant material was mixed with 9ml of sterile distilled water and shaken. The suspension was serially diluted (10^0 - 10^{-6}) and 100µL aliquots of 10^{-6} dilution were plated in nutrient agar and incubated at 37°C for 24 h for bacterial endophytes

isolation. The endophytes were then sub-cultured until pure cultures were obtained. In an in-vitro experiment, pure endophytes were evaluated for plant growth promotion by determining their ability to fix biological nitrogen, solubilised insoluble phosphate, produced indole acetic acid and siderophore. The ability of bacterial endophytes to fix biological nitrogen was determined as described by (Hamza et al., 2017) on nitrogen free media (Burk's media) containing magnesium sulphate (0.2g), dipotassium phosphate (0.8g), monopotassium phosphate (0.2g), calcium sulphate (0.13g), ferric chloride (0.00145g), sodium molybdate (0.000253g), sucrose (20g) and bacteriological agar (15g) in 1 L. P-solubilisation of bacterial endophytes were evaluated qualitatively on Pikovskaya agar medium (PVK) enriched with 0.5% of insoluble tricalcium phosphate (TCP) $\text{Ca}_3(\text{PO}_4)_2$ as indicated by (Pikovskaya, 1948). Indole acetic acid production by endophytes was determined using Salkowski's reagent (150 mL concentrated sulfuric acid (H_2SO_4), 250 ml distilled water, and 7.5 ml of 0.5 M FeCl_3) in the presence of 1% of L-tryptophan as described by Putrie et al. (2017). Lastly, to determine the ability of the endophytes to produce siderophores, the Chrome Azural S (CAS) agar plate method was used, as described by Mukherjee & Dutta (2019).

6.2.2. Molecular identification of bacterial endophytes from *Lessertia frutescens* plants

Based on their in vitro plant growth-promoting N-fixation, P-solubilisation, IAA, and siderophore production, the two most efficient strains that tested positive for all the plant growth in-vitro tests were selected for the greenhouse experiment. Prior to the greenhouse experiment, the two endophytes were identified using SANGER sequencing as described by Palanichamy et al., (2018) with modifications. Bacterial genomic DNA from each pure endophytic strain was extracted using Fungal/Bacterial DNA miniPrep™ kit (Zymo Research Corporation, USA) following manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify the V3-V4 region of bacteria's 16S rDNA gene using universal forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The SANGER sequencing was done at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa) and the sequences were compared with available sequences in the National Center for Biotechnology Information (NCBI) database by the Basic Local Alignment Search Tool (BLAST) program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The best two isolates were identified as *Bacillus paralicheniformis* and *B. velezensis*.

6.2.3. Whole genome sequencing

DNA extraction and sequencing

Bacterial genomic DNA from pure *B. paralicheniformis* and *B. velezensis* endophytes was extracted using Fungal/Bacterial DNA miniPrep™ kit (Zymo Research Corporation, USA) following manufacturer's instructions. The genomic DNA libraries were prepared, sequenced on an Illumina NovaSeq platform and 150 bp paired-end reads were generated.

Preparation of the genome assembly and annotation

The total sequenced reads generated from *B. paralicheniformis* and *B. velezensis* were filtered to remove adapter sequence and low-quality fragments using Trimmomatic v0.36 (Bolger et al., 2014) with a minimum quality score of 15 and a minimum sequence length of 70. The adapter regions were clipped using a mismatch value of 2, a palindrome clip threshold of 30, and a simple clip threshold of 10. The assembly was performed with the quality reads using SPAdes v3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020). Completeness of the assembled genome and presence of undesired sequence fragments were assessed using CheckM v1.0.18. The genome assembly was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v6.7 (Li et al., 2021) and Rapid Annotations using Subsystems Technology (RAST) with SEED viewer v. 2.0 (Overbeek et al., 2014). Mapping of the entire circular genome was visualised using Proksee (Grant et al., 2023) while the CRISPR array and Cas clusters were determined by CRISPR/Cas finder, and CARD resistance genes Identifier was used to identify antimicrobial resistance genes within the genome.

6.2.4. Compatibility test between *Bacillus paralicheniformis* and *Bacillus velezensis*

Compatibility testing was used to establish whether the selected strains could coexist. With modifications, compatibility testing between *B. paralicheniformis* and *B. velezensis* was carried out in accordance with Barbarossa et al. (2018). Pure *B. paralicheniformis* and *B. velezensis* endophytes were streaked perpendicularly to one another on a nutrient agar plate. The agar plates were then incubated for 72 h at 30 °C, and zones of inhibition were checked. The ability of these strains to grow across each other without producing a halo zone of inhibition suggested their compatibility.

6.2.5. Experimental design

Lessertia frutescens was propagated in a greenhouse experiment at North-West University in Potchefstroom campus using ten-week-old *L. frutescens* seedlings that were obtained at Die Blomwinkeltjie nursery in Potchefstroom. The seedlings were transplanted into pots filled with sterile coconut peat, and the inoculation process was initiated after one week of growth. Two strains (*B. paralicheniformis* and *B. velezensis*) that demonstrated the ability to promote plant development in an in vitro test were used in the greenhouse. Bacterial endophytes were inoculated in nutrient broth and incubated for 24 h at 37 °C to prepare the inoculum. The inoculums' OD values were assessed, and 1.0 OD value were diluted to 1 using sterile nutrient broth. The V-1100 D Spectrophotometer of Labex (Pty) Ltd was used to detect the absorbance at a wavelength of 540nm. Four treatments were replicated six times in a completely randomised design. The treatments were: Treat-Control (control = 0 inoculum), Treat-CL (CL = *B. paralicheniformis* inoculum), Treat-CR (CR = *B. velezensis* inoculum), and Treat-AB (AB = *B. paralicheniformis* + *B. velezensis* inoculum). For nutritional supplementation, all treatments were fertilised with 100ml of Hoagland solution once a week and watered every other day. The greenhouse temperatures were maintained at 30 °C during the day and 18 °C at night, with a steady 70% humidity level. Three months after the inoculation, the plants were harvested and the following parameters were measured: plant height, wet and dry total biomass, wet and dry root biomass, and wet and dry shoot biomass. The dry biomass of the plants was measured after freeze-drying them with liquid nitrogen. Thereafter, the leaf samples were analysed for nutritional content and metabolites.

6.2.6. Nutritional analysis

The plant leaves were sent to the Agricultural Research Council Institute for Industrial Crops (ARC-IIC) in Rustenburg for nutritional analysis. Due to inadequate leaf biomass, the samples from each treatment were combined to make a single composite sample, and the analysis was conducted in four samples, i.e. one from each treatment. The chemical analysis at ARC-IIC was carried out using the following methods: Sulfuric acid and selenium dioxide were used in the micro-Kjeldahl acid digestion method to determine nitrogen (N) and phosphorus (P) from the samples using a segmented flow analyser (SEAL AA3) (Trikilidou et al., 2022). To determine N, the samples were reacted with salicylate and sodium hypochlorite solution to form a blue compound at 660nm wavelength, and nitroprusside was used as a catalyst.

Phosphorus, on the other hand, was determined using a colourimetric approach in which the interaction of phosphate with molybdovanadate in an acidic solution produces a yellow colour and the phospho-molybdovanadate complex was read at 420nm. To determine the amount of sulfur in plant samples, the nitric-perchloric acid wet digestion method was employed. Using a Novaspec Spectrophotometer, the quantity of sulfur in the digested samples were measured. At a wavelength of 420nm, the device was calibrated using calibration standards that were within the working range of 0–50mg/l. Samples for measurement of calcium (Ca), magnesium (Mg), and micronutrients (Cu, Fe, Zn, Mn, and B) were prepared using the dry ashing method in a muffle furnace at 550°C, followed by digestion with aqua regia on a steam bath (Paul *et al.*, 2017). An Atomic Absorption Spectrophotometer (AAS) was used to measure Fe, Cu, Zn, and Mn, and the AAS was tuned at different wavelengths for each micronutrient. To determine Fe, the instrument was calibrated with calibration standards containing 3, 6, and 9mg/l Fe at a wavelength of 248.3nm. To determine Cu, calibration standards containing 0.5, 1.0, and 1.5mg/l Cu at 324.8nm were used. Calibration for Zn was performed using standards containing 1, 2, and 3mg/l Zn at 213.9nm. Mn was determined using calibration standards containing 1, 2, and 4mg/l Mn at 279.5nm. Boron was determined using the colorimetric method with a segmented flow autoanalyser. The equipment was calibrated using calibration standards containing 0.5, 1.0, 1.5, 2.0, and 2.5mg/l B at 420nm wavelength.

6.2.7. Extraction of metabolites from bacterial endophytes

With modifications, extraction of metabolites from bacterial endophytes was performed as described by (Shanthakumar *et al.*, 2015; Sulistyani & Narwanti, 2015). Pure culture of bacterial endophytes was inoculated into 50 ml LB and incubated at 37 °C for 48 h. After 48 hours, equal volumes (25 ml) of ethyl acetate and chloroform were added to the culture and ultrasonicated for 30 min to disrupt the bacterial cells and release the intracellular metabolites into the solvent. After ultrasonication, the mixture was allowed to settle before the organic portion was centrifuged at 10 000 rpm for 20 min. The clean supernatant was stored at -80 °C overnight before being freeze-dried for 5 days until it was powdered. The powdered form was used to determine the metabolite production of these endophytes.

6.2.8. Analysis of metabolites from bacterial endophytes and *Lessertia frutescens* plant leaves

Following the technique of Kim et al. (2010) with some adjustments, approximately 100mg of *L. frutescens* leaf samples were suspended in 1 mL D₂O (99% deuterated, Aldrich) (Kempton Park, South Africa) and vortexed until all material was wetted. The samples were then sonicated for 30 min at ambient temperature. The liquid of each sample was aspirated and transferred into a clean tube. These samples were placed in a centrifuge for 15 min at 10 000 rpm. The supernatant was transferred to standard 5mm NMR tubes for analysis. A 600 MHz Bruker Avance NEO spectrometer (Bruker BioSpin, Ettlingen, Germany) was employed for NMR analysis. A direct-observation, two-channel broadband iProbe (Bruker) was fitted to the equipment. ¹H spectra were recorded at 600 MHz at a set temperature of 30 °C. Water signal suppression was performed utilising selective, multiple presaturation during the relaxation delay and mixing times. About 512 scans were recorded to produce data for processing. All NMR operations and data processing were performed with TopSpin 4.4.0 (Bruker). The same method was utilised for bacterial endophytes metabolite analysis.

6.2.9. Multivariate data analysis

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), and the correction of phasing and baseline, normalisation and peak alignment were done manually on the ¹H NMR spectrum. All processed data were divided into 0.04 ppm bins, representing 0 – 10 ppm and converted to Excel comma-separated values (CSV) file format for pattern recognition multivariate data analysis. Transformed data were statistically analysed, and all the imported data were Pareto scaled in the soft independent modelling of class analogy (SIMCA) software (SIMCA, Version 14.0, Umetrics, Umeå, Sweden). Similar and non-similar samples were statistically discriminated using the Principal Component Analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA). Annotations of compounds were performed using the Chenomx software (NMR suite, version 10.1) and published NMR data.

6.2.10. Other Statistical analyses

The effect of *B. paralicheniformis* and *B. velezensis* on *L. frutescens* growth parameters in a greenhouse were determined statistically. The obtained data was subjected to one-way analysis of variance (ANOVA) to evaluate differences among different treatment groups. The Shapiro-

Wilk Test ($\alpha=0.05$) was used to validate the normality assumption. The tests were set at a 5% significance level ($P < 0.05$). Pairwise comparisons were made using post hoc tests, Fisher's least significant difference (LSD), and Tukey's Honest Significance Difference (HSD).

6.3. RESULTS

Basic statistical features of the genomes are stated in Table 1 for *B. paralicheniformis* and *B. velezensis*.

Table 1: Statistical features of the genome assemblies for both strains

Organisms	Genome size	Contigs	N50	L50	GC	CDS	Completeness
<i>B. paralicheniformis</i>	4,343,421	22	414808	4	45.8	4629	99.71%
<i>Bacillus velezensis</i>	3,931,901	123	836455	3	46.5	4091	99.45%

6.3.1. Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* plant growth promotion capacity

Table 2 shows the putative gene functions associated with *B. paralicheniformis* and *B. velezensis* nitrogen regulation and availability. The genome of *B. paralicheniformis* contains gene functions involved in different stages of the nitrogen cycle, such as nitric oxide reductase activation protein (*NorQ* and *NorD*), nitrate/nitrite transporter (*NarT*), and nitrate reductase. On the other hand, *B. velezensis* nitrogen-based gene functions included nitrate reductase, nitrogen regulation protein (*NtrC*), nitrate/nitrite transporter (*NarT*), nitrate transporter (*NasA*), and *NifU*. With regard to plant growth-promoting ability of these strains, Table 3 shows the putative gene functions associated with *B. paralicheniformis* and *B. velezensis* phosphate solubilisation. *Bacillus paralicheniformis* possessed genes responsible for phosphate solubilisation, regulation, and transportation, including *PpaX*, *RapK*, *YcsE*, and *PstA*, *PstB*, *PstC*, *PstS*. With reference to phosphate solubilisation, phosphate regulation and transportation, *B. velezensis* contained genes such as *PhoR*, *PstA*, *PstB*, *PstC* and *PstS*. Furthermore, *B. paralicheniformis* and *B. velezensis* contained gene functions indole biosynthesis pathway which were alpha and beta tryptophan synthase as well as Indole-3-glycerol phosphate synthase as shown in Table 4. *Bacillus paralicheniformis* and *Bacillus velezensis* are significant producers of siderophores due to the presence of several gene functions involved in siderophore synthesis and iron uptake such as *Fur*, *Feu A*, *Fec B*, *FeuB*, *FeuC*, *FeoA*, *FeoB*, and *FhuB*, *FhuC*, and *FhuG* as shown in Table 5.

Table 2: Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* nitrogen regulation and availability

Gene functions	<i>Bacillus paralicheniformis</i>			<i>Bacillus velezensis</i>		
	Fragment position	Size (bp)	DNA strand	Fragment position	Size (bp)	DNA strand
Nitrate/nitrite transporter <i>NarT</i>	658039 – 659223	1 184	Forward	321201 – 322391	1190	Forward
Nitrate transporter <i>NasA</i>	445988 – 449671	3 683	Forward	95583 – 96788	1205	Forward
Nitrogen regulation protein NR(I), GlnG (=NtrC)	-	-	-	738447 – 737455	992	Reverse
Nitrogen regulatory protein P-II	300156 – 300506	350	Forward	-	-	-
<i>NtrC</i> family Transcriptional regulator, ATPase domain	389857 – 392643	2 786	Forward	-	-	-
<i>NifU</i> -like domain protein	-	-	-	819830 – 819594	236	Reverse

Table 3: Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* phosphate solubilization

Gene functions	<i>Bacillus paralicheniformis</i>			<i>Bacillus velezensis</i>		
	Fragment position	Size (bp)	DNA strand	Fragment position	Size (bp)	DNA strand
Alkaline phosphatase (EC 3.1.3.1)	96633 – 98294	1 661	Forward	70245 – 68866	1 379	Reverse
Phosphatase <i>YcsE</i>	259516 – 260271	755	Forward	-	-	-
Alkaline phosphatase synthesis transcriptional regulatory protein <i>PhoP</i>	-	-	-	101079 – 101798	719	Forward
Phosphate regulon sensor protein <i>PhoR</i> (SphS) (EC 2.7.13.3)	-	-	-	101791 – 103530	1 739	Forward
Pyrophosphatase <i>PpaX</i> (EC 3.6.1.1)	131860 – 131213	647	Reverse	-	-	-
Phosphate ABC transporter, permease protein <i>PstA</i> (TC 3.A.1.7.1)	555695 – 556582	887	Forward	390734 – 391618	884	Forward
Phosphate ABC transporter, ATP-binding protein <i>PstB</i> (TC 3.A.1.7.1)	556602 – 557429	827	Forward	391637 – 392443	806	Forward
Phosphate ABC transporter, permease protein <i>PstC</i> (TC 3.A.1.7.1)	554766 – 555695	929	Forward	389805 – 390734	929	Forward
Phosphate ABC transporter, substrate-binding protein <i>PstS</i> (TC 3.A.1.7.1)	553773 – 554690	917	Forward	388854 – 389756	902	Forward

Table 4: Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* indole acetic and tryptophan production

Gene functions	<i>Bacillus paralicheniformis</i>			<i>Bacillus velezensis</i>		
	Fragment position	Size (bp)	DNA strand	Fragment position	Size (bp)	DNA strand
Indole-3-glycerol phosphate synthase (EC 4.1.1.48)	283758 – 284516	758	Forward	658483 – 659235	752	Forward
Tryptophan synthase beta chain (EC 4.2.1.20)	285153 – 286379	1 226	Forward	659874 – 661076	1 202	Forward
Tryptophan synthase alpha chain (EC 4.2.1.20)	286348 – 287151	803	Forward	661069 – 661866	797	Forward

Table 5: Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* siderophore production

Gene functions	<i>Bacillus paralicheniformis</i>			<i>Bacillus velezensis</i>		
	Fragment position	Size (bp)	DNA strand	Fragment position	Size (bp)	DNA strand
Ferric uptake regulation protein <i>Fur</i>	198586 – 199035	449	Forward	-	-	-
Fe-bacillibactin uptake system <i>FeuC</i> @ ABC-type Fe ³⁺ -siderophore transport system, permease component	321243 – 320224	1 019	Reverse	9373 – 8351	1 022	Reverse
Fe-bacillibactin uptake system <i>FeuB</i> @ ABC-type Fe ³⁺ -siderophore transport system, permease 2 component	322240 – 321236	1 004	Reverse	10370 – 9363	1 007	Reverse
Fe-bacillibactin uptake system <i>FeuA</i> , Fe-bacillibactin binding	323214 – 322258	956	Reverse	11346 – 10390	956	Reverse
Siderophore synthetase superfamily, group C @ Siderophore synthetase component, ligase	223003 – 221189	1 814	Reverse	-	-	-
Siderophore biosynthesis protein, monooxygenase	224363 – 223020	1 343	Reverse	-	-	-
Siderophore synthetase small component, acetyltransferase	224892 – 224338	554	Reverse	-	-	-
Siderophore synthetase superfamily, group A @ Siderophore synthetase large component, acetyltransferase	226652 – 224889	1 763	Reverse	-	-	-
Siderophore biosynthesis L-2,4-diaminobutyrate	228160 – 226649	1 511	Reverse	-	-	-

decarboxylase								
Siderophore biosynthesis diaminobutyrate--2-oxoglutarate aminotransferase (EC 2.6.1.76)	229490 – 228144	1 346	Reverse	-	-	-		
Ferrichrome transport system permease protein <i>FhuB</i>	30807 – 31871	1 064	Forward	719204 – 720271	1 067	Forward		
Ferrichrome transport system permease protein <i>FhuG</i>	31871 – 32884	1 013	Forward	720271 – 721281	1 010	Forward		
Ferrichrome transport ATP-binding protein <i>FhuC</i>	32903 – 33715	812	Forward	721300 - 722103	803	Forward		

6.3.2. Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus velezensis* metabolites production

The genes shown in Table 6 are involved in the production, control, translocation, and consumption of some of the metabolites that *B. paralicheniformis* produces. The genes that *B. paralicheniformis* possessed included *TatAy*, *TatCy*, *TatAd*, and *TatCd* for twin-arginine translocation protein, *ArgR* for arginine pathway regulatory protein, *ArtP M*, *ArtP*, *ArtQ*, and *RocB* for arginine utilisation, among others that are involved in arginine biosynthesis, transportation, translocation, and utilisation. Also included were *SufS* genes for the biosynthesis of cysteine desulfurase, and the LacI family, the repressor of the sucrose operon *ScrR*, multiple sugar transporter genes (*MsmE*, *MsmF*, and *MsmG*), proline-producing genes (*ProP* and *PutP*), and the enzyme trehalose-6-phosphate hydrolase, which is a component of the trehalose biosynthesis pathway.

Table 6: Putative gene functions associated with *Bacillus paralicheniformis* metabolites production capacity

Gene function	Fragment position	Size (bp)	DNA strand
Twin-arginine translocation protein <i>TatAy</i>	7402 – 7575	173	Forward
Twin-arginine translocation protein <i>TatCy</i>	7582 – 8358	776	Forward
Arginine pathway regulatory protein <i>ArgR</i> , repressor of arg regulon	64396 – 64845	449	Forward
Arginine-binding extracellular protein <i>ArtP</i>	85197 – 85964	767	Forward
Arginine transport system permease protein <i>ArtQ</i>	86051 – 86713	662	Forward
Arginine transport ATP-binding protein <i>ArtM</i>	86700 – 87428	728	Forward
Arginine utilization protein <i>RocB</i>	111567 – 113171	1 604	Forward
Twin-arginine translocation protein <i>TatAd</i>	11466 – 11678	212	Forward
Twin-arginine translocation protein <i>TatCd</i>	11754 – 12488	734	Forward
Cysteine desulfurase (EC 2.8.1.7) => <i>SufS</i>	101908 – 103128	1 220	Forward
Ribose ABC transporter, ATP-binding protein <i>RbsA</i> (TC 3.A.1.2.1)	253087 – 254568	1 481	Forward
Ribose ABC transporter, permease protein <i>RbsC</i> (TC 3.A.1.2.1)	254570 – 255532	962	Forward
Ribose ABC transporter, substrate-binding protein <i>RbsB</i> (TC 3.A.1.2.1)	255547 – 256461	914	Forward

Fructose-1,6-bisphosphatase, <i>GlpX</i> type (EC 3.1.3.11)	371372 – 370407	965	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmG</i>	574964 – 574116	848	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmF</i>	575899 – 574961	938	Reverse
Multiple sugar ABC transporter, substrate-binding protein <i>MsmE</i>	577209 – 575911	1 298	Reverse
Pyrroline-5-carboxylate reductase (EC 1.5.1.2), <i>ProG</i>	25532 – 24720	812	Reverse
Proline/sodium symporter <i>PutP</i> (TC 2.A.21.2.1) @ Propionate/sodium symporter	110110 – 111540	1 430	Forward
Trehalose operon transcriptional repressor	5035 – 4319	716	Reverse
Trehalose-6-phosphate hydrolase (EC 3.2.1.93)	6745 – 5057	1 688	Reverse

Numerous genes and enzymes involved in metabolic pathways are present in *B. velezensis*, as shown in Table 7. The most notable ones were for metabolite production, transportation, and utilisation of arginine. Among the genes that *B. velezensis* possessed were those involved in arginine biosynthesis, transportation, translocation, and utilisation: *RocR* for arginine utilisation, *ArtP*, *ArtP*, *ArtQ*, and *RocB* for other arginine processes, and *TatAy*, *TatAd*, and *TatCd* for twin arginine translocation protein. Gene functions for sugar compounds (xylose, fructose, sucrose, and glucose) were also present in *B. velezensis* as well as multiple sugar transport genes, including *MsmGE*, *MsmGF*, and *MsmG*. The whole genome sequence results also revealed the presence of the genes *ProP* and *PutP*, which are involved in the transportation and synthesis of proline.

Table 7: Putative gene functions associated with *Bacillus velezensis* metabolites production capacity

Gene functions	Fragment position	Size (bp)	DNA strand
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Alanine racemase (EC 5.1.1.1)	449316 – 448135	1 181	Reverse
Arginine-binding extracellular protein <i>ArtP</i>	474426 – 475193	767	Forward
Arginine transport system permease protein <i>ArtQ</i>	475255 – 475914	659	Forward
Arginine transport ATP-binding protein <i>ArtM</i>	475907 – 476629	722	Forward
Arginine utilization regulatory protein <i>RocR</i>	32477 – 31062	1 415	Reverse
Arginine permease <i>RocE</i>	35230 – 36630	1 400	Forward
Arginine utilization protein <i>RocB</i>	275890 – 277572	1 682	Forward
Twin-arginine translocation protein <i>TatAy</i>	10647 – 10817	170	Forward
Twin-arginine translocation protein <i>TatAd</i>	28957 – 29154	197	Forward
Twin-arginine translocation protein <i>TatCd</i>	29216 – 29941	725	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyJ</i>	678648 – 679460	812	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyK</i>	679486 – 680298	812	Forward
L-cystine ABC transporter, permease protein <i>TcyL</i>	680321 – 681043	722	Forward
L-cystine ABC transporter, permease protein <i>TcyM</i>	681059 – 681766	707	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyA</i>	66982 – 67779	797	Forward
L-cystine ABC transporter, permease protein <i>TcyB</i>	67781 – 68470	689	Forward
L-cystine ABC transporter, ATP-binding protein <i>TcyC</i>	68483 – 69226	-743	Forward
Xylose-responsive transcription regulator, ROK family	849409 – 848255	1 154	Reverse
Xylose isomerase (EC 5.3.1.5)	849673 – 851010	1 337	Forward
Fructose-1,6-bisphosphatase, <i>GlpX</i> type (EC 3.1.3.11)	347281 – 348246	965	Forward
Sucrose operon repressor <i>ScrR</i> , <i>LacI</i> family	579051 – 578077	974	Reverse
Glucose: proton symporter <i>GlcU</i>	28164 – 27301	863	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmG</i>	35015 – 34194	821	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmF</i>	35926 – 35012	914	Reverse

Multiple sugar ABC transporter, substrate-binding protein <i>MsmE</i>	37200 – 35923	1 277	Reverse
L-Proline/Glycine betaine transporter <i>ProP</i>	68440 – 69843	1 403	Forward
Proline/sodium symporter <i>PutP</i> (TC 2.A.21.2.1) @ Propionate/sodium symporter	82481 – 83911	1 430	Forward

6.3.3. Metabolites from endophytes

Figure1 shows the ¹H NMR spectra of several metabolites produced by *B. paralicheniformis* endophyte (A), *B. velezensis* (B) and the combination (*B. paralicheniformis* + *B. velezensis*) (C). *Bacillus paralicheniformis* fingerprints' entire ¹H NMR spectra (δ 0.5–8.5 ppm) revealed two distinct spectra regions: aliphatic region of δ 0–5 ppm and the region from δ 3–5 ppm which displays resonance signals associated with the sugar region (Figure1A). *Bacillus velezensis* had high variation of metabolites compared to *B. paralicheniformis* and their combination (Figure 1 B). *Bacillus velezensis* fingerprints' complete ¹H NMR spectra (δ 0.5–8.5 ppm) revealed two distinct spectra regions: the sugar region of δ 3.0–5 ppm, with high signal overlapping and the region from δ 0–3 ppm, which displays resonance signals associated with the aliphatic region (Figure 1 B). The combination of bacterial strains produced the least metabolites as showed by fewer peaks and their fingerprints' entire ¹H NMR spectra (δ 0.5–8.5 ppm) revealed spectra regions: the sugar region of δ 3.0–5 ppm and the aliphatic regions from δ 0–3 ppm. From the visual comparison of the spectra, there are obvious similarities between the metabolic profiles of *B. paralicheniformis* (Figure1A) and the combination (Figure1C) of these two endophytes. This suggests the possible competition between these strains when combined.

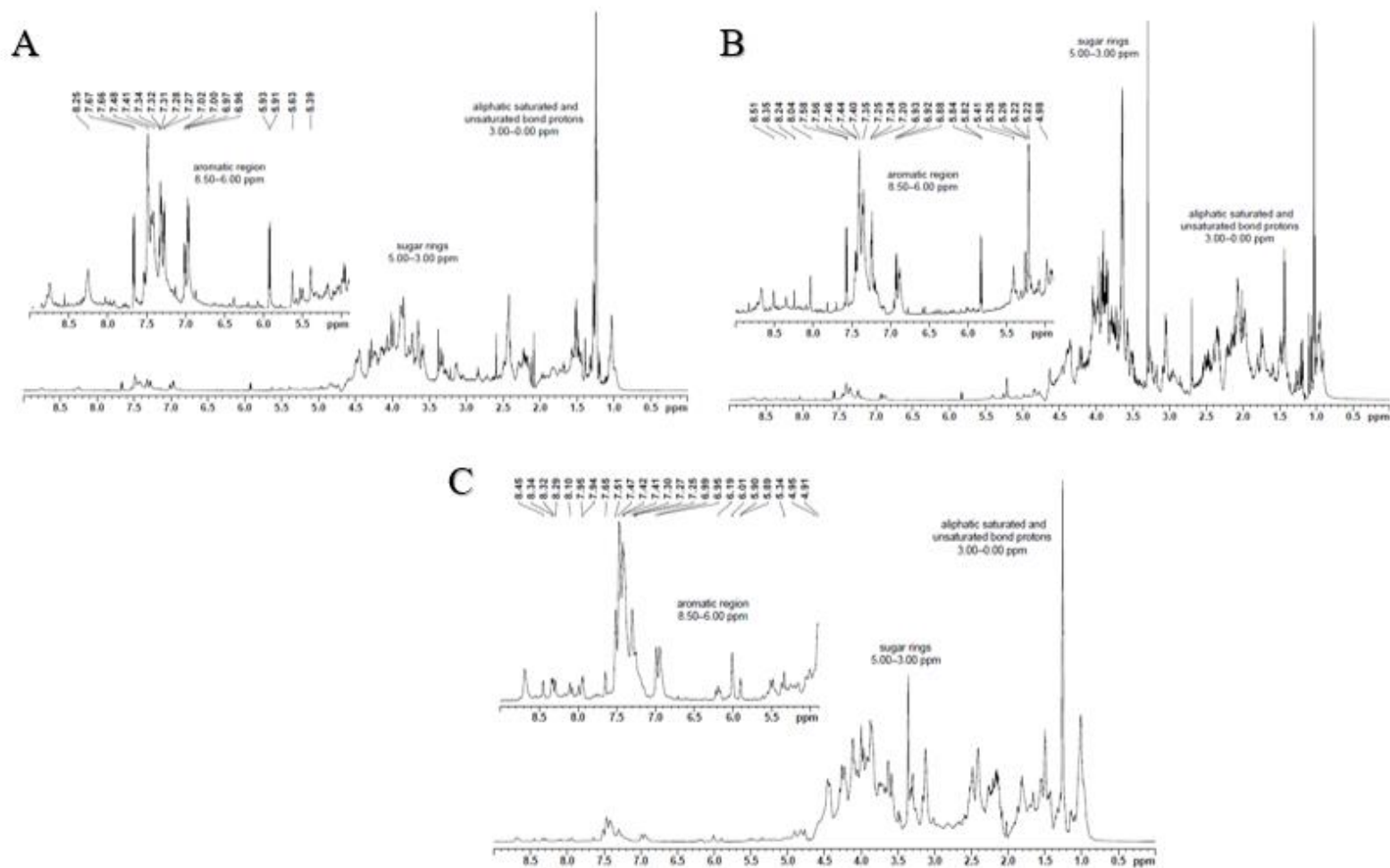


Figure 1: The ^1H NMR spectra of several metabolites of *B. paralicheniformis* endophyte (A), *B. velezensis* (B) and the combined effect of (*B. paralicheniformis* + *B. velezensis*)

Table 8 displays Chenomx-assisted annotations of differentially produced metabolites from *B. paralicheniformis* and *B. velezensis* endophytes and their co-culture. *Bacillus paralicheniformis* and *Bacillus velezensis* endophytes and their combination produced more comparable metabolites. Metabolites from *B. paralicheniformis* had higher concentrations than those from *B. velezensis*. co-culturing boosted the quantity of metabolites generated, as shown by the high concentrations.

Table 8: Chenomx-assisted annotated metabolites from bacterial endophytes

Sample ID	<i>B. paralicheniformis</i>		Co-culture		<i>B. velezensis</i>	
Metabolites	Concentration (mM)	Chemical shifts (ppm)	Concentration (mM)	Chemical shifts (ppm)	Concentration (mM)	Chemical shifts (ppm)
Alanine	2.6266	3.77 (d), 1.47 (d)	2.4107	3.77 (d), 1.47 (d)	2.9836	3.77 (d), 1.47 (d)
β-Alanine	3.0310	3.17 (t), 2.54 (t)	8.6902	3.17 (t), 2.54 (t)	1.9912	3.17 (t), 2.54 (t)
Arabinose	12.1166	5.29 (d), 4.51 (d), 3.93 (s), 3.80 (dd)	11.6032	5.29 (d), 5.23 (d), 4.51 (d)	0.4911	5.29 (d), 4.10 (m), 3.99 (d), 3.83 (q)
Arginine	6.2462	3.76 (t), 3.23 (t), 1.88 (m)	14.8894	3.76 (t), 3.23 (t), 1.91 (m), 1.71 (m)	3.2153	3.76 (t), 3.23 (t), 1.88 (m), 1.71 (m)
S-Sulfocysteine	6.5232	4.16 (q), 3.67 (dd), 3.46 (q)	9.8292	4.16 (q), 3.67 (dd), 3.46 (q)	3.7558	4.16 (q), 3.67 (dd), 3.46 (q)
Trehalose	-	-	2.4595	5.18 (d), 3.84 (m), 3.64 (q)	0.0959	3.84 (m), 3.64 (q)
Cysteine	7.8150	3.97 (t), 3.09 (dd), 3.02 (dd)	8.8493	3.97 (t), 3.09 (dd), 3.02 (dd)	5.2524	3.97 (t), 3.09 (dd), 3.02 (dd)
Cystine	8.3227	4.10 (q), 3.37 (dd), 3.17 (q)	15.2556	4.10 (q), 3.37 (dd), 3.17 (q)	2.2995	4.10 (q), 3.37 (dd), 3.17 (q)
Fructose	6.9494	4.09 (d), 3.99 (t) 3.88 (q), 3.69 (m)	16.8859	4.11 (d), 4.01 (t), 3.88 (q), 3.55 (m)	12.3957	4.11 (d), 4.01 (t), 3.88 (q), 3.70 (m), 3.55 (m)
Fucose	2.8698	5.26 (d), 4.54 (d), 3.85 (m),	3.9440	4.54 (d), 4.07 (m),	0.3968	4.54 (d), 4.07 (m), 3.84 (dd), 3.79 (m),
Glucose	10.1075	4.63 (d), 3.89 (dd), 3.71 (m), 3.48 (m)	10.2192	5.22 (d), 4.63 (d), 3.89 (dd), 3.82 (m), 3.71 (m), 3.48 (m)	0.3728	5.22 (d), 4.63 (d), 3.89 (dd), 3.82 (m), 3.71 (m), 3.48 (m)

Xylose	10.4057	5.19 (d), 4.57 (d), 3.91 (q), 3.66 (m), 3.51 (q)	11.5376	5.19 (d), 4.57 (d), 3.91 (q), 3.66 (m), 3.51 (q)	0.4133	5.19 (d), 4.57 (d), 3.91 (q), 3.66 (m), 3.51 (q)
Ribose	23.9688	5.37 (s), 4.92 (d), 4.20 (t), 4.09 (s), 3.91 (m), 3.67 (m)	36.3798	5.37 (d), 5.24 (s), 4.44 (d), 4.09 (s), 3.91 (m), 3.67 (m)	3.0256	5.37 (s), 4.92 (d), 4.20 (t), 4.09 (s), 3.91 (m), 3.67 (m)
Ornithine	3.9336	3.77 (t), 3.05 (t), 1.93 (m), 1.82 (m), 1.73 (m)	4.6567	3.77 (t), 3.05 (t), 1.93 (m), 1.82 (m), 1.73 (m)	2.9550	3.77 (t), 3.05 (t), 1.93 (m), 1.82 (m), 1.73 (m)
Proline	12.8916	4.12 (q), 3.41 (m), 3.32 (m), 2.33 (m)	18.7386	4.12 (q), 3.41 (m), 3.32 (m), 2.33 (m)	1.6904	4.12 (q), 3.41 (m), 3.32 (m), 2.33 (m)
Peak multiplicity (s=singlet; d= doublet; dd= doublet of doublet; t= triplet; q= quartet; m = multiplet)						

6.3.4. Influence of endophytes on *Lessertia frutescens* plant growth

Endophytic bacteria significantly affected *L. frutescens* plant height growth ($P = 0.0000000000459$ i.e. ($P \leq 0.05$)) (Figure 2). Compared to the control (without inoculum) with the lowest height, the combined treatment (Treat-AB) resulted in the highest growth (Figure 2). Similarly, the individual treatment with *B. paralicheniformis* and *B. velezensis* significantly promoted plant height growth.

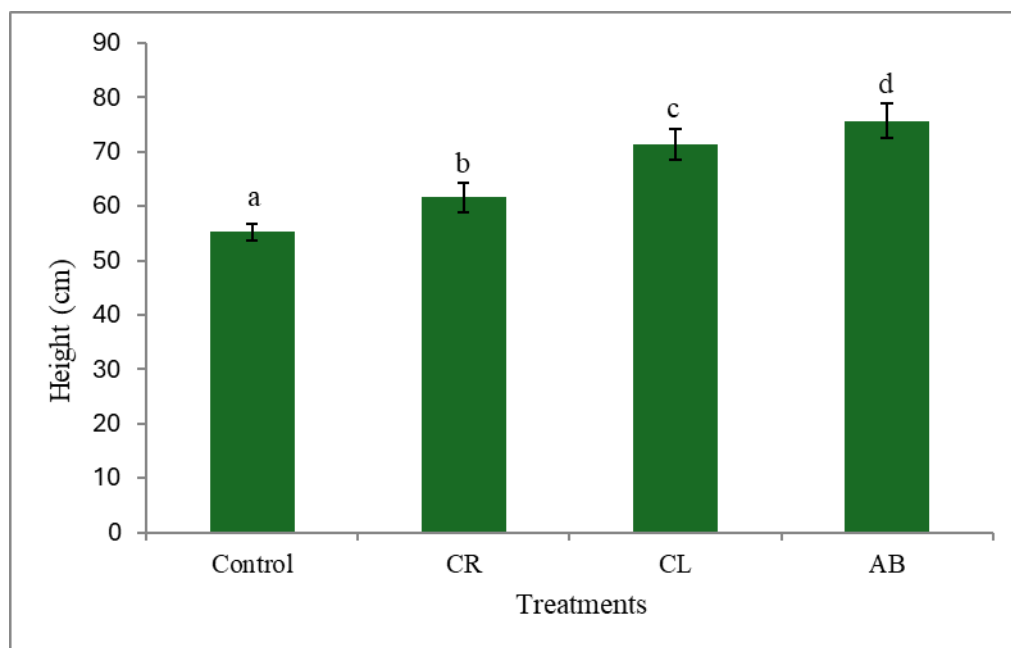


Figure 2: The significant effect of endophytes on *Lessertia frutescens* medicinal plant height. Error bars represent standard deviation ($n = 6$). Different letters on the upper portion of each bar indicate significant differences between treatments. $P = 0.0000000000459$ i.e. ($P \leq 0.05$). CL = *B. paralicheniformis*, CR = *B. velezensis* and AB = *B. paralicheniformis* + *B. velezensis*

Endophytic bacteria significantly affected *L. frutescens* plant total wet and dry biomass (Figure 3) and the P-values for wet and dry biomass were $P = 0.000000000000000111$ and $P = 0.0000000000000000777$ i.e. ($P \leq 0.05$), respectively. Compared to the control (without inoculum) with the lowest total wet biomass, the combined treatment (Treat-AB) resulted in the highest total wet (Figure 3 A) and dry (Figure 3 B) biomass. Similarly, the individual treatment with *B. paralicheniformis* and *B. velezensis* significantly promoted plant total wet (Figure 3 A) and dry biomass (Figure 3B).

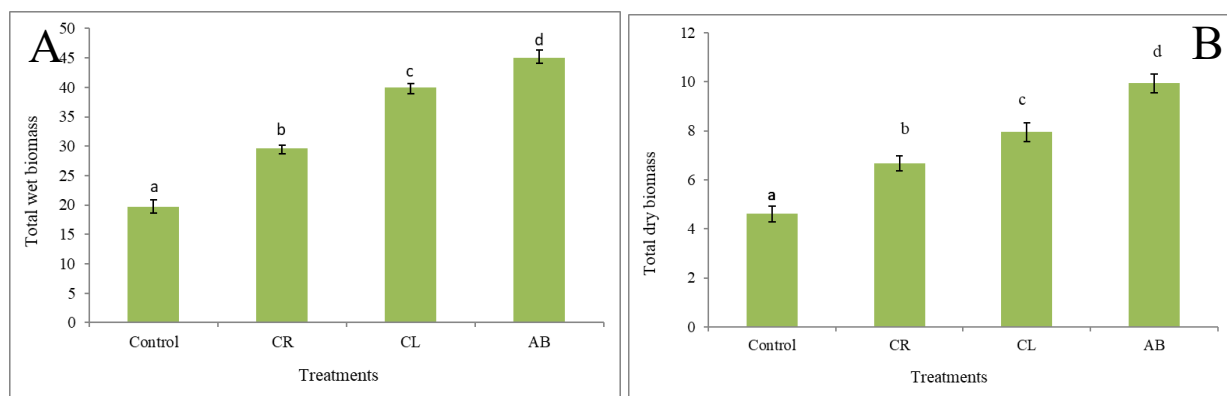


Figure 3: The significant effect of endophytes on *Lessertia frutescens* medicinal plant total wet (A) and dry (B) biomass. Error bars represent standard deviation ($n = 6$). Different letters on the upper portion of each bar indicate significant differences between treatments. P-values for wet and dry biomass were $P = 0.0000000000000000111$ and $P = 0.0000000000000000777$ i.e. ($P \leq 0.05$), respectively. CL = *B. paralicheniformis*, CR = *B. velezensis* and AB = *B. paralicheniformis* + *B. velezensis*.

Endophytic bacteria significantly affected *L. frutescens* plant shoot wet and dry biomass (Figure 4) and the the P-values of wet and dry shoot biomass were $P = 0.0000000000000000333$ and $P = 0.0000000000000000559$ ($P \leq 0.05$), respectively. The combined treatment (Treat-AB) resulted in the highest wet (Figure 4A) and dry (Figure 4B) shoot biomass when compared to the control (without inoculum) and other treatments with single isolates. Similarly, the individual treatment with *B. paralicheniformis* and *B. velezensis* significantly promoted plant wet (Figure 4A) and dry (Figure 4B) shoot biomass.

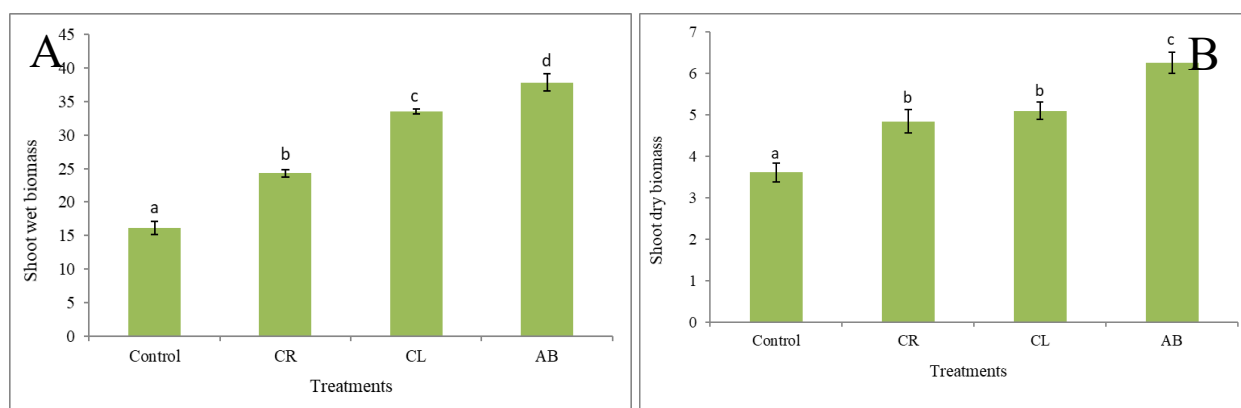


Figure 4: The significant effect of endophytes on *Lessertia frutescens* medicinal plant wet (A) and dry (B) shoot biomass. Error bars represent standard deviation (n = 6). Different letters on the upper portion of each bar indicate significant differences between treatments. The P-values of wet and dry shoot biomass were $P = 0.000000000000000333$ and $P = 0.0000000000000559$ ($P \leq 0.05$), respectively. CL = *B. paralicheniformis*, CR = *B. velezensis* and AB = *B. paralicheniformis* + *B. velezensis*.

Endophytic bacteria significantly affected *L. frutescens* plant root wet and dry biomass (Figure 5) and the P-values for wet and dry biomass were $P = 0.000000000000203$ and $P = 0.00000000000000444$ i.e. ($P \leq 0.05$), respectively. Compared to the control (without inoculum) with the lowest root wet biomass, the combined treatment (Treat-AB) resulted in the highest root wet (Figure 5A) and dry biomass (Figure 5B). Similarly, the individual treatment with *B. paralicheniformis* and *B. velezensis* significantly promoted plant root wet (Figure 5A) and dry biomass (Figure 5B).

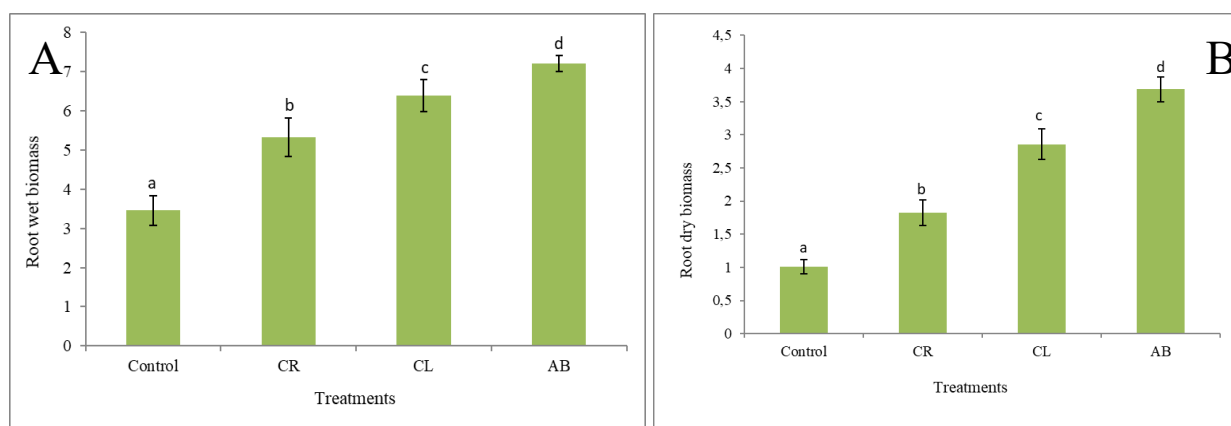


Figure 5: The significant effect of endophytes on *Lessertia frutescens* medicinal plant root wet (A) and dry (B) biomass. Error bars represent standard deviation (n = 6). Different letters on the upper portion of each bar indicate significant differences between treatments. P-values for wet and dry biomass were $P = 0.000000000000203$ and $P = 0.00000000000000444$ i.e. ($P \leq 0.05$), respectively. CL = *B. paralicheniformis*, CR = *B. velezensis* and AB = *B. paralicheniformis* + *B. velezensis*.

6.3.5. Influence of endophytes on *L. frutescens* macronutrients

Figure 6 illustrates the influence of endophytes on the macronutrient (N, P, Mg, Ca and S) content of *L. frutescens* leaves. Treat-CL enhanced the accumulation of nitrogen, phosphorus, calcium, magnesium and sulfur in *L. frutescens* leaves.

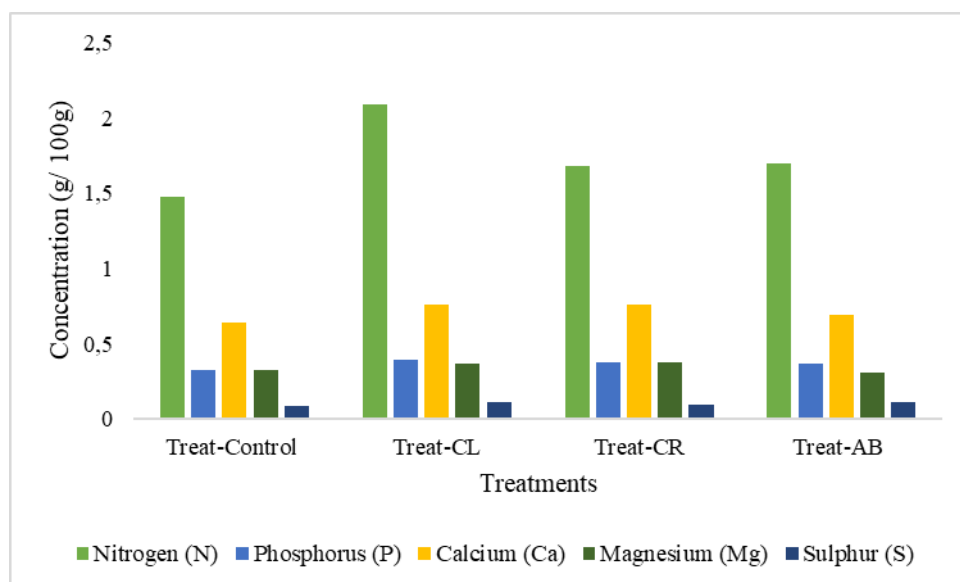


Figure 6: Influence of endophytes on macro-nutrient (N, P, Mg, Ca and S) content of *Lessertia frutescens* leaves. Treat-control (no-inoculum), Treat-CL= inoculated with *B. paralicheniformis*, Treat-CR= inoculated with *B. velezensis* and Treat-AB= inoculated with combination of *B. paralicheniformis* + *B. velezensis*.

6.3.6. Influence of endophytes on *Lessertia frutescens* micronutrients

Figure 7 illustrates the influence of endophytes on micronutrient (Fe, Cu, B, Zn and Mn) content of *L. frutescens* leaves.

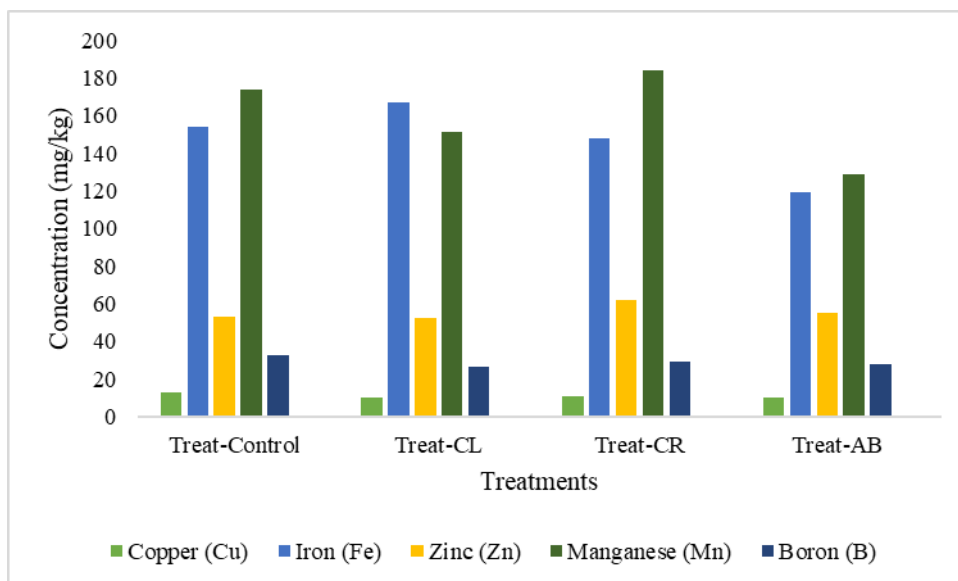


Figure7: Influence of endophytes on micro-nutrient (Fe, Cu, B, Zn and Mn) content of *Lessertia frutescens* leaves. Treat-control (no-inoculum), Treat-CL= inoculated with *B. paralicheniformis*, Treat-CR= inoculated with *B. velezensis* and Treat-AB= inoculated with combination of *B. paralicheniformis* + *B. velezensis*.

6.3.7. Influence of endophytes on *Lessertia frutescens* metabolites production

Figure 8 displays the ¹H NMR spectra of different metabolites identified in *L. frutescens* leaves under different treatments. *L. frutescens* leaves inoculated with endophytes produced more peaks than the control (without inoculum), implying that they contain more metabolites. Treatment control had the least influence on *L. frutescens* metabolites production (Figure 8A). The visual comparison of the spectra reveals significant similarities between the metabolic profiles of different treatments with inoculation, particularly metabolites between the aliphatic and sugar portions of the spectra, implying the potential of identical substances (Figure 8B, 8C and 8D).

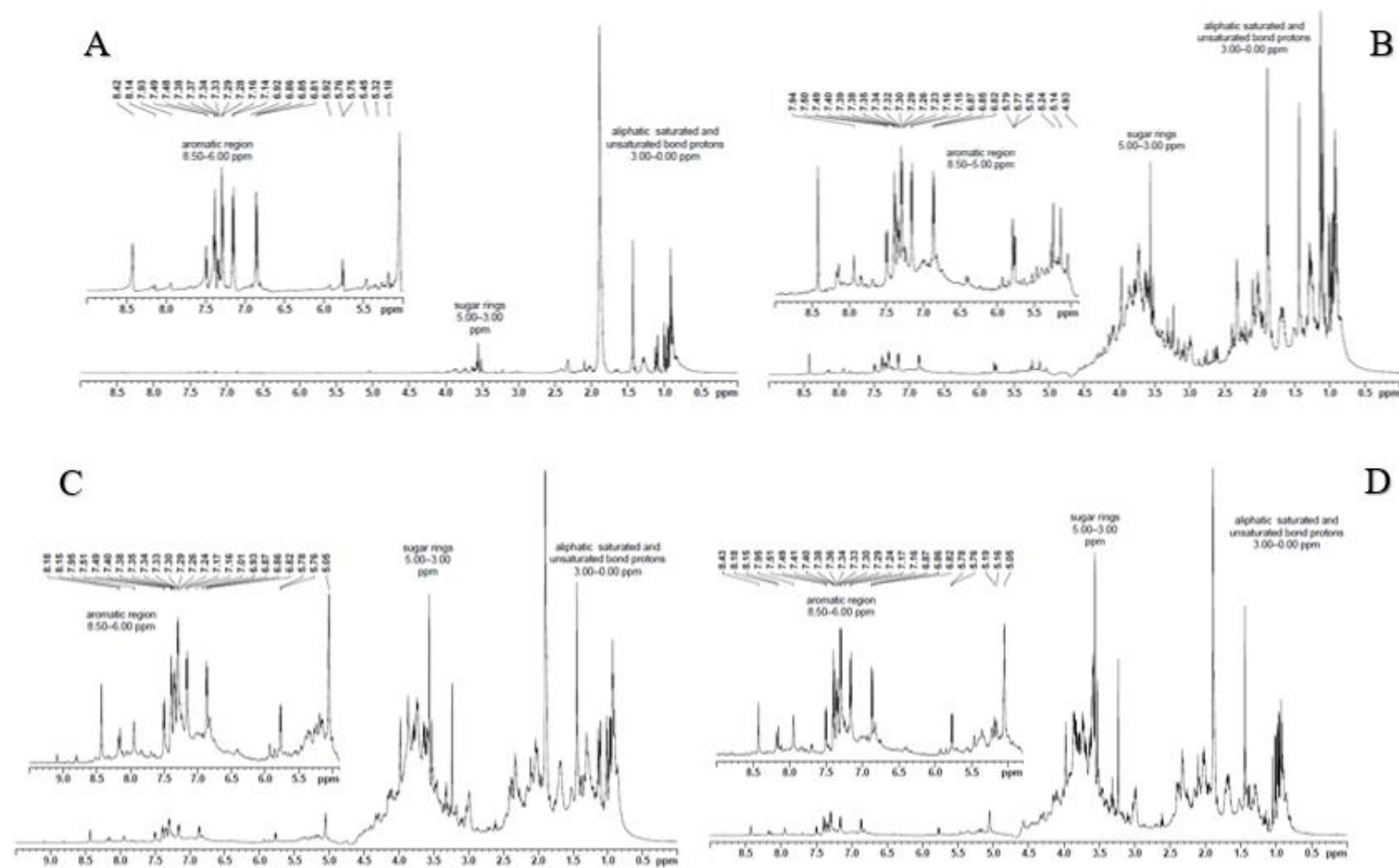


Figure 8: ¹H NMR representative spectra showing the effect of endophytes on *Lessertia frutescens* metabolites production. A= Treat-control (no-inoculum), B=Treat-CL (inoculated with *B. paralicheniformis*), C=Treat-CR (inoculated with *B. velezensis*) and D=Treat-AB (inoculated with combination of *B. paralicheniformis* + *B. velezensis*)

Table 9 displays Chenomx-assisted annotations of differentially produced metabolites from *L. frutescens* leaves under various treatments. Treat-control had the least metabolites (Figure 8A). Inoculating *L. frutescens* with a combination of *B. paralicheniformis* + *B. velezensis* (treatment-AB) influenced the production amino acids and sugar compounds. The *Bacillus paralicheniformis* (treatment-CL) had a greater impact on *L. frutescens*' synthesis of aliphatic compounds. *B. paralicheniformis* not only influenced the production of these metabolites, but also increased their quantity, the largest recorded quantity (concentration) of these compounds having been found in *L. frutescens* inoculated with *B. paralicheniformis*. Lastly, inoculating *L. frutescens* with *B. velezensis* (treatment-CR) influenced the production of amino acids and sugar compounds.

Table 9: Chenomx-assisted annotations of differentially produced metabolites from *Lessertia frutescens* leaves under different treatments

Metabolites	Treat-Control			Treat-AB			Treat-CL			Treat-CR		
	Conc. (mM)	Chemical (ppm)	shift	Conc.(m M)	Chemical (ppm)	shift	Conc. (mM)	Chemical (ppm)	shift	Conc. (mM)	Chemical (ppm)	shift
Alanine	-	-		-	-		74.4541	3.78 q, 1.47 d		1.9716	3.78 q	
β-Alanine	10.5470	3.17 s, 2.54 s					126.8154	3.17 t, 2.54 t				
γ-Glutamylphenylalanine	-	-		-	-		-	-		0.3838	3.57 m, 3.21 dd, 2.32 m	
Phenylalanine	-	-		-	-		1.1092	7.42 t, 7.37 t		-	-	
Arginine	-	-		3.8490	3.23 t, 1.92 m, 1.72 m		133.0523	3.76 t, 1.88 m		1.8851	1.92 m, 1.72 m	
Indole-3-acetate	-	-		0.5969	7.23 t		11.6896	7.61 d, 7.24 t		-	-	
Tryptophan	-	-		-	-		16.8223	7.27 q, 7.19 dd		-	-	
Cysteine	-	-		4.9283	3.97 t, 3.09 q, 3.03 dd		209.2971	3.97 t, 3.10 m, 3.02 m		4.3051	3.97 t, 3.09 q, 3.03 dd	
Threonine	-	-		-	-		7.3030	4.25 m, 3.58 d		-	-	
Ornithine	11.9771	3.05s		-	-		-	-		2.3629	3.78 t, 3.05 t, 1.82 m	

Ribose	50.1157	5.38 s, 5.24 s, 4.92 s, 4.09 s, 3.82 m, 3.52 m	15.5201	5.24 d, 4.20 t, 4.09 s, 3.98 m, 3.91 m, 3.67 m	354.0672	3.98 s, 3.92 m, 3.67 m, 3.52 s	2.6899	3.98 m, 3.87 m, 3.81 m, 3.60 m
Xylose	15.9632	5.19 s, 3.19 s, 3.62 s, 3.43 s, 3.23 s	2.1987	3.91 q, 3.69 m, 3.60 m	0.9343	3.91 m, 3.67 m, 3.60 m, 3.51 dd, 3.31 t	0.1408	3.91 m, 3.67 m, 3.60 m
Sucrose	3.7053	5.40 s	-	-	-	-	-	-
Fucose	20.1759	5.19s, 4.54 s	1.0289	5.26 d, 4.19 q, 3.86 m, 3.63 dd	167.3134	4.07 m, 4.00 m, 3.24 m	0.2036	4.07 m
Fructose	44.0863	4.09 s, 3.99 d, 3.79 s, 3.56 d	11.7741	4.11 d, 4.01 s, 3.89 dd, 3.70 m	408.0017	4.11d, 4.01 t, 3.79 m, 3.55 m	3.1580	3.89 dd, 3.79 m, 3.70 m, 3.55 m
Proline	56.4016	4.11 s, 3.41 s	12.0274	4.12 q	374.9204	4.12 t, 3.41 m		
Valine	-	-	11.7100	3.60d, 2.26 m	-	-	3.2102	3.60 d, 2.26 m, 1.03 d
Ibuprofen	2.9525	7.25 d, 3.60 s	-	-	-	-	-	-
Ferulate	-	-	1.1360	7.32 s, 6.92 d	-	-	-	-
myo-Inositol	-	-	4.1034	4.05 t, 3.61 t	4.8425	4.06 t, 3.61 t, 3.56 dd, 3.27 t	-	-

6.3.8. Multivariate data analysis for *Lessertia frutescens* leaves from different treatments

Figure 9 portrays the Principal Component Analysis (PCA) (A) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (B) scores of *L. frutescens* leaves, demonstrating metabolite relativity among the samples per treatment. An unsupervised Principal Component Analysis (PCA) plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *L. frutescens* samples (Figure 9A). There was no distinctive clustering on the samples from different treatments. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), which is the supervised recognition analysis that allows the algorithm to expose discrimination between the groups, was applied to the dataset (Figure 9 B). The samples are clustered according to their treatments (Figure 9B).

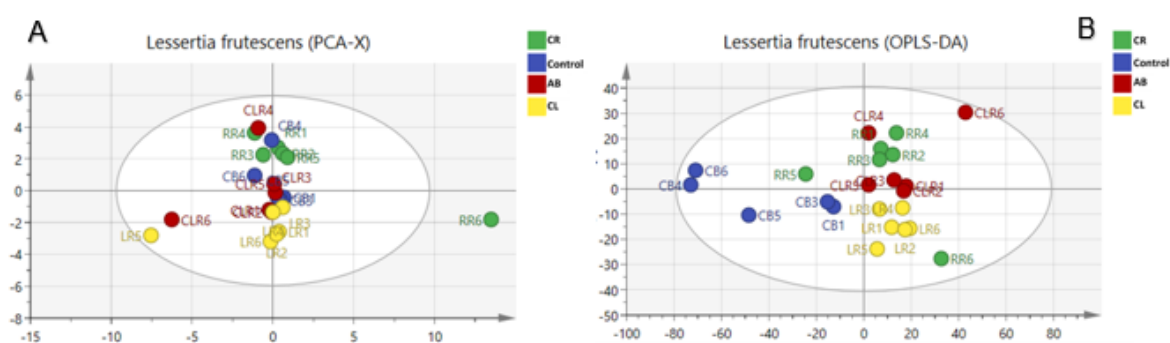


Figure 9: Principal Component Analysis (PCA) (A) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (B) scores of *Lessertia frutescens* leaves showing metabolite relativity of the samples per treatment. CA=control, CL = *B. paralicheniformis*, CR = *B. velezensis* and AB = *B. paralicheniformis* + *B. velezensis*

The statistical interference analysis was used to further validate the built OPLS-DA discriminant model (Figure 10). The model's dependability and degree of overfitting were determined by looking at the intercept of the fitting-line created by the estimated values R2X and Q2 for all samples on the y-coordinate axis. The higher the value of Q2, the more predictive the model becomes, and the higher the value of R2X, the more robust the explanatory ability. With a Q2 regression line intercept of -0.692 (Figure 10A), the developed OPLS-DA discriminant model was shown to be well predictive and not over-fit. The predictive residual was cross validated (p-value < 0.05) and the Receiver Operated

Characteristic (ROC), which computes the area under the curve (AUC), was shown (Figure 10B).

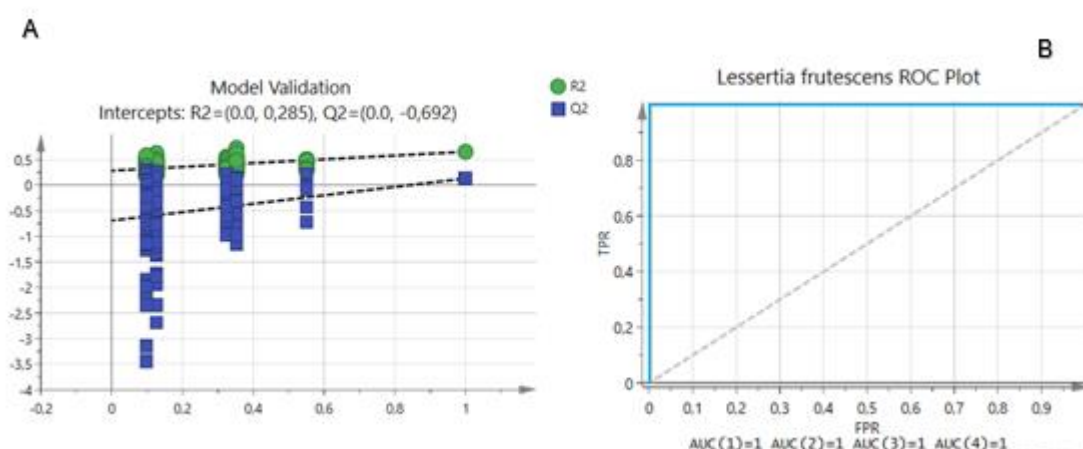


Figure 10: Statistical validation of the OPLS-DA model by permutations (A) and diagnostic performance through ROC (AUC=0.999) (B) analysis. Q² intercept of -0.692 is less than 0.05 indicating a valid model.

6.4. DISCUSSION

6.4.1. Plant growth promoting genes of *Bacillus paralicheniformis* and *Bacillus velezensis* endophytes

Comparing endophyte genetic characteristics with their various functional activities is crucial. (Kaul et al., 2016). It also aids in understanding the various metabolite gene clusters that regulate the production of metabolites and other essential bioactive substances by endophytes (Kaul et al., 2016). Genomic methods, such as whole genome sequencing, can aid in the identification of possible genes, networks, and pathways involved in the manufacture of endophytes' bioactive substances, as well as in experimental validation. For example, when plant growth-promoting qualities are examined in an in vitro experiment, genomic approaches can be used to validate them by identifying functional genes. Ammonium is used by plants as a reduced form of nitrogen and different genes are involved in biological conversion of nitrogen by microbes, including endophytes (Ali, 2020). For instance, in the current study, the *NtrC*, *NarT*, *NasA*, and *NifU* genes were among the genes from *Bacillus paralicheniformis* and *Bacillus velezensis* that are involved in the biological conversion of

nitrogen from one form to another (Table 2). *Nor* genes are among the functional markers used to evaluate the processes of nitrification and denitrification (Sun et al., 2021). Nitrate, which was also present in the current study, can be used by microorganisms as an electron acceptor in anaerobic environments or as a substrate for N assimilation (Alvarez et al., 2019). Nitrate assimilation is one of the most important processes for optimal plant growth and development, and is regulated by nitrate reductase (NR) and nitrite reductase (NiR) enzymes (Ali, 2020). NifS is a cysteine desulfurase that produces sulfur from L-cysteine, while *nifU* is an acceptor of iron and sulfur to construct Fe-S gene clusters, which are extensively distributed in natural environments (Kumeta *et al.*, 2007). The synthesis of active nitrogenase depends on several Nif proteins that are encoded by molecular scaffold protein genes and *nif* genes. It has been proposed that *nif* genes can partially replace their genomic function, and it has been shown that *nif* genes or functions vary among microbes based on the host's physiology and environmental niche (Li & Chen, 2020). For instance, the *nifU* is a gene responsible for the production of Mo-Fe nitrogenase, which is important in electron transport and redox supply (Zhang et al., 2023). Moreover, the *nifU* gene is also crucial for the FeS cluster assembly (Li & Chen, 2020)). Thus, there are wide variations in the organization and complexity of *nif* genes in microorganisms (Shamseldin, 2013).

Endophytes also play a role in nutrient acquisition and the exchange of essential nutrients such as phosphorus by releasing immobile, insoluble phosphate for plant usage (Hassan, 2017). They assist phosphorus mobilization through numerous processes such as acidifying, chelation, and other enzymatic activities (Jayakumar et al., 2020). Among the genes involved in phosphate solubilisation that *Bacillus paralicheniformis* and *Bacillus velezensis* carried was the *phoR* gene (Table 3). In inorganic phosphorus soils, phosphate solubilisation occurs by enzymatic degradation, in which *pho* genes promote the production of related enzymes to hydrolyse organic P, releasing inorganic P for plant use (Pan & Cai, 2023). Upregulating the genes, involved in the phosphorus starvation response (*phoR*) under P-deficient environments, may increase the production of phosphatase (Dai et al., 2020). Additionally, endophytes such as *B. velezensis* establish stable relationships with plants, promoting plant growth and preventing soil-borne plant diseases. It achieves this by forming biofilms and producing quorum-quenching enzymes, like phytase, that aid in the solubilisation of phosphorus (Ghosh et al., 2021; Rabbee et al., 2019). Chemical fertilisers are known to induce microbial shifts in the soil, therefore using endophytes for crop production has an

excellent chance of guaranteeing a high ecological balance of natural microflora (Chouhan et al., 2022).

Endophytes can also stimulate plant growth by producing siderophores. Siderophores are crucial for crop productivity and agricultural sustainability (Dhuldhaj & Pandya, 2021). They promote plant growth by acquiring insoluble iron from iron rich environments and convert it to soluble form which is usable by plants (Roskova et al., 2022). The two primary pathways for siderophores synthesis are dependent NRPS (non-ribosomal peptide synthetases) and independent NRPS (non-ribosomal peptide synthetases), both of which are controlled by multiple genes and enzymes involved in iron acquisition and regulation (Ghosh et al., 2020). For example, the *fhu* genes play a role in bacterial cell iron (III) hydroxamate uptake (Mademidis & Köster, 1998). Several genes (*Fur*, *Feu A*, *FeuB*, *FeuC*, *FeoA*, *FeoB*, and *FhuA*, *FhuB*, *FhuC*, *FhuG*) involved in the manufacture and transportation of siderophores were found in *Bacillus paralicheniformis* and *Bacillus velezensis* in this study (Table 5). The *fhuA* gene encodes ferrichrome's outer membrane receptor. By contrast, *fhuB*, *fhuC*, and *fhuD* play a significant role in the translocation of ferrichrome and other ferric hydroxamate molecules into the inner membrane (Mademidis & Köster, 1998). Besides their significance for plant growth, siderophore-producing endophytes present a significant possibility in the production of effective medications and disease management in the medicinal sector. For example, cancer cells are thought to acquire more iron than normal cells to survive and thrive and iron chelation with siderophores can aid in slowing their growth, multiplication, and development (Khan et al., 2018). Desferrioxamine, an inhibitor of iron-dependent activities, for example, has been discovered to drastically diminish a variety of cancers (Petrik et al., 2021). It is, possible, therefore, to use any siderophore that outcompetes cancer cells for iron to stop the growth of cancer cells (Swayambhu et al., 2021).

6.4.2. Gene functions related to metabolites production by endophytes

Biosynthetic gene clusters have been found to encode a wide range of metabolites found in microorganisms including endophytes (Dinesh et al., 2017). This was demonstrated in the current study since *B. paralicheniformis* and *B. velezensis* both possessed gene functions responsible for metabolite biosynthesis, transportation, regulation, and use, as indicated in Tables and 5 and 6. *Bacillus paralicheniformis* and *B. velezensis* had several genes involved in arginine production, transportation, translocation, and use, including *TatAy* and *TatCy*. *TatAd*, *TatCD*, *ArtP*, *ArtQ*, *RocB*, and *RocR*. *Bacillus paralicheniformis* is a facultative

anaerobic bacterium and, under anaerobic circumstances, derives energy from nitrate respiration, glucose fermentation, or L-arginine degradation (Maghnouj et al., 1998). Thus, it has been noted that the arginine deiminase pathway is one of the key components that allow *B. paralicheniformis* species to thrive under anaerobic environments (Maghnouj et al., 1998). In the presence of arginine, *B. paralicheniformis* *ArgR* was found to behave as a repressor of the anabolic ornithine carbamoyltransferase and an activator of the arginine deiminase pathway (Maghnouj et al., 1998). Bacteria's arginine and ornithine intracellular levels are modulated by arginase in response to the demands and growth conditions of the bacteria (Hernández et al., 2021). A wide range of bacteria use arginine and ornithine as nitrogen and carbon sources (Hernández et al., 2021). Furthermore, the breakdown of arginine produces $\text{NADH} + \text{H}^+$, which is required for ATP synthesis. *B. paralicheniformis* also included trehalose-6-phosphate hydrolase, an enzyme involved in the trehalose production process as shown in Table 6. Trehalose 6-phosphate is an intermediate for trehalose biosynthesis (Lunn et al., 2014). Trehalose protects microorganisms and plants from osmotic, thermal, and cold stress (Avonce et al., 2006). It has also been shown to be important in the regulation of carbon metabolism and photosynthesis (Iturriaga et al., 2009). Besides, it has a function in transporting sugar compounds, notably sucrose (Fichtner & Lunn, 2021). Sucrose contributes to the growth of seeds, roots, and flowers in plants (Fichtner & Lunn, 2021).

6.4.3. Endophytes as a source of metabolites

Visual comparison of the ^1H NMR spectra reveals significant similarities between the metabolic patterns of *B. paralicheniformis* (Figure 1A), *B. velezensis* (Figure 1B), and the combination of these two endophytes (Figure 1C). These similarities were validated by the annotated chemicals in Table 8, as there were more similar compounds between the two strains and their combination. The amount (concentration) of metabolites increased when these endophytes were co-cultured. Bacterial endophytes have been shown to contain a wide variety of novel or unidentified therapeutic compounds that are encoded by cryptic biosynthetic gene clusters (BGCs) (Dinesh et al., 2017; Saati-Santamaría et al., 2022). However, the possibilities of rediscovery are higher with little to no chances of new discoveries, as the media can be selective, favouring some expressions over others, due to some of the unexpressed BGCs requiring a distinct set of conditions. Thus, it is challenging to cover all the potential BGCs under a single set of conditions in the laboratory. However, the use of genomic methods like whole genome sequencing can help to provide a greater and deeper understanding of them, increasing the likelihood of their being isolated or extracted

(Atanasov et al., 2021). These BGCs can then be activated, allowing them to be examined for improved chances of new medication discovery (Dror et al., 2020). However, endophytes' silent biosynthetic gene clusters can be activated and examined using genomic-based techniques, resulting in production of compounds that are undetectable in typical laboratory conditions (Li & Lou, 2018). Thus, it has been suggested that with regard to searching for novel compounds for development of drugs from microorganisms, genome-based strategy may be more efficient and profitable compared to screening methods, including high throughput screening (Saati-Santamaría et al., 2022). Genome-based approaches are superior because they allow for a greater in-depth search for uncharacterised BGCs that aid in the finding of encoded metabolic chemicals with fewer chances of rediscovery (Saati-Santamaría et al., 2022). This stands in contrast to high throughput screening, where sample preparation includes culturing microbes in culture media, and BGC expression is more likely to be affected by the components and composition of the culture media used (Atanasov et al., 2021). However, before manipulating a certain pathway, genes involved in that pathway need to be identified, because different genes are responsible for the biosynthesis of different metabolic compounds. Thus, genomic approaches are necessary to reveal endophytes' larger potential for development of new agricultural and pharmaceutical products (Li & Lou, 2018).

6.4.4. Influence of endophytes on *Lessertia frutescens* plant growth

One of the most significant worldwide challenges is increasing crop productivity while safeguarding the environment. The use of high-yielding cultivars and agricultural chemical-based products no longer results in a corresponding rise in crop yield (Pal et al., 2022). Therefore, a sustainable method with high productivity is required. Research has shown that endophytes' interactions with plants has a good impact by enhancing plant health and their individual (Pal et al., 2022) and such was proven in this study through enhancement of medicinal plant growth. Native strains have been shown to be more successful in terms of plant growth promotion (Masenya et al., 2022). In the present study, native strains (*B. paralicheniformis* and *B. velezensis*) significantly promoted *L. frutescens* growth through promotion of height, total wet and dry biomass, wet and dry shoots biomass as well as wet and dry root biomass shown in Figure 2, 3, 4, and 5. The consortium gave the highest agronomic parameters compared to all other treatments, while controlled had the lowest. Similar results were obtained by Masenya et al. (2022), whereby *L. frutescens* native strain enhanced the growth characteristics of *L. frutescens*, including plant height, dry shoot mass, root length, total plant biomass, and several chemical components like nitrogen and protein

content. Additionally, shoots are typically utilised to make traditional medicines and to extract metabolites for the synthesis of drugs. It is noteworthy in this regard that *B. paralicheniformis* and *B. velezensis* were successful in promoting medicinal plant *L. frutescens* plant biomass yield.

6.4.5. Influence of endophytes on *Lessertia frutescens* macronutrients composition

Plant nutrition is one of the key determinants of plant success, and an insufficient supply of critical nutrients can inhibit plant growth and development. Essential nutrients, for example, increase plants' resistance to biotic or abiotic stressors (Hajiboland, 2012). Essential nutrients are those elements without which a plant cannot complete its life cycle, and whose function cannot be replaced by another element. Furthermore, the element should be directly involved in the plants' metabolism (Hajiboland, 2012). Essential plant elements include macronutrients (N, P, K, Ca, Mg, and S) and micronutrients (B, Cl, Cu, Fe, Mn, Mo, Ni, and Zn). It has been reported that macronutrients represent about 0.1-5% of dry plant tissue, whereas micronutrients represent approximately 0.025% (Jones & Jacobsen, 2005; White & Greenwood, 2013). Through enhancing cycling, intake of plant nutrients such as N, P, Fe, and production of auxins, mainly IAA, endophytes regulate and promote plant growth. *Bacillus paralicheniformis* and *Bacillus velezensis* tested positive for N-fixation, P-solubilisation and production of IAA and siderophore, and it is thus not surprising that these endophytes enhanced growth. In the present study, endophytes improved the macronutrient accumulation in *L. frutescens* leaves (Figure 6). Also in the present study, inoculating *L. frutescens* with *B. paralicheniformis* (Treat-CL) enhanced accumulation of Nitrogen in *L. frutescens* leaves while Treat-Control had the least influence on N content. Higher accumulation of N and other elements in the leaves is an indication of high availability of those nutrients in the soil solution for plant use (Nagaveni *et al.*, 2018). One of the well-studied plant-endophyte interactions is a symbiotic relationship between *Rhizobium* bacteria and leguminous plants, in which plants are supplied with biological nitrogen for metabolism (Papik *et al.*, 2020). Plants, in turn, provide endophytes with photosynthate, which serve as an energy and carbon source for endophyte survival (Via *et al.*, 2016). Therefore, it is not unexpected that *B. paralicheniformis* (Treat-CL), which displayed the ability to fix nitrogen in an *in vitro* test, increased the amount of N in *L. frutescens* leaves. The capacity of *B. paralicheniformis* (Treat-CL) to promote nitrogen build-up in plants can help reduce nitrogen emissions into the environment, and aid in climate change mitigation. Nitrogen fixation has been indicated to be directly affected by P availability (Vandamme *et al.*, 2014).

Furthermore, the N fixation process requires a lot of Adenosine Triphosphate (ATP) per reaction for the production of two NH₃ molecules (Schulze et al., 1999; Thuynsma et al., 2014). Production of ATP is a high P-requiring reaction, and hence, lack of phosphorus has a direct impact on N fixation (Thuynsma et al., 2014; Valentine et al., 2011). The ability of *B. paralicheniformis* to solubilise P could be attributed to its ability to enhance P accumulation on *L. frutescens* leaves as seen in Figure 6. Phosphorus is one of the limiting elements for crop growth (Uchida, 2000). A low content of available P in plants could lead to poor root development and growth stunting (Kushwaha et al., 2020). This is because P is one of the elements that play an important part in enzymatic activities responsible for plant physiological functions such as growth (Ahemad, 2015). The ability of *B. paralicheniformis* (Treat-CL) to enhance P availability in *L. frutescens* could help in reducing dependence on the use of P chemical fertiliser. This would be useful because, globally, the efficacy of phosphate fertilisation is between 10-25%, with a major portion being fixed in the soil or washed off (Khan et al., 2009). Fixed phosphate is not available for plant use, but can be made available by phosphate solubilising microorganisms through different mechanisms (Kaur & Reddy, 2014). On the other hand, *B. paralicheniformis* (Treat-CL) and *B. velezensis* (Treat-CR) both increased calcium accumulation in *L. frutescens* leaves (Figure 6). Calcium serves as a messenger in various plant biological processes, particularly under stressful conditions (Ghosh et al., 2022). It functions as a signalling molecule, thereby playing a part in signalling pathways in plants for the production of diverse bioactive compounds (Ghosh et al., 2022). *Bacillus paralicheniformis* (Treat-CL) and *Bacillus velezensis* (Treat-CR) also had a comparable effect on Mg content and the Treat-AB caused elevated Mg build-up in *L. frutescens* leaves (Figure 6). Magnesium is essential for plant enzyme activity and the structural stability of nucleic acids, ensuring optimal functionality (Sreedhara & Cowan, 2002). It also plays a significant role in plant protection against abiotic and biotic conditions and the formation of metabolites, particularly amino acids and sugars (Gerendás & Führs, 2013). Similarly, sulfur ameliorates plant biotic and abiotic stress by interacting with biomolecules such as nitric oxide and polyamine (Narayan et al., 2023). It is a structural component of amino acids and protein disulfide and serves as a cofactor in the production of a range of bioactive compounds. It influences the production of amino acids and metabolites such as glutathione and methionine, as well as vitamins like thiamine and biotin (Q. Li et al., 2020). Its deficiency leads to reduced plant development and loss (Narayan et al., 2023). From the present study, inoculating *L. frutescens* with *B. velezensis* (Treat-CL) and the

combination of *B. paralicheniformis* and *B. velezensis* (Treat-AB) had a positive influence on accumulation of sulfur in *L. frutescens* leaves (Figure 6).

6.4.6. Influence of endophytes on *Lessertia frutescens* micronutrients

Producing siderophores, which are essential for iron chelation, and to increase iron availability for plant usage, is another method by which endophytes promote plant growth (Kaur et al., 2020). A significant portion of the soil's iron is insoluble (Fe^{3+}) and (Fe^{2+}) used by plants (Chowdappa et al., 2020). However, the presence of siderophore-producing microorganisms, such as endophytes, can chelate and make iron available in iron-limited environments. Nitrogen and other nutrients, such as sulfur, can be employed as donor atoms for iron siderophore chelation, even though the affinity of Fe (III) is less, compared to when oxygen atoms are used (Roskova et al., 2022). In the present study, inoculation of *L. frutescens* with *B. paralicheniformis* (Treat-CL) enhanced accumulation of iron in *L. frutescens* leaves (Figure 7). This strain tested positive for siderophore production in an in vitro experiment. This strain's ability to positively impact accumulation of components such as N and S may have contributed to its ability to promote Fe accumulation, as N and S have been noted to play a role in chelation of Fe by siderophores (Bhatia & Singh, 2021; Sun et al., 2022). Siderophore-producing endophytes are thus critical in sustainable agriculture and crop production because of their role in iron acquisition, and providing plants with ecological fitness under abiotic and biotic stress conditions (Cui et al., 2022). On the other hand, *B. velezensis* (Treat-CR) increased the accumulation of zinc in *L. frutescens* leaves, whereas Treat-control had the lowest level of zinc (Figure 7). Zn plays a role in plant activities such as plant metabolic and protein biosynthesis as well as in activation of some plant enzymes (Navarro-León et al., 2016). Zn availability has been indicated to have an influence on IAA production which in turn affect plant growth and development (Navarro-León et al., 2016). IAA is the major auxin, and its key functions include cell elongation and division according to a study conducted by (Navarro-León et al., 2016). Improved nutrient availability can help to improve crop quality. Therefore, using endophytes such as *B. paralicheniformis* could play a significant role in ensuring food security and crop productivity.

6.4.7. Influence of endophytes on *Lessertia frutescens* metabolites production

Over the years, there has been a severe decline in medicinal plant bioactive substances, particularly amino acid production in *L. frutescens* medicinal plants. Some of the reasons for this decrease include environmental changes as a result of climate change. *L. frutescens*

exhibits never-ending drastic metabolomics variations, hence it is highly unlikely that their production of metabolites will follow the pattern (Albrecht et al., 2012). Interestingly, presence of endophytes can induce the production of secondary metabolites, which play crucial roles in plant defence mechanisms and have therapeutic applications in humans (Van Wyk & Albrecht, 2008). Such was proven in this study through enhancement of plant growth and metabolite production by *L. frutescens*. The visual comparison of the spectra reveals significant similarities between the metabolic profiles of different treatments with inoculation (Figure 8 B, C and D). The PCA (Figure 9A), which revealed no clustering of samples from these treatments, confirmed the possibility of similarities in metabolic profiles between the treatments. Nevertheless, (OPLS-DA) (Figure 9B) displayed clustering, with samples grouped according to their treatments, suggesting potential metabolic differences between the treatments. In a similar experiment, propagation of *L. frutescens* in an in vitro experiment also produced beneficial results, as it promoted the accumulation of metabolites (Nosov et al., 2023). Chemomx metabolite annotation demonstrated that *B. paralicheniformis* (Treat-CL) had a stronger influence on *L. frutescens*' production of aliphatic chemicals and amino acids. *B. paralicheniformis* (treatment-CL) not only increased the number of metabolite production in *L. frutescens* but also influenced the quality, as the maximum concentration was reported on plants inoculated by this endophyte (Table 9). The influence of *Rhizobium* (*Bradyrhizobium* and *Rhizobium*) inoculation on *L. frutescens*'s phytochemical profiles was investigated by (Makgato et al., 2020) and the results showed significant enhancement on plants' total phenolic and flavonoids compounds as well as its antioxidative activity. There were variations in the distribution of metabolites among the treatments, as influence by different endophytes having more metabolite production influenced by *B. paralicheniformis* compared to *B. velezensis* (Figure 8 and Table 9). Metabolite distribution is usually uneven and patchy in plants (Wink, 2013). Patchy distribution can be explained by either plant-convergent evolution, or the kind of endophytes that they interact with, which is a driving factor in the early acquisition of critical genes required for metabolite synthesis (Wink, 2013). Endophytes are well-known biological agents that help plants stay healthy by sustaining their metabolisms even in harsh environments. They do this by developing plant tolerance under such conditions through the accumulation of metabolites (Ogbe et al., 2020). It is well known that the relationship between plants and endophytes is complex and extends well beyond the defence-to-virulence balance; hence, simple processes are insufficient to adequately describe it (Ogbe et al., 2020). Endophytes' different mode of actions to influence metabolite production in plants and their influence starts at colonisation. Plant colonisation

by endophytes is largely dependent on interactive colonisation processes, which are controlled by communication between plants and endophytes (Chaturvedi et al., 2016). In the course of colonisation, plants identify the molecular patterns of endophytes, including peptidoglycan, porins, flagellin, and lipoteichoic acids (Bodenhausen et al., 2014). The release of endophytic molecules, such as organic molecules and signalling hormones, affects the expression of plant genes that regulate biosynthesis and the build-up of metabolites in plants (Wang et al., 2023). The process, initiated by the introduced strain, occurs only once the elicitor has been identified. For some plants, it takes longer than others, depending on the defence systems available and the strain's ability to adapt to a new environment (Shasmita et al., 2018). This can lead to variations on how each endophyte influences metabolites in plants. In addition, cellular activity in plants and microbes determines their responsiveness, survivability, and adaptability to environmental changes (F. Chen et al., 2016). Following signal transmission through cell membranes, changes in ionic fluxes through changed influxes of ions such as calcium (Ca^{2+}) and potassium (K^{+}) across the plasma membrane result in biochemical and physical changes in plants (Zhao et al., 2005). Alternatively, endophytes function as inducers to modify plant metabolite biosynthesis pathways (Wang et al., 2023). In addition, endophytes can either modify their host's metabolic reaction to promote the synthesis of specific metabolites or collaborate with their host to complete the same signal pathway which usually yields essential enzymes responsible for metabolites production. This can result in the production of similar metabolites by plants and endophytes (Khattab & Farag, 2022). Ideally, combining two or more endophytes should lead to the enhanced production of metabolites, but this was not the case in the present study as Treat-AB had less influence on *L. frutescens* metabolite production compared to Treat-CL and Treat-CR. It has been noted that combining two or more strains could lead to competition for resources by those strains, and when strains compete, the plant into which they are inoculated may pick the best competitors (Hlongwane et al., 2023). This was observed by (Gerding et al., 2014) when *Mesorhizobium* and *Rhizobium* were used to propagate *L. frutescens*.

6.5. CONCLUSION

Bacillus paralicheniformis and *Bacillus velezensis* promoted *L. frutescens* plant growth in a pot trial experiment as evidenced by increased plant height, total wet and dry biomass, shoot wet and dry biomass, as well as root wet and dry biomass. Inoculating *L. frutescens* with *B. paralicheniformis* and *B. velezensis* resulted in variation in metabolites production by *L.*

frutescens. In addition to influencing the accumulation of metabolites, *B. paralicheniformis* increased their quantity; the highest concentration of most metabolites was from *L. frutescens* inoculated with *B. paralicheniformis* (treatment-CL). Lastly, compared to *L. frutescens*, endophytes produce a wider variety of metabolites, and the quality (concentration) of those produced metabolites is improved when a consortium of endophytes is used, as compared to a single isolate. Due to their high turnover rate for the synthesis of the metabolites of choice, endophytes appear to be a better choice to produce metabolites.

7. CHAPTER SEVEN: GENOMIC AND METABOLOMIC ANALYSES OF ENDOPHYTIC *BACILLUS* SPECIES REVEALS ENHANCED GROWTH AND METABOLITE PRODUCTION IN *ARTEMISIA AFRA*

7.1. ABSTRACT

The notable role of endophytes in promoting host plant growth stems from their ability to enhance nutrient acquisition, induce stress tolerance, and stimulate the biosynthesis of bioactive compounds, such as alkaloids and flavonoids. Research shows that these compounds not only serve as natural defenses against herbivores and pathogens but also possess various therapeutic properties, including antioxidants and antimicrobial activities. Beyond this, some endophytes produce metabolites that are chemically similar to those synthesized by their host plants, revealing a complex interplay between these organisms. This study aimed to evaluate the influence of two endophytes previously obtained from *Artemisia afra*: *Bacillus paralicheniformis* KSW1 and *Bacillus subtilis* KSW7, on the growth and metabolite production of *A. afra* in the pot trial experiment. In addition, the study investigated the quality and quantity of metabolites produced by the two *Bacillus* species. The pot trial experiment was conducted for a duration of 3 months and consisted of four treatments with six replicates in a completely randomised design. The treatments include inoculation with *B. paralicheniformis* KSW1 only; *B. subtilis* KSW6 only; combination of *B. paralicheniformis* KSW1 and *B. subtilis* KSW6, and control with no inoculum. Metabolites from *A. afra* leaves and pure bacterial endophytes were analysed using NMR. The entire genome of both isolates was assessed for genes related to their metabolites production and plant-growth promotion. *Bacillus paralicheniformis* KSW1, *Bacillus subtilis* KSW6 and their combination significantly increased *A. afra* plant growth as evidenced by increased plant height, wet and dry total biomass, wet and dry shoot and root biomass. The endophytes also had a positive effect on macro- and micronutrients in *A. afra* leaves. *Bacillus paralicheniformis* (Treat-AR) had the greatest impact on *Artemisia afra*'s nutrient composition because it promoted the accumulation of micronutrients (Fe, Cu, Zn, and Mn) and macronutrients (N, P, Mg, Ca, and S). In comparison to the control (without inoculum), *A. afra* produced more metabolites when inoculated with *B. paralicheniformis* and *B. subtilis*.

Inoculated *A. afra* and pure bacterial endophytes produced comparable metabolites. Whole genome sequencing revealed that *B. paralicheniformis* and *B. subtilis* contain genes that promote plant growth, such as those involved in the biological conversion of nitrogen from one form to another (*NtrC*, *NarT*, *NorD* and *NifU*), also phosphorus solubilisation, uptake, and transportation (*PhoH*, *PhoP*, *PhoR*, *pstA*, *pstB* and *pstC*), as well as genes (*FhuC*, *FhuC*, *FhuG*, *Feu A*, *Feu B*, and *FeuC*) involved in siderophore production. Endophyte inoculation presents a promising avenue for enhancing the productivity and health of medicinal plants while contributing to sustainable agricultural practices. Ongoing research and innovation in this field are essential for unlocking the full potential of endophytes in both agriculture and medicine, offering a pathway toward more resilient and effective cultivation methods.

7.2. INTRODUCTION

It is undeniable that both rapid and gradual environmental changes pose a great threat to agricultural productivity and sustainability, worsening food insecurity issues. Even though plants develop coping mechanisms, for example, the production of metabolites for such changes, the effect of these changes is still massive. Plant conservation is critical because it seeks to preserve plant species, in order to ensure a steady supply of essential products while also improving plant biodiversity (Das et al., 2016; Llauroadó Maury et al., 2020). Even for medicinal plants that are not endangered, such as *Artemisia afra*, extraction of good quality and quantity of metabolites is not assured, as most medicinal plants, if not all, contain complex mixtures (Street & Prinsloo, 2013). Understanding the biological controls of metabolism in relation to changing conditions is essential (Akula & Ravishankar, 2011). Additionally, the depletion of medicinal plants is increasing globally due to large-scale, unsustainable harvesting from the wild, and this may affect their potential economic contribution (Golinska et al., 2015; Palanichamy et al., 2018). Therefore, it is critically important to conserve medicinal plants in view of their usefulness in different industries, notably pharmaceuticals. To solve the problems with low quality and quantity production of metabolites by medicinal plants, endophytes as biological enhancers can expand their therapeutic range. The introduction of intermediates, such as beneficial endophytes, has been proven to enable plants to produce higher quality and quantity metabolites (Julsing et al., 2006). To date, different strategies ranging from elicitation, metabolic engineering, hairy root cultures, plant cell immobilisation and biotransformation have been investigated for their

efficacy in improving quality and quantity of metabolites produced by medicinal plants. However, microbial elicitation has been proven to be a more effective solution to such issues (Shasmita et al., 2018). The use of plant growth- promoting microorganisms is perhaps one of the most common and easiest strategies used to promote plant growth. Microorganisms, such as endophytes, regulate plant growth through cell growth and elongation, help with cell regeneration and differentiation, and encourage metabolites production (Ali et al., 2017). The implications of this interaction are profound, as they suggest potential strategies for improving agricultural productivity through natural means while reducing reliance on chemical fertilizers and pesticides (Z. Li et al., 2022). Endophytes' shared metabolic pathways with their host plants indicate a coevolutionary relationship that allows them to thrive in a symbiotic capacity. This mutualism not only benefits the plants by enhancing their defenses and stress tolerance but also presents opportunities for bioprospecting novel compounds that could be harnessed for pharmaceutical applications (Chowdhury et al., 2015). Therefore, the ability of certain endophytes to produce metabolites that are chemically similar to those synthesized by their host plants underscores the ecological and biochemical relationships that exist in plant microbiomes and has significant implications for agriculture and medicine, particularly in the realms of sustainable practices and drug discovery (Aswani, Jishma, et al., 2020; Singh & Jain, 2022). Use of endophytes as biofertilizers has a potential of ensuring high quality, safe, and pollution free herbal drugs produced from medicinal plants (Rajasekharan & Wani, 2020; Shahrajabian et al., 2019). Economically, propagation of medicinal plants could lead to greater business opportunities, high market rates, social stability, optimum quality, and economic returns. (Shahrajabian et al., 2019). The ultimately purpose is employing endophytes to stimulate the production of high quality medicinal plants with sustained levels of bioactive compounds (Julsing et al., 2006). Additionally, endophyte-produced metabolites are useful sources of compounds in the pharmaceutical and agricultural industries due to their various biological activities (Eshboev et al., 2024). Certain gene clusters in the endophytic bacteria genome control the synthesis of these compounds (Kliebenstein & Osbourn, 2012). The gene clusters coordinate the expression of genes involved in the generation of these metabolites and encode the enzymes involved in their biosynthesis (Eshboev et al., 2024). Discovering the genes responsible for biologically active endophytic metabolites, and linking them to their functions through genomic research, are therefore essential (Eshboev et al., 2024). Thus, there are two aims of this study. The first was to evaluate the effect of *Bacillus paralicheniformis* and *Bacillus subtilis* endophytes on *Artemisia afra* growth and metabolites production. Secondly, the study

also investigated the quality and quantity of metabolite production by both *B. paralicheniformis* and *B. subtilis*.

7.3. METHODS

7.3.1. Isolation and assessment of bacterial endophytes

Plant samples of *Artemisia afra* were collected from the Agricultural Research Council-Roodeplaat Vegetable, Industrial, and Medicinal Plants (ARC-VIMP) in Pretoria, South Africa, and Eden Nursery in Klerksdorp, South Africa. The plants were surface-sterilised with 95% ethanol, followed by a 2.5% sodium hypochlorite solution, and then rinsed in distilled water (Palanichamy et al., 2018). The effectiveness of surface sterilisation was tested by plating a small portion of each sample on nutrient agar and incubating at 37 °C for 48 hours. The sterile plant samples (root, stem, and leaf) were crushed in liquid nitrogen, serially diluted in distilled water, plated on nutrient agar, and incubated at 37 °C for 24 hours to isolate bacterial endophytes. Each pure bacterial isolate was evaluated for plant growth-promotion through in vitro assessment of its biological nitrogen-fixing capability, using nitrogen free Burke's medium (Hamza et al., 2017), phosphate solubilisation on Pikovskaya agar enriched with 0.5% insoluble tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) (Pikovskaya, 1948), indole acetic acid production using Salkowski's reagent (Putrie et al., 2017), and siderophore production on chrome azural s (CAS) agar (Mukherjee & Dutta, 2019).

7.3.2. Pot trial experiment for plant growth promotion

Based on the results of the *in-vitro* plant growth-promoting assessment, two highly effective endophytic strains were selected for the pot trial experiment. The experiment was conducted with ten-week-old *Artemisia afra* seedlings sourced from Die Blomwinkeltjie nursery in Potchefstroom. The seedlings were transplanted into pots filled with sterile coconut peat and inoculated with a 24-hour-old standardised bacterial inoculum one week later. Four treatments, each replicated six times in a completely randomised design, were applied. The treatments were labelled as Treat-AR, Treat-CR, Treat-AB, and Treat-Control, representing *B. paralicheniformis* KSW1, *Bacillus subtilis* KSW6, a combination of both *B. paralicheniformis* KSW1 and *B. subtilis* KSW6, and the control, respectively. All treatments

were supplemented with 100ml of Hoagland solution weekly and irrigated every other day. After one month, the irrigation frequency increased to a daily basis. The plant growth facility was maintained at 30 °C during the day and 18 °C at night, with a consistent humidity of 70%. Three months post-inoculation, the plants were harvested, and several parameters were measured: plant height, total wet and dry biomass, wet and dry biomass of the root, and those of the shoot. The dry biomass was determined after freeze-drying the plants with liquid nitrogen. Additionally, leaf samples were analysed for nutritional content and metabolites.

7.3.3. Analyses of the nutritional contents

Leaf samples from similar replicates were combined to create a single composite sample, and the analysis was conducted for each treatment. The nitrogen (N) and phosphorus (P) content of the samples were determined using the micro-Kjeldahl acid digestion method, which involved the use of sulfuric acid and selenium dioxide. A segmented flow analyser (SEAL AA3) was then employed for the analysis (Trikilidou et al., 2022). To determine the nitrogen content, the samples were reacted with salicylate and sodium hypochlorite solution, forming a blue compound that was measured at a wavelength of 660 nm, with nitroprusside serving as a catalyst. Phosphorus content was determined via a colorimetric method, where phosphate interacts with molybdovanadate in an acidic solution, producing a yellow colour. The phospho-molybdovanadate complex was then measured at 420 nm.

The nitric-perchloric acid wet digestion method was used to measure sulfur content in the plant samples, and the resulting digests were analysed using a Novaspec Spectrophotometer. The instrument was calibrated at 420nm with calibration standards in the range of 0–50mg/l to quantify the sulfur content. For the analysis of calcium (Ca), magnesium (Mg), and micronutrients (Cu, Fe, Zn, Mn, and B), dry ashing method was employed by heating the samples in a muffle furnace at 550 °C. The digested samples were then treated with Aqua Regia on a steam bath (Paul et al., 2017). Atomic Absorption Spectrophotometer (AAS) was used to measure the concentrations of Fe, Cu, Zn, and Mn at respective wavelength. Iron (Fe) was measured at 248.3 nm, using calibration standards of 3, 6, and 9 mg/l Fe. Copper (Cu) at 324.8nm, with calibration standards of 0.5, 1.0, and 1.5mg/l Cu. Zinc (Zn) at 213.9nm, using standards containing 1, 2, and 3mg/l Zn, while manganese (Mn) was analysed at 279.5 nm, with standards of 1, 2, and 4mg/l Mn. Boron (B) was determined using a colorimetric method with a segmented flow auto-analyser, calibrated with standards containing 0.5, 1.0, 1.5, 2.0, and 2.5mg/l B, measured at 420nm.

7.3.4. Extraction of metabolites from bacterial endophytes

Extraction of metabolites from the bacterial endophytes was performed as described by (Shanthakumar et al., 2015; Sulistyani & Narwanti, 2015) with slight modifications. Pure culture of bacterial endophytes was inoculated into 50 ml LB and incubated at 37 °C for 48 h. Then equal volumes (25 ml) of ethyl-acetate and chloroform were added to the culture and ultrasonicated for 30 minutes to disrupt the bacterial cells and release the intracellular metabolites into the solvent. The mixture was allowed to settle before the organic portion was centrifuged at 10 000 rpm for 20 minutes. The clean supernatant was stored at -80 °C overnight before being freeze-dried. The powdered extract was used to determine the metabolite production.

7.3.5. Analysis of metabolites from bacterial endophytes and *Artemisia afra* plant leaves

Following the technique of Kim et al. (2010) with some adjustments, approximately 100mg of *Artemisia afra* leaf samples were suspended in 1 mL D₂O (99% deuterated, Aldrich Kempton Park, South Africa) and vortexed until all material was wetted. The samples were then sonicated for 30 min at ambient temperature. The liquid of each sample was aspirated and transferred into a clean tube and thereafter centrifuged for 15 min at 10 000 rpm. The supernatant was transferred to standard 5mm NMR tubes for analysis. A 600 MHz Bruker Avance NEO spectrometer (Bruker BioSpin, Ettlingen, Germany) was employed for NMR analysis. A direct observation two-channel broadband iProbe (Bruker) was fitted to the equipment. ¹H spectra were recorded at 600 MHz at a set temperature of 30 °C. Water signal suppression was performed utilising selective, multiple pre-saturation during the relaxation delay and mixing times. About 512 scans were recorded to produce data for processing. All NMR operations and data processing were performed with TopSpin 4.4.0 (Bruker). The same method was used for bacterial endophytes metabolite analysis.

7.3.6. Multivariate data analysis

Data analysis and processing were performed using MestReNova software (10.0.1, Mestrelab Research, Spain), and the correction of phasing and baseline, normalisation and peak alignment were done manually on the ¹H NMR spectrum. All processed data were divided into 0.04 ppm bins, representing 0 – 10 ppm and converted to Excel comma-separated values (CSV) file format for pattern recognition multivariate data analysis. Transformed data were

statistically analysed, and all the imported data were Pareto scaled in the soft independent modelling of class analogy (SIMCA) software (SIMCA, Version 14.0, Umetrics, Umeå, Sweden). Similar and non-similar samples were statistically discriminated using the Principal Component Analysis (PCA) and orthogonal projections to latent structure discriminant analysis (OPLS-DA). Annotations of compounds were performed using the Chenomx software (NMR suite, version 10.1) and published NMR data.

7.3.7. Whole genome assessment of the two isolates

Bacterial genomic DNA was extracted from the two endophytic bacteria using Fungal/Bacterial DNA miniPrepTM kit (Zymo Research Corporation, USA) following manufacturer's instructions. The libraries were prepared using Illumina Nextera DNA flex library prep kit and sequenced on an Illumina NovaSeq platform. The total sequenced reads of 9,340,034 and 10,740,324 were generated for *B. paralicheniformis* KSW1 and *B. subtilis* KSW6 respectively with 300-bp paired-end read length.

The reads quality was assessed with fastQC v0.12.1 and the low-quality reads and adapter sequences were filtered using Trimmomatic v0.36 (Bolger et al., 2014) with a minimum quality score of 15 and a minimum sequence length of 70. The adapter regions were clipped using a mismatch value of 2, a palindrome clip threshold of 30, and a simple clip threshold of 10. The assembly was made with the quality reads using SPAdes v3.15.3 (Bankevich et al., 2012; Prjibelski et al., 2020). Completeness of the assembled genome and presence of contaminants were assessed with CheckM v1.0.18. The genome assembly was annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v6.7 (Li et al., 2021) and Rapid Annotations, using Subsystems Technology (RAST) with SEED viewer v. 2.0 (Overbeek et al., 2014).

The taxonomy of the genome assembly was determined for both genomes using combination of the genome taxonomy database GTDB-Tk v2.3.2 (Rodriguez-R et al., 2018) and the genome blast distance phylogeny (GBDP) applying the distance formula d5 using the TYGS database (Meier-Kolthoff & Goker, 2019). Circular mapping of the entire genome was visualised using Proksee (Grant et al., 2023), while the CRISPR array and Cas clusters were determined by CRISPR/Cas finder, and CARD resistance genes Identifier was used to identify antimicrobial resistance genes within the genome.

7.3.8. Statistical analysis

The effect of *Bacillus paralicheniformis* and *Bacillus subtilis* on *Artemisia afra* growth parameters in pot trial experiment was determined statistically. The obtained data were subjected to one-way analysis of variance (ANOVA) to evaluate differences among different treatment groups. The Shapiro-Wilk Test ($\alpha=0.05$) was used to validate the normality assumption. The tests were set at a 5%-significance level ($P < 0.05$). Pair wise comparisons were made using post hoc tests, Fisher's least significant difference (LSD), and Tukey's Honest Significance Difference (HSD).

7.4. RESULTS

Basic statistical features of the genomes are stated in Table 1.

Table 1: Statistical features of the genome assemblies for both strains

Organisms	Genome size	Contigs	N50	L50	GC	CDS	Completeness
<i>Bacillus subtilis</i> KSW6	4,163,027	13	2,136,222	1	43.62	4419	99.59%
<i>B. paralicheniformis</i> KSW1	4,269,291	18	1,062,212	2	45.88	4496	99.59%

7.4.1. Putative gene functions associated with *Bacillus paralicheniformis* and *Bacillus subtilis* plant growth promotion capacity

Table 2 shows the putative gene functions associated with *B. paralicheniformis* and *B. subtilis* nitrogen regulation and availability. Nitrate reductase, nitric oxide reductase activation protein (NorD), nitrogen regulation protein (*NtrC*), nitrate/nitrite transporter (*NarT*), and *NifU* nitrogen cluster protein are among the enzymes, proteins, and genes involved in various stages of the nitrogen cycle recovered from *B. paralicheniformis*. Nitrate reductase, nitrite reductase [NAD(P)H], ammonium transporter, and *NifU* nitrogen cluster protein are among the enzymes, proteins, and genes involved in various stages of the nitrogen cycle recovered from *B. subtilis*. Table 3 shows the putative gene functions associated with *B. paralicheniformis* and *B. subtilis* phosphate solubilisation. *B. paralicheniformis* contained genes such *PhoP*, *PhoR*, *Pst ABCS*, and *YcsE* that are involved in phosphate solubilisation, control, and transportation. *PhoP*, *PhoR*, *PhoH*, *YcsE*, and *PstA*, *PstB*, and *PstC* were among the genes that *B. subtilis* carried in relation to phosphate solubilisation, phosphate regulation, and phosphate transportation. Table 4 shows the putative gene functions associated with *B. paralicheniformis* and *B. subtilis* indole acetic and tryptophan production. For *Bacillus*

paralicheniformis, the only enzymes involved in the synthesis of indole were alpha and beta tryptophan synthase. The only enzymatic functions involved in indole synthesis for *B. subtilis* were tryptophanyl-tRNA synthetase, indole-3-glycerol phosphate synthase, and alpha and beta tryptophan synthase. Lastly, Table 5 shows the putative gene functions associated with *B. paralicheniformis* and *Bacillus subtilis* siderophore production and iron regulation. Several genes (*FhuB*, *FhuC*, *FhuG*, *Feu A*, *Feu B*, and *FeuC*), involved in siderophore production and transport, were retrieved in relation to the ability of *B. paralicheniformis* to promote plant development. *Bacillus subtilis* also contained a variety of genes involved in siderophore synthesis and iron transfer. *Feu A*, *FeuB*, *FeuC*, and *FhuB*, *FhuC*, *FhuG* were among the genes present.

Table 2: Putative gene functions associated with *Bacillus paralichenformis* and *Bacillus subtilis* nitrogen regulation and availability

Gene functions	<i>Bacillus paralichenformis</i>			<i>Bacillus subtilis</i>		
	Fragment position	Size (pb)	DNA strand	Fragment position	Size (pb)	DNA strand
Nitrogen regulatory protein P-II	-	-	-	485793 – 485443	350	Reverse
Ammonium transporter	-	-	-	487019 – 485805	1 214	Reverse
NtrC family Transcriptional regulator, ATPase domain	390915 – 393701	2786	Forward	449355 – 452171	2 816	Forward
Nitrogen regulation protein NR(I), GlnG (=NtrC)	930551 – 929550	1001	Reverse	-	-	-
Nitric oxide reductase activation protein <i>NorD</i>	974254 - 976167	1915	Forward	-	-	-
Nitrate/nitrite transporter <i>NarT</i>	1224975 - 1226159	1184	Forward	-	-	-
<i>NifU</i> -like domain protein	133621 - 133860	239	Forward	961066 – 960830	236	Reverse
Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4)	-	-	-	264039 – 266456	2 417	Forward
Nitrite reductase [NAD(P)H] small subunit (EC 1.7.1.4)	-	-	-	266488 – 266808	320	Forward

Table 3: Putative gene functions associated with *Bacillus paralichenformis* and *Bacillus subtilis* phosphate solubilization

Gene functions	<i>Bacillus paralichenformis</i>			<i>Bacillus subtilis</i>		
	Fragment position	Size (pb)	DNA strand	Fragment position	Size (pb)	DNA strand
Alkaline phosphatase synthesis transcriptional regulatory protein <i>PhoP</i>	169891 - 170613	722	Forward	195296 – 196018	722	Forward
Phosphodiesterase/alkaline phosphatase D	-	-	-	338261 – 336510	1 751	Reverse
Phosphate regulon sensor protein <i>PhoR</i> (SphS) (EC 2.7.13.3)	170606 - 172351	1745	Forward	196011 – 197750	1 739	Forward
Phosphate ABC transporter, permease protein <i>PstC</i> (TC 3.A.1.7.1)	557697 - 558626	929	Forward	619117 – 620046	929	Forward
Phosphate ABC transporter, permease protein <i>PstA</i> (TC 3.A.1.7.1)	558626 - 559513	887	Forward	620046 – 620930	884	Forward
Phosphate ABC transporter, ATP-binding protein <i>PstB</i> (TC 3.A.1.7.1)	559533 - 560360	827	Forward	620951 – 621760	809	Forward
Phosphatase <i>YcsE</i>	104887 - 105642	755	Forward	166153 – 165404	749	Reverse
Phosphate starvation-inducible protein <i>PhoH</i> , predicted ATPase	-	-	-	585338 – 586297	959	Forward

Table 4: Putative gene functions associated with *Bacillus paralichenformis* and *Bacillus subtilis* indole acetic and tryptophan production

Gene functions	<i>Bacillus paralichenformis</i>			<i>Bacillus subtilis</i>		
	Fragment position	Size (pb)	DNA strand	Fragment position	Size (pb)	DNA strand
Tryptophan synthase beta chain (EC 4.2.1.20)	852901 - 854127	1226	Forward	-	-	-
Tryptophan synthase alpha chain (EC 4.2.1.20)	854096 - 854899	803	Forward	-	-	-
Tryptophanyl-tRNA synthetase (EC 6.1.1.2)	-	-	-	1868164 – 1869156	992	Forward
Tryptophan synthase beta chain (EC 4.2.1.20)	-	-	-	826460 – 827662	1 202	Forward
Tryptophan synthase alpha chain (EC 4.2.1.20)	-	-	-	827655 – 828458	803	Forward
Indole-3-glycerol phosphate synthase (EC 4.1.1.48)	-	-	-	825075 – 825827	752	Forward

Table 5: Putative gene functions associated with *Bacillus paralichenformis* and *Bacillus subtilis* siderophore production and iron regulation

Gene functions	<i>Bacillus paralichenformis</i>			<i>Bacillus subtilis</i>		
	Fragment position	Size (pb)	DNA strand	Fragment position	Size (pb)	DNA strand
Ferrichrome transport ATP-binding protein <i>FhuC</i>	241054 - 240242	812	Reverse	855148 – 855957	809	Forward
Ferrichrome transport system permease protein <i>FhuG</i>	242086 - 241073	1013	Reverse	854123 – 855133	1 010	Forward
Ferrichrome transport system permease protein <i>FhuB</i>	243150 - 242086	1064	Reverse	852924 – 854123	1 199	Forward
Fe-bacillibactin uptake system <i>FeuC</i> @ ABC-type Fe ³⁺ -siderophore transport system, permease component	589018 - 587999	1019	Reverse	437201 – 438211	1 010	Forward
Fe-bacillibactin uptake system <i>FeuB</i> @ ABC-type Fe ³⁺ -siderophore transport system, permease 2 component	590015 - 589011	1004	Reverse	436204 – 437208	1 004	Forward
Fe-bacillibactin uptake system <i>FeuA</i>	590989 - 590033	956	Reverse	435232 – 436185	953	Forward

7.4.2. Putative gene functions associated with *B. paralicheniformis* and *B. subtilis* metabolites production

Table 6 illustrates the putative gene functions associated with *B. paralicheniformis* metabolites production. The most notable ones were for the synthesis, transportation, and utilisation of metabolites like arginine. *Bacillus paralicheniformis* possessed several genes involved in arginine biosynthesis, transportation, translocation, and utilisation, including *RocB* for arginine utilisation, Arginine pathway regulatory protein (*ArgR*), *TatAy*, *TatCy*, *TatAd*, and *TatCd* for twin-arginine translocation protein, and *ArtQ*, and *RocE* for other arginine processes. Moreover, *B. paralicheniformis* had gene activities for the sugar compounds, xylose, fructose, sucrose, and glucose. There were also several sugar transport genes, including *MsmGE*, *MsmGF*, and *MsmG*, as well as sugar-transporting systems, including fructose specific IIA, IIB, IIC, IID, and the PTS system. On the other hand, ribose transporters were encoded *RbsA*, *RbsB* and *RbsC* genes. Also restored was the function of cysteine desulfurase, which is thought to have a stress reduction effect.

Table 6: Putative gene functions associated with *Bacillus paralicheniformis* metabolites production

Gene functions	Fragment position	Size (bp)	DNA strand
Twin-arginine translocation protein <i>TatAy</i>	7402 - 7575	173	Forward
Twin-arginine translocation protein <i>TatCy</i>	7582 - 8358	776	Forward
Arginine pathway regulatory protein <i>ArgR</i> , repressor of arg regulon	625287 - 625736	449	Forward
Arginine transport system permease protein <i>ArtQ</i>	646919 - 647581	662	Forward
Arginine transport ATP-binding protein <i>ArtM</i>	647574 - 648296	722	Forward
Arginine utilization protein <i>RocB</i>	672436 - 674040	1604	Forward
Twin-arginine translocation protein <i>TatAd</i>	98104 - 98316	212	Forward
Twin-arginine translocation protein <i>TatCd</i>	98392 - 99126	734	Forward
Arginine utilization regulatory protein <i>RocR</i>	20155 - 18668	1487	
Arginine permease <i>RocE</i>	21697 - 23142	1445	Forward
Cysteine desulfurase (EC 2.8.1.7)	129636 - 130781	1145	Forward
L-cystine ABC transporter, ATP-binding protein <i>TcyC</i>	17039 - 16296	743	Reverse
L-cystine ABC transporter, permease protein <i>TcyB</i>	17759 - 17055	704	Reverse
L-cystine ABC transporter, substrate-binding protein <i>TcyA</i>	18552 - 17746	806	Reverse
PTS system, fructose-specific IIA component (EC 2.7.1.202)	393902 - 394339	437	Forward
PTS system, fructose-specific IIB component (EC 2.7.1.202)	394339 - 394830	491	Forward
PTS system, fructose-specific IIC component	394849 - 395652	803	Forward
PTS system, fructose-specific IID component	395674 - 396501	827	Forward

Fructose-1,6-bisphosphatase, GlpX type (EC 3.1.3.11)	639156 - 638191	965	Reverse
Sucrose operon repressor <i>ScrR</i> , <i>LacI</i> family	850761 - 851735	974	Forward
Ribose ABC transporter, ATP-binding protein <i>RbsA</i> (TC 3.A.1.2.1)	519867 - 521348	1481	Forward
Ribose ABC transporter, permease protein <i>RbsC</i> (TC 3.A.1.2.1)	521350 - 522312	962	Forward
Ribose ABC transporter, substrate-binding protein <i>RbsB</i> (TC 3.A.1.2.1)	522327 - 523241	914	Forward
Multiple sugar ABC transporter, permease protein <i>MsmG</i>	848282 - 847434	848	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmF</i>	849217 - 848279	938	Reverse
Multiple sugar ABC transporter, substrate-binding protein <i>MsmE</i>	850527 - 849229	1298	Reverse
Trehalose-6-phosphate hydrolase (EC 3.2.1.93)	22376 - 24061	1 685	Forward
Trehalose operon transcriptional repressor	24089 - 24799	710	Forward

Table 7 illustrates putative gene functions associated with *B. subtilis* metabolites production. Some of the arginine genes and pathways include *TatCy* and *TatAy* for twin-arginine translocation protein, *ArgR* for arginine pathway regulatory repression, *ArtQ* and *ArtM* genes, as well as *RocB* for arginine utilisation. *B. subtilis* also contained *SufS* for cysteine desulfurase production. *TcyA*, *TcyB*, *Tcy*, *TcyJ*, *TcyK*, *TcyL*, *TcyM*, and *TcyN* genes involved in L-cystine transport-related mechanisms. *Bacillus subtilis* also contained multiple sugar transporters, *MsmGE*, *MsmF* and *MsmG*.

Table 7: Putative gene functions associated with *Bacillus subtilis* metabolites production

Gene Functions	Fragment position	Size (bp)	DNA strand
Alanine--anticapsin ligase (EC 6.3.2.49) => <i>BacD</i>	369818 - 371236	1 418	Forward
D-alanine--poly(phosphoribitol) ligase ACP subunit (EC 6.1.1.13)	282570 - 282334	236	Reverse
Arginine permease <i>RocE</i>	108472 - 109875	1 403	Forward
Arginine utilization protein <i>RocB</i>	363012 - 364712	1 700	Forward
Twin-arginine translocation protein <i>TatCy</i>	338163 - 337408	755	Reverse
Twin-arginine translocation protein <i>TatAy</i>	338352 - 338179	173	Reverse
Arginine pathway regulatory protein <i>ArgR</i> , repressor of arg regulon	676988 - 677437	449	Forward
Arginine transport system permease protein <i>ArtQ</i>	707811 - 708470	659	Forward
Arginine transport ATP-binding protein <i>ArtM</i>	708463 - 709185	722	Forward
Arginine utilization protein <i>RocB</i>	724083 - 725726	1 643	Forward
Cysteine desulfurase (EC 2.8.1.7) => <i>SufS</i>	912692 - 913912	1 220	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyA</i>	210764 - 211570	806	Forward

L-cystine ABC transporter, permease protein <i>TcyB</i>	211557 – 212261	704	Forward
L-cystine ABC transporter, ATP-binding protein <i>TcyC</i>	212275 – 213018	743	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyJ</i>	165581 – 166390	809	Forward
L-cystine ABC transporter, substrate-binding protein <i>TcyK</i>	166425 – 167216	791	Forward
L-cystine ABC transporter, permease protein <i>TcyL</i>	167238 – 167957	719	Forward
L-cystine ABC transporter, permease protein <i>TcyM</i>	167987 – 168694	707	Forward
L-cystine ABC transporter, ATP-binding protein <i>TcyN</i>	168691 – 169470	779	Forward
myo-inositol operon protein <i>IolS</i>	162802 – 161870	932	Reverse
Proline/sodium symporter <i>PutP</i> (TC 2.A.21.2.1) @ Propionate/sodium symporter	274998 – 273649	1 349	Reverse
Sucrose operon repressor <i>ScrR</i> , <i>LacI</i> family	490495 – 491469	974	Forward
Multiple sugar ABC transporter, permease protein <i>MsmG</i>	73777 – 72947	830	Reverse
Multiple sugar ABC transporter, permease protein <i>MsmF</i>	74685 – 73774	911	Reverse
Multiple sugar ABC transporter, substrate-binding protein <i>MsmE</i>	75958 – 74678	1 280	Reverse

7.4.3. *Bacillus paralicheniformis* and *Bacillus subtilis* metabolites production

Figure 1 shows the ¹H NMR spectra of several metabolites recovered from *B. paralicheniformis* KSW1, *B. subtilis* KSW6 and their combination. *Bacillus paralicheniformis* KSW1 produced more sugars and aliphatic compounds than aromatic compounds. The *Bacillus paralicheniformis* ¹H NMR spectra (δ 0.5–8.5 ppm) revealed three distinct spectra regions: the sugar region of δ 3.0–5 ppm, with high signal overlapping and the region from δ 0-3 ppm with high signal overlapping, also displaying the resonance signals-associated aliphatic region, and lastly, the δ 6-8.5 ppm displaying the resonance signals-associated aromatic region (Figure 1A). *Bacillus subtilis* produced more sugars and aliphatic compounds than aromatic compounds. The *Bacillus subtilis* ¹H NMR spectra (δ 0.5–8.5 ppm) revealed three distinct spectra regions: the sugar region of δ 3.0–5 ppm, with high signal overlapping and the region from δ 0-3 ppm with high signal overlapping, also displaying the resonance signals-associated aliphatic region, and lastly, the δ 6-8.5 ppm displaying the resonance signals-associated aromatic region (Figure 1B). Combining these endophytes encouraged the production of sugar and aliphatic compounds more than

aromatic compounds. The combination of these strains' ^1H NMR spectra (δ 0.5–8.5 ppm) revealed three distinct spectra regions: the sugar region of δ 3.0–5 ppm, with high signal overlapping and the region from δ 0–3 ppm, which displays resonance signals-associated aliphatic region, as well as resonance signals associated with aromatic region (δ 6–8.5 ppm) (Figure 1C).

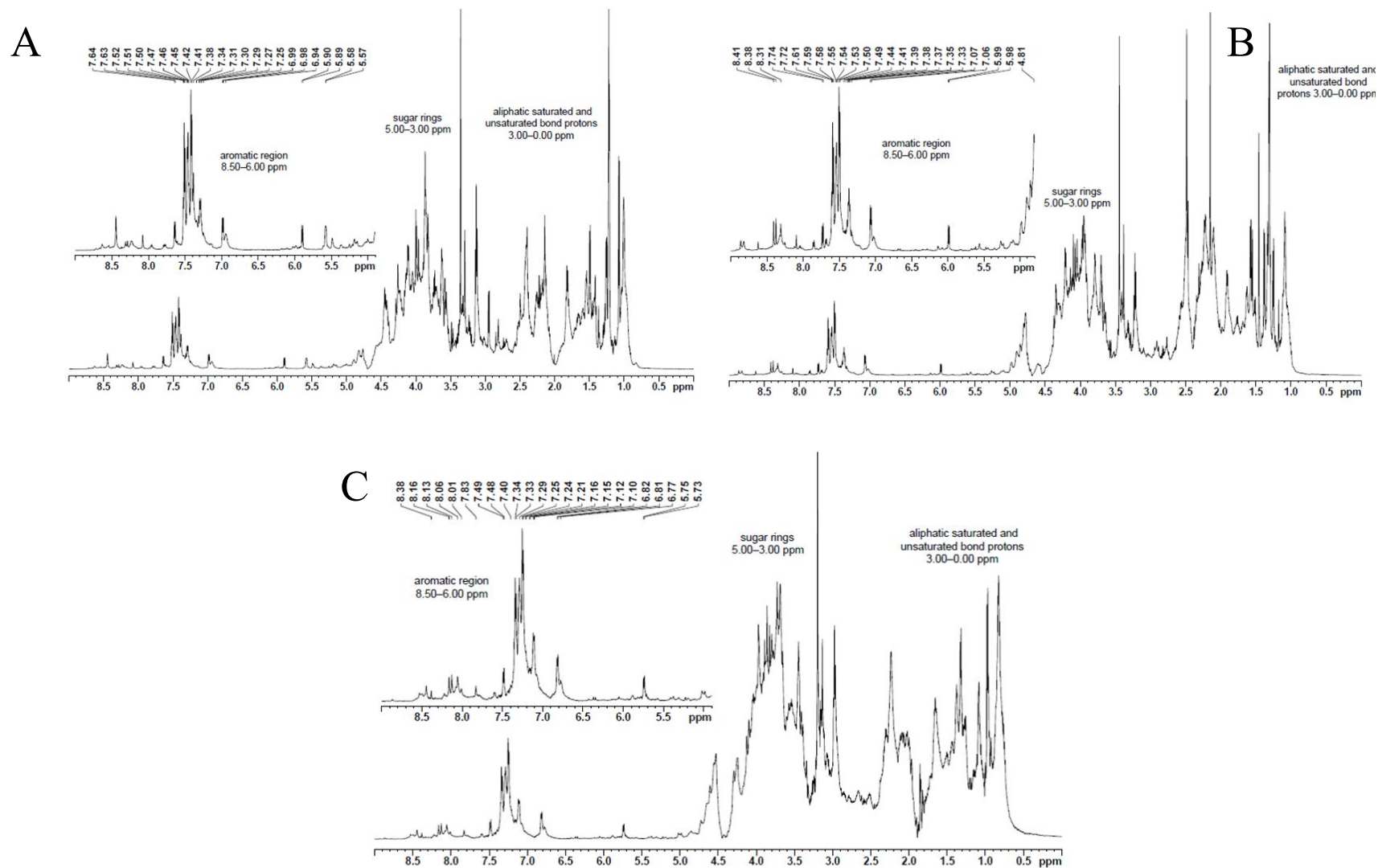


Figure 1: The ^1H NMR spectra of several metabolites found in *B. paralichenformis* KSW1 (A), *B. subtilis* KSW6 (B) and their combination (C).

Table 8 shows Chenomx-annotated metabolites from *B. paralicheniformis* KSW1 and *B. subtilis* KSW6 endophytes and their co-culture. *Bacillus paralicheniformis* and *Bacillus subtilis* endophytes and their co-culture produced comparable metabolites. *Bacillus subtilis* produced more metabolites compared to *Bacillus paralicheniformis* and their co-culture.

Table 8: Chenomx annotated metabolites from *Bacillus paralichenformis* KSW1 and *Bacillus subtilis* KSW6 endophytes and their co-culture

Metabolites	<i>B. paralichenformis</i>			Co-culture			<i>B. subtilis</i>	
	Conc (mM)	Chemical (ppm)	shift	Conc (mM)	Chemical shift (ppm)		Conc (mM)	Chemical shift (ppm)
Alanine	4.1176	1.47 (d)		5.1843	3.77 (q), 1.47 (d)		11.2322	3.77 (q), 1.47 (d)
Tryptophan	-	-		0.4718	7.72 (s), 7.53 (d), 7.31 (s)		0.1294	4.05 (q), 3.47 (dd)
Indole-3-acetate	1.0842	7.23 (s)		0.6851	7.61 (d), 7.50 (d), 7.15 (t)		0.1896	7.61 (d), 7.50 (d),
β-Alanine	5.9291	3.17 (t)		3.5841	3.17 (t), 2.54 (t)		5.3425	3.17 (t), 2.54 (t)
Arabinitol	7.8147	3.68 (m), 3.57 (dd)		8.0627	3.92 (m), 3.83 (dd), 3.68 (m)		8.6107	3.92 (m), 3.83 (dd), 3.68 (m)
Arginine	5.2375	1.88 (m)		5.6101	3.76 (t), 3.23 (t), 1.88 (m), 1.71 (m)		5.8987	3.23 (t), 1.91 (m), 1.71 (m)
Ornithine	-	-		4.7279	3.37 (t), 3.05 (t), 1.93 (m)		10.0909	3.77 (t), 3.05 (t), 1.93 (m), 1.82(m)
Trehalose	0.4591	3.64 (q)		1.1107	5.18 (d), 3.85 (m), 3.64 (q)		5.4933	5.18 (d), 3.85 (m), 3.64 (dd), 3.44 (t)
S-Sulfocysteine	-	-		9.1119	4.16 (q), 3.67 (dd), 3.49 (q)		11.7830	4.16 (q), 3.67 (t), 3.49 (q)
Proline	0.7257	3.32 (m)		35.5405	4.12 (q), 3.41 (m), 3.04 (m), 2.33 (m), 2.05 (m), 1.97 (m)		37.2278	4.12 (q), 3.41 (m), 3.32 (m), 2.33 (m), 2.05 (m)
Cysteine	13.3534	3.50 (dd)		9.7605	3.97 (t), 3.09 (q), 3.02 (dd)		18.5787	3.97 (t), 3.09 (q), 3.02 (dd)
Cystine	14.5313	3.65 (dd), 3.63 (q)		9.4827	4.10 (q), 3.37 (dd), 3.17 (q)		13.8844	4.10 (q), 3.37 (dd), 3.17

						(q)
myo-Inositol	5.3581	3.66 (t)	6.9753	4.05 (t), 3.61 (t), 3.52 (dd)	9.0232	4.05 (t), 3.61 (t), 3.52 (dd), 3.27 (t)
Fructose	13.8591	3.88 (dd), 3.70 (m), 3.59 (m)	16.7399	4.11 (d), 4.01 (t), 3.88 (q), 3.79 (m), 3.70 (m), 3.55 (m)	12.6660	4.11 (d), 4.01 (t), 3.88 9dd), 3.70 (m), 3.55 (m)
Fucose	2.3848	3.63 (dd), 3.43 (t), 1.23 (m)	8.1784	4.07 (m), 3.96 (t),	6.7707	4.18 (m), 4.00 (m), 3.56 (m)
Glucose	-	-	5.3443	4.63 (d), 3.89 (dd), 3.71 (m), 3.52 (m), 3.40 (m)	23.2872	5.22 (d), 4.63 (d), 3.89 (dd), 3.75 (m), 3.48 (m), 3.39 (m)
Ribose	6.2822	3.91 (m), 3.67 (m), 3.64 (m)	16.9722	5.37 (d), 4.92 (d), 4.09 (s), 3.91(m), 3.67 (m)	32.3400	5.37 (d), 5.24 (s), 4.09 (s), 3.98 (m), 3.67 (m)
Sucrose	-	-	-	-	5.1656	4.20 (d), 4.04 (t), 3.88 (m), 3.68 (s)
Xylose	2.2007	3.91 (q), 3.66 (m), 3.61 (m), 3.31 (t)	6.0596	5.19 (d), 4.57 (d), 3.91 (q), 3.66 (m), 3.51 (q), 3.42 (t), 3.31 (t), 3.21 (t)	27.2441	5.19 (d), 4.57 (d), 3.91 (q), 3.66 (m), 3.60 (m)
Ibuprofen	2.6231	7.25 (d), 7.21 (d)	0.1527	3.60 (d), 1.82 (m)	0.1431	3.60 (q), 2.46 (d)
Cis, cis-Muconate	-	-	0.0925	5.96 (m)	0.0526	5.96 (m)

NB: Peak multiplicity (s=singlet; d= doublet; dd= doublet of doublet; t= triplet; q= quartet; m = multiplet)

7.4.4. The influence of *Bacillus paralicheniformis* and *Bacillus subtilis* endophytes on the growth and nutrient acquisition of *Artemisia afra*

The bacteria endophytes demonstrated significant influence on the growth rate of *Artemisia afra*, considering apparent factors such as plant height, observable leaf scorching, and leaf loss (Figure 2 and 3). As shown in Figure 2, the effect of endophytes on *A. afra* plant height was significant between the treatments ($P = 0.0000000000000000222$ i.e. $P \leq 0.05$).

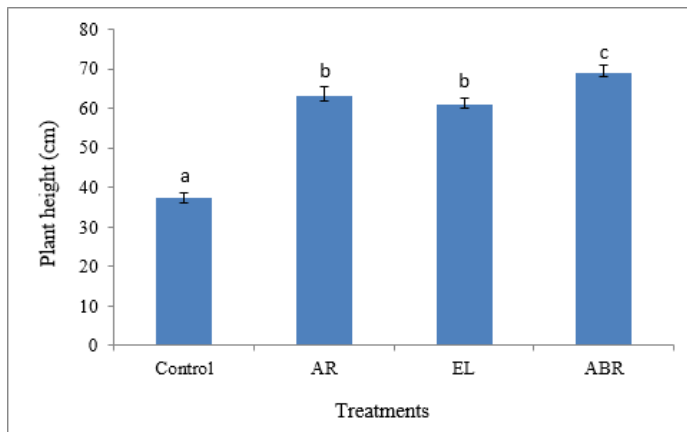


Figure 2: The effect of endophytes on *Artemisia afra* plant height. Error bars represent standard deviation ($n=6$). Different letters on each bar indicates significant differences between treatments. $P = 0.0000000000000000222$ i.e. $P \leq 0.05$. AR= *B. paralicheniformis*, EL= *B. subtilis* and ABR= *B. paralicheniformis* and *B. subtilis*.

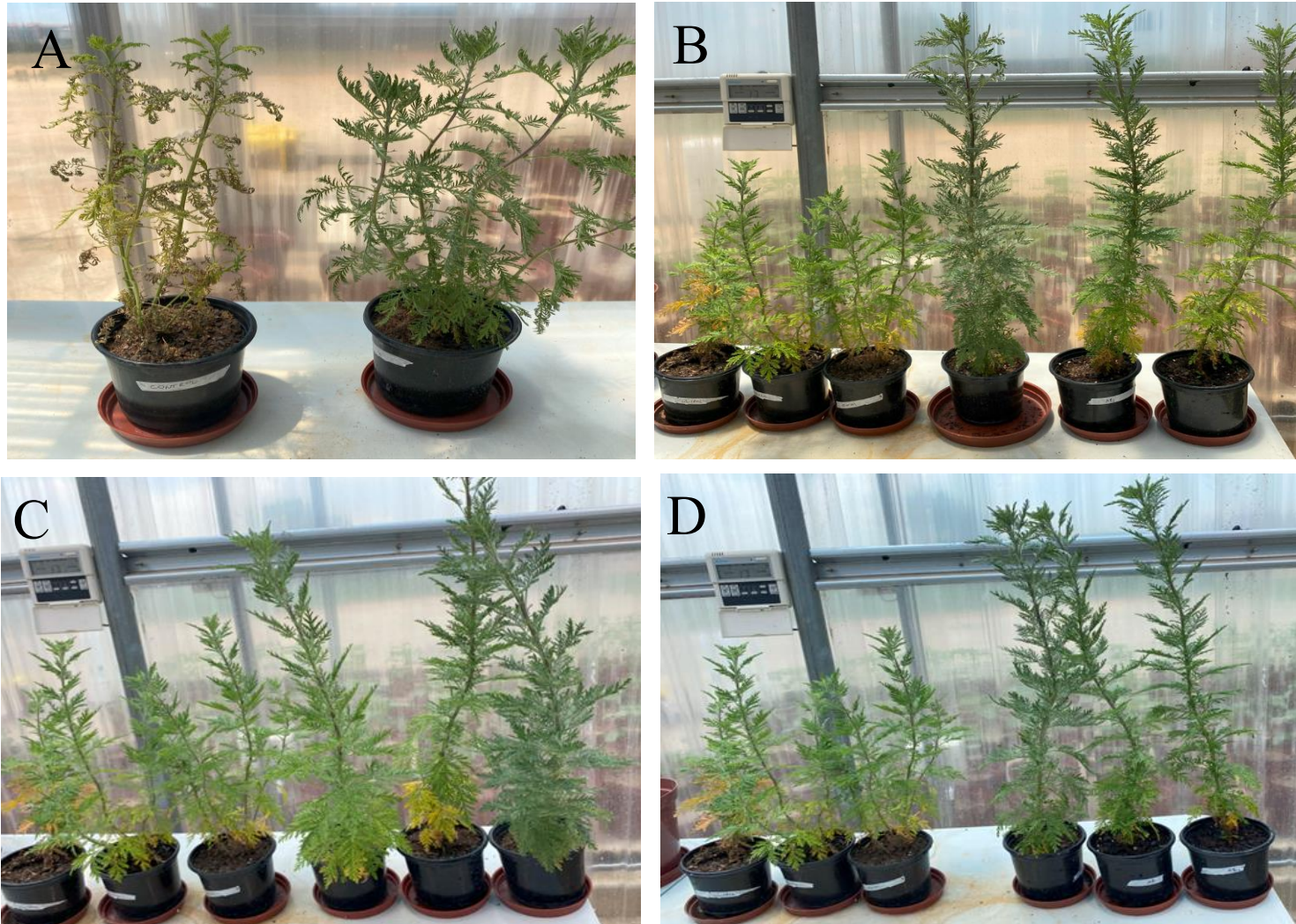


Figure 3: Cultivation of the *Artemisia afra* in a greenhouse under the influence of the endophytes as compared to the control setup. (A) Control (with some plants suffering from possible water stress as seen by loss of leaves), (B) Control and AR (*B. paralichenformis*) (C) Control and EL (*B. subtilis*) and (D) Control and ABR (*B. paralichenformis* and *B. subtilis*).

Other factors, such as total wet and dry biomass (Figure 4), shoot biomass (Figure 5) and root biomass (Figure 6) were also measured. While all three treatments independently aided the plant growth, the combined treatment (ABR) appeared to be the most effective across all the observed parameters. Endophytes significantly influenced *A. afra* plant, total wet and dry biomass, the P-values for wet and dry biomass having been $P = 0$ ($P \leq 0.05$) and $P = 0.00000000000000003331$; ($P \leq 0.05$), respectively (Figure 4). Moreover, endophytes significantly influenced *A. afra* wet and dry shoot biomass and their P-values were $P = 0.0000000000000002109$ and $P = 0.0000000000006377$; ($P \leq 0.05$), respectively (Figure 5). Lastly, endophytes significantly influenced *A. afra* plant root wet and dry biomass, and P-values for wet and dry root biomass were $P = 0.000000000000000111$ and $P = 0.00000000000001478$; ($P \leq 0.05$), respectively (Figure 6).

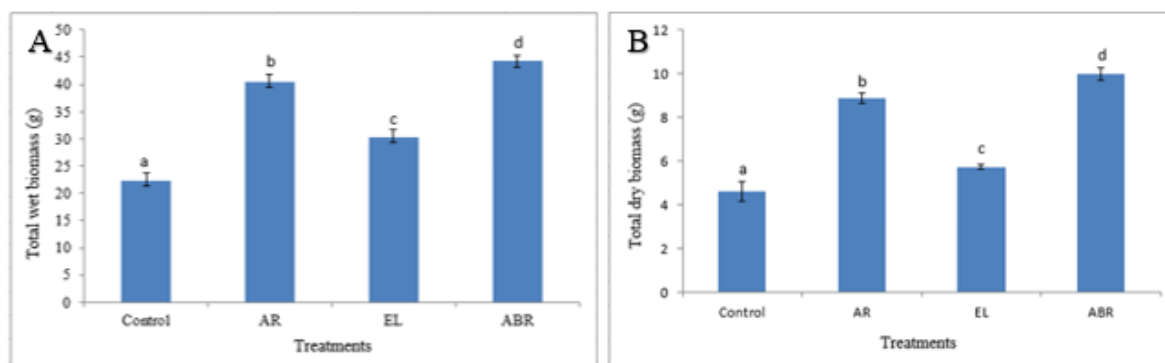


Figure 4: The effect of endophytes on *Artemisia afra* plant total wet (A) and dry (B) biomass. Error bars represent standard deviation (n=6). Different letters on each bar indicate significant differences between treatments. The P-values for wet and dry biomass were $P = 0$ ($P \leq 0.05$) and $P = 0.00000000000000003331$; ($P \leq 0.05$), respectively. AR= *B. paralichenformis*, EL= *B. subtilis* and ABR= *B. paralichenformis* and *B. subtilis*.

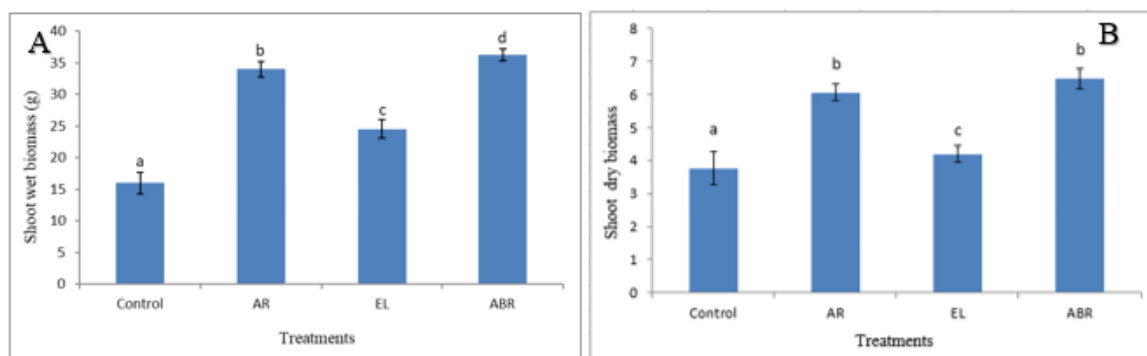


Figure 5: The effect of endophytes on *Artemisia afra* wet (A) and dry (B) shoot biomass. Error bars represent standard deviation (n=6). Different letters on each bar indicate significant differences between treatments. $P = 0.000000000000002109$ and $P = 0.0000000000006377$; ($P \leq 0.05$), respectively. AR= *B. paralichenformis*, EL= *B. subtilis* and ABR= *B. paralichenformis* and *B. subtilis*.

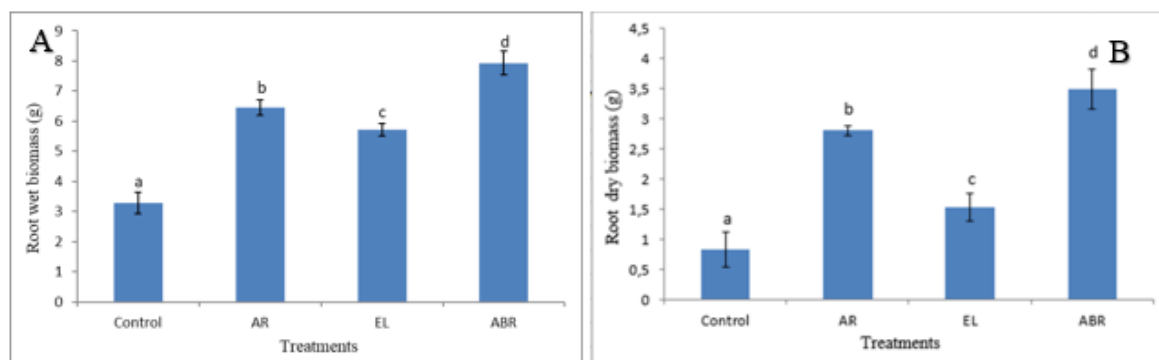


Figure 6: The effect of endophytes on *Artemisia afra* plant root wet (A) and dry (B) biomass. Error bars represent standard deviation (n=6). Different letters on each bar indicate significant differences between treatments. P-values for wet and dry root biomass were $P = 0.000000000000000111$ and $P = 0.00000000000001478$; ($P \leq 0.05$), respectively. AR= *B. paralichenformis*, EL= *B. subtilis* and ABR= *B. paralichenformis* and *B. subtilis*.

On the other hand, accumulation of specific macro- (N, P, Mg, Ca, and S) and micronutrients (Fe, Cu, B, Zn and Mn) for all the treatment, as compared with the control, was also determined (Figure 7 and 8).

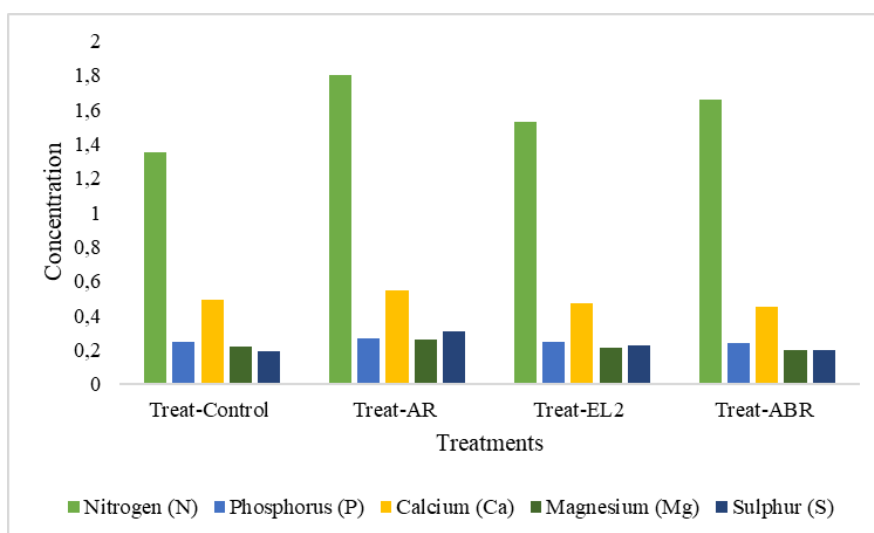


Figure 7: Influence of endophytes on macro-nutrient (N, P, Mg, Ca, and S) content of *Artemisia afra* leaves. Treat-AR = *B. paralichenformis*, Treat-EL2 = *B. subtilis* and Treat-ABR = combination of *B. paralichenformis* and *B. subtilis*.

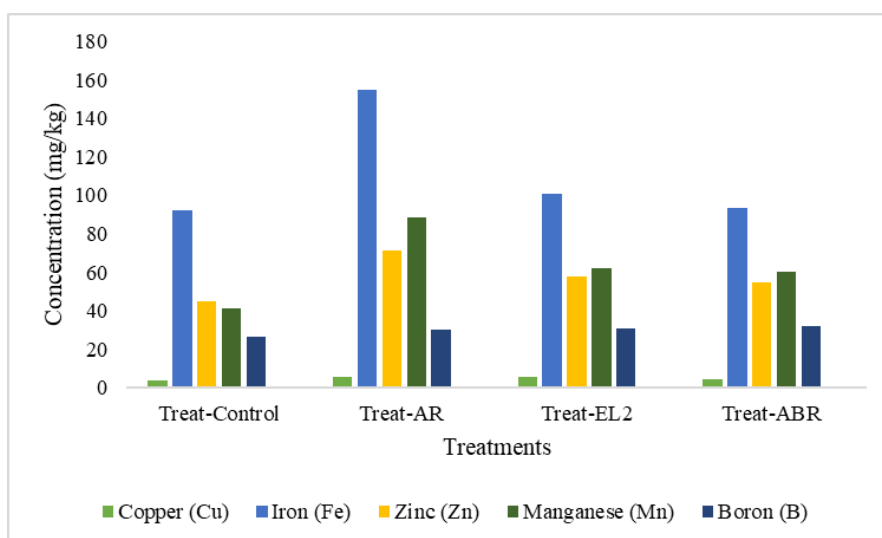


Figure 8: The influence of endophytes on micro-nutrient content of *Artemisia afra* leaves. Treat-AR = *B. paralichenformis*, Treat-EL2 = *B. subtilis* and Treat-ABR = *B. paralichenformis* and *B. subtilis*.

7.4.5. Influence of endophytes on *Artemisia afra* metabolites production

Figure 9 shows the ¹H NMR spectra of several metabolites produced by *Artemisia afra* leaves as influenced by treatments. *Artemisia afra* inoculated with endophytes produced more peaks than the control, showing that they contain more metabolites. *Bacillus paralichenformis* KSW1 (AR) had the greatest impact on metabolites accumulation in *A. afra*. The ¹H NMR spectra (δ 0.5–8.5 ppm) for the *A. afra* inoculated with *B. paralichenformis* KSW1 revealed two distinct spectra

regions: the sugar region of δ 3.0–5 ppm, with high signal overlapping, and the region from δ 0–3 ppm, that displays resonance signals-associated aliphatic region (Figure 9B). *Bacillus subtilis* KSW6 increased sugar accumulation in *A. afra* and had minimal effect on aliphatic compounds. The *Artemisia. afra* ^1H NMR spectra (δ 0.5–8.5 ppm) revealed two distinct spectra regions: the sugar region of δ 3.0–5 ppm, which proved quite complex due to high signal overlapping, and the region from δ 0–3 ppm displaying resonance signals-associated aliphatic region (Figure 9C). On the other hand, the combination strains influence accumulation of sugars more than aliphatic metabolites (Figure 9D). Amongst other treatments, combined treatment (ABR) has a promising effect on aromatic compounds, even though the influence was minute.

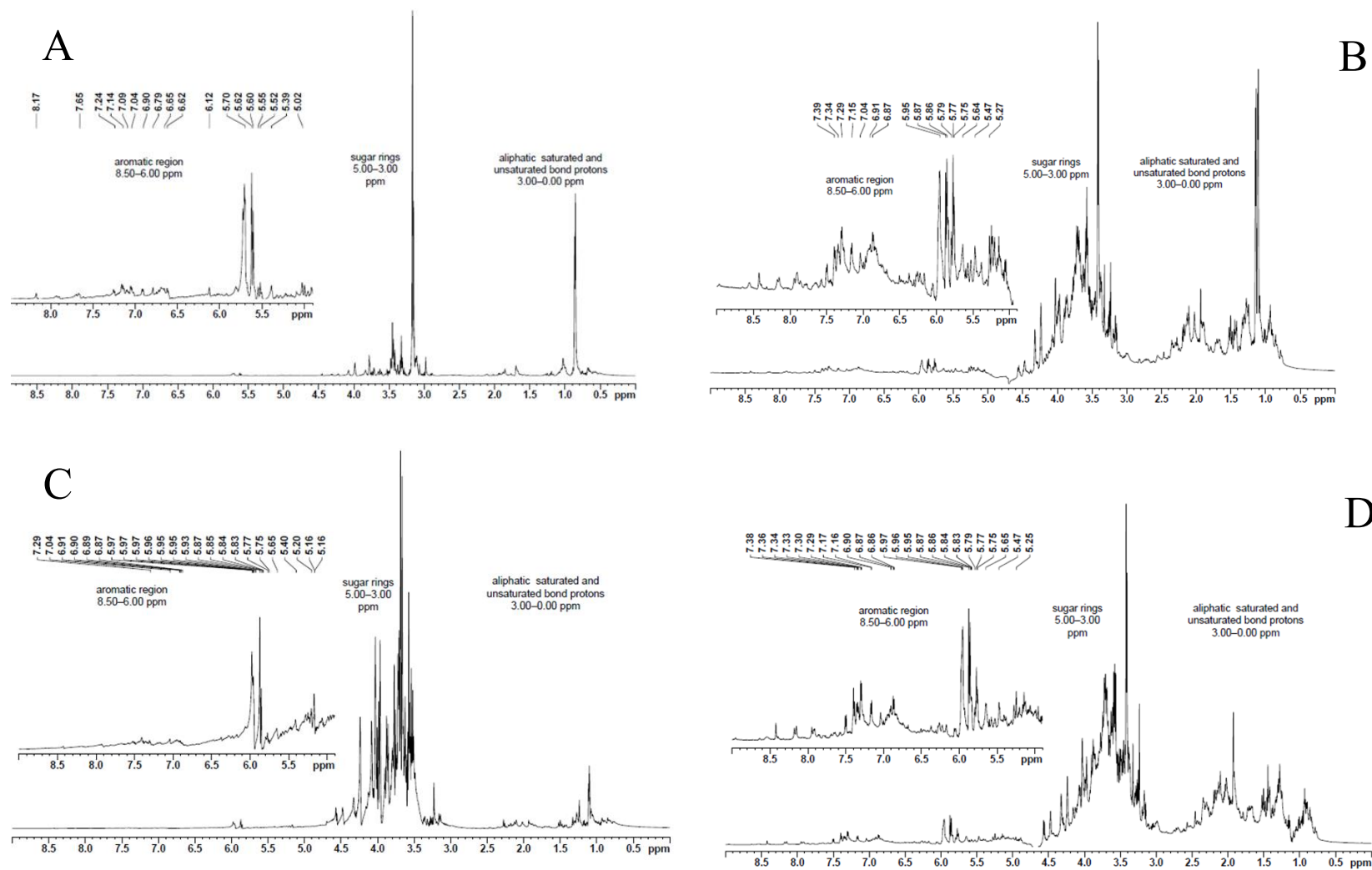


Figure 9: The ^1H NMR representative spectra showing the effect of *B. paralichenformis* and *B. subtilis* endophytes on *Artemisia afra* metabolites production. A = Control B = *B. paralichenformis* C = *B. subtilis* and D = *B. paralichenformis* and *B. subtilis*.

Table 9 shows Chenomx-assisted annotations of differentially produced metabolites from *A. afra* leaves under different treatments. Combined treatment (treatment-ABR) influenced the production of amino acids and sugar compounds. *Bacillus paralicheniformis* KWS1 (Treat-AR) had a positive influence on accumulation of metabolites in *A. afra* leaves, especially of aliphatic and sugar compounds. Lastly, *Bacillus subtilis* KSW6 inoculation (Treat-EL) influenced the accumulation of aliphatic compounds and sugar compounds in *A. afra*.

Table 9: Chenomx-assisted annotations of differentially produced metabolites from *Artemisia afra* leaves under different treatments

Metabolites	Treat-Control			Treat-ABR			Treat-AR1			Treat-EL2		
	Conc. (mM)	Chemical (ppm)	shift	Conc.(m M)	Chemical (ppm)	shift	Conc. (mM)	Chemical (ppm)	shift	Conc. (mM)	Chemical (ppm)	shift
Tryptophan	3.4140	7.72 d, 7.53 d, 7.31 s	-	-	-	-	0.4291	4.05 m, 3.48 dd	-	2.1924	3.74 t, 3.59 t	-
Indole-3-lactate	-	-	-	-	-	-	7.2860	3.97 m, 3.21 t	-	0.3077	4.34 q, 3.25 dd,	-
Alanine	3.7079	1.47 d	-	-	-	-	-	-	-	3.0918	1.47 d	-
β-Alanine	2.1046	3.17 t, 2.54 t	-	-	-	-	-	-	-	1.8044	2.53 q	-
Arginine	-	-	-	1.9414	3.76 t, 3.23 d, 1.92 m, 1.72 m, 1.64 m	-	21.0917	3.76 t, 3.24 t, 1.92 m, 1.72 m	-	6.8078	3.76 t, 3.24 t, 1.89 m, 1.64 d	-
Ornithine	-	-	-	-	-	-	-	-	-	0.8109	1.93m, .82m, 1.74m	-
Trehalose	-	-	-	-	-	-	10.8905	3.77 q, 3.68 m, 3.63 m	-	1.8767	3.85 m, 3.76 q, 3.45 q	-
Cystine	-	-	-	-	-	-	4.0978	4.12 q, 3.38 dd, 3.18 q	-	-	-	-
Cysteine	-	-	-	-	-	-	1.0907	3.99 q, 3.27 dd	-	2.2469	4.97 q, 3.09 q, 3.03	-

									dd
S-Sulfocysteine	-	-	13.7992	4.16 q 3.67 dd, 3.49 q	10.3875	4.17q, 3.67 dd, 3.50 q	-	-	-
Proline	-	-	26.5350	4.12 t, 3.41m,3.32m, 2.34m	1.8409	4.12q, 3.41m, 3.32m,	10.8731	4.12 q, 3.41m	
Xylose	10.4297	5.19 d, 4.57 d, 3.92 q, 3.69 q, 3.62 m, 3.21 t	1.3276	3.92 m, 3.67 m	3.4306	3.91 q, 3.69 dd, 3.61 m	2.4588	5.19 d, 4.57 d, 3.91 q, 3.69 t, 3.62 m, 3.52 m, 3.31 m	
Ribose	92.1220	5.37 d, 4.20 t, 4.09 s, 3.98 m, 3.91 m	-	-	16.9517	5.24 s, 4.20 t, 3.99 m, 3.67 m, 3.51 m	-	-	-
Glucose	11.1405	5.22d, 4.62 d, 3.89 dd,3.75 m, 3.45 m	7.6069	5.22 d, 4.64 d, 3.82 m, 3.48 m	3.3677	3.89 dd, 3.82 m, 3.71 m, 3.53 dd	-	-	-
Fucose	-	-	-	-	-	-	6.0274	5.27 d, 4.18 q, 3.85 dd, 3.79 m, 3.63 dd,	
Fructose	34.5399	4.09 d, 4.01 t, 3.88 dd, 3.55 m	46.6062	4.11 d,4.0 dd 3.79 s,3.71 m, 3.55 m	1.5694	4.11 d	4.4939	4.11 d, 3.79 q, 3.71 q	
Sucrose	11.4648	5.40 d, 4.21 d, 4.04	-	-	-	-	-	-	-

		t,							
Lactose	-	-	7.9087	3.95 m, 3.66 m	-	-	-	-	-
Chlorogenate	-	-	-	-	7.1540	3.88dd, 2.19m,	-	-	-
						2.13 dd, 2.03 t			
Imidazole	-	-	-	-	-	-	0.8780	8.28 s	
Ibuprofen	0.4380	7 d, 7.20 d	-	-	8.2569	3.61 q, 1.83 m,	0.2503	3.60 q	
						1.37 d			

Peak multiplicity (s=singlet; d= doublet; dd= doublet of doublet; t= triplet; q= quartet; m = multiplet)

7.4.6. Multivariate data analysis for *Artemisia afra* leaves from different treatments

Figure 10 illustrates the Principal Component Analysis (PCA) (A) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (B) scores of *A. afra* leaves, showing metabolite relativity of the samples per treatment. An unsupervised Principal Component Analysis (PCA) plot was constructed to determine the intraspecies variation and clustering patterns within the dataset of the *A. afra* samples (Figure 10A). There was no clustering of the samples from different treatments, suggesting similarity on their metabolic profiles. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), which is the supervised recognition analysis that allows the algorithm to expose discrimination between the groups, was applied to the dataset. The samples clustered according to their treatments (Figure 10B).

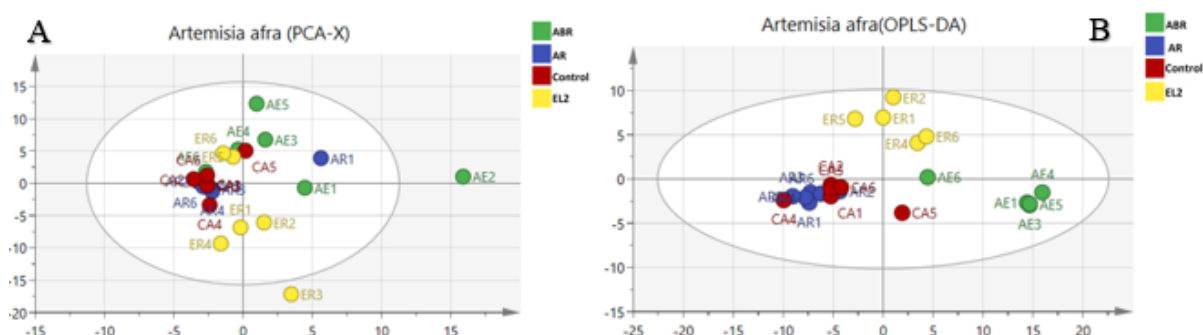


Figure 10: Principal Component Analysis (PCA) (A) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) (B) scores of *Artemisia afra* leaves showing metabolite relativity of the samples per treatment. CA= control, AR= *B. paralichenformis*, EL= *B. subtilis* and ABR= *B. paralichenformis* and *B. subtilis*.

The statistical interference analysis was used to further validate the built OPLS-DA discriminant model (Figure 11). The model's dependability and degree of over-fitting were determined by looking at the intercept of the fitting-line created by the estimated values R2X and Q2 for all samples on the y-coordinate axis. The higher the value of Q2, the more predictive the model is, and the higher the value of R2X, the more robust the explanatory ability. With a Q2 regression line intercept of -0.753 (Figure 11A), the developed OPLS-DA discriminant model was shown to be well predictive and not over-fit. The predictive residual was cross validated (p-value < 0.05) and the receiver operated characteristic (ROC), which computes the area under the curve (AUC), was shown (Figure 11B).

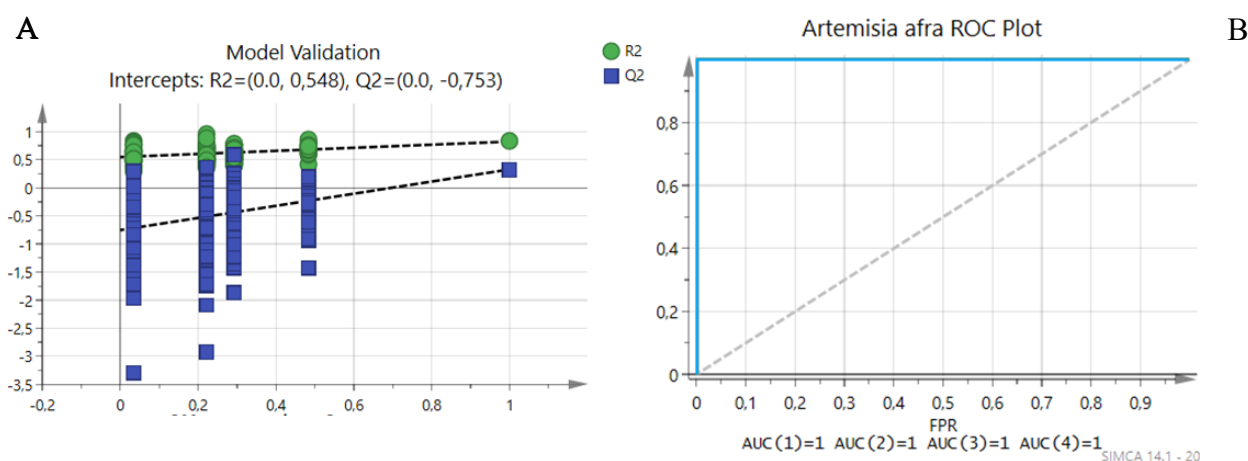


Figure 11: Statistical validation of the OPLS-DA model by permutations (A) and diagnostic performance through ROC (AUC=0.999) (B) analysis. Q2 intercept of -0.753 is less than 0.05, indicating a valid model.

7.5. DISCUSSION

7.5.1. Gene functions associated with plant growth promotion

It is essential to comprehend the various ways endophytes promote plant growth, particularly when choosing the best strains for the manufacture of biofertilizer. Genes involved in a variety of functions, including plant growth stimulation, bioremediation, biotic and abiotic stress tolerance, phytohormone production, and antibacterial potential for both endophytes and plants, including medicinal plants, can be found through plant-endophyte genomic investigations (Berg et al., 2017; Burragoni & Jeon, 2021). Transformation of nitrogen from one form to another is the basis of the biogeochemical nitrogen cycle and various enzymes and genes are involved in N transformation (Simon & Klotz, 2013). Membrane-bound nitrate reductase (*Nar*), periplasmic nitrate reductase (*Nap*), cytochrome nitrite reductase (*NirS*), copper nitrite reductase (*NirK*), nitric oxide reductase (*Nor*), nitrous oxide reductase (*Nos*), and cytochrome c nitrite reductase (*Nrf*) are some of the enzymes and genes involved in the various N-cycle processes (Simon & Klotz, 2013). The maintenance of the N-cycle is greatly sustained by bacteria. The presence of some of these enzymes and genes on the endophytes investigated in the present study signifies their role in the nitrogen cycle. The following genes were identified from this investigation from *B. paralicheniformis* and *B. subtilis* as being involved in the biological conversion of nitrogen from one form to another: *NtrC*, *NarT*, *NorD* and *NifU* (Table 2). Nitrogen fixation is an energy intensive process that requires approximately 16 moles of ATP per mole of fixed nitrogen, and has been seen as a limiting factor in crop productivity (Sun et al., 2021). Nitrogen regulatory protein C (*NtrC*) regulates the transcription of genes necessary for nitrogen metabolism in some microorganisms (Douceff et al., 2005). Nitrogen fixing and metabolic gene, *ntrC* is one of the most prevalent genes found in endophytes

(Mengistu, 2020). On the other hand, microbes fix biological nitrogen using nitrogenase enzymes, and all nitrogen fixers have *nif* genes that encode nitrogenase complexes (Sun et al., 2021). The *nif* gene cluster contains genes responsible for nitrogen fixation. For instance, the *nifU* gene, which was present in our study, encodes proteins involved in the production of iron-sulfur (Fe-S) metalloclusters that play a role in N-fixation (Hwang et al., 1996). On the other hand, *Nrt* is one of the genes encoding nitrate transporters (Ali, 2020). In bacteria, nitrate, as a fixed form of nitrogen, is essential as an electron acceptor and a source of nitrogen (Kraft et al., 2011). Additionally, nitrate is the primary component of nitrogen cycle pathways, and consequently certain microbial processes, such as dissimilatory nitrate reduction to ammonium, compete for it (Kraft et al., 2011). However, the mechanism of nitrate reduction might be respiratory, dissimilatory, or assimilatory (Simon, 2002). Nitrate reduction assimilation contributes to the synthesis of ammonium and is therefore utilised as a source of nitrogen (Simon, 2002). It is catalysed by cytoplasmic nitrite reductase, which uses NAD(P)H as an electron donor, and this can occur in both bacteria and plants (Simon, 2002).

Endophytes also enhance plant growth by solubilising insoluble phosphate and making it accessible to the plant. In the current investigation, phosphorus uptake and transport (*pstA*, *pstB* and *pstC*) genes from *B. paralicheniformis* and *B. subtilis* were recovered, as shown in Tables 3. Alkaline phosphatase, which is in charge of producing alkaline phosphatase genes like *PhoA* and *PhoC* genes, as well as the genes *PhoH*, *PhoP*, and *PhoR* were also found in *B. paralicheniformis* and *B. subtilis*. The *phoR* is one of the genes that regulates phosphorus response and is closely associated with P uptake and transport genes (*pst* genes) (Pan & Cai, 2023). The presence of these genes in microbes is essential because they also regulate the expression of genes encoding alkaline phosphatases, especially under P deficient conditions (Dai et al., 2020). In this study, *Bacillus paralicheniformis* was also found to produce alkaline phosphatase D (Table 3). Different gene carriers in phosphate-solubilising microorganisms express distinct enzymes, including alkaline phosphatases (possessing *phoA* and *phoD* genes), and acid phosphatases (possessing *phoC* gene), with the alkaline phosphatases (possessing *phoA* and *phoD* genes), being the most prevalent among microbes (Guo et al., 2022). More than 30% of bacteria have been found to have at least one alkaline phosphatase gene (Guo et al., 2022). Through nutrient cycling, the presence of the mentioned genes may significantly contribute to the strains' agricultural significance (Wan et al., 2020). Shown by an increase in the number of copies of the corresponding genes, Wan et al. (2020) found that inoculating plants with phosphate soluble strains increased the abundance of *phoD* genes. This demonstrates the immense potential of *Bacillus* sp.

Tryptophan is one of the essential amino acids that play a crucial role in plant development (Xie et al., 2001). The most prevalent tryptophan enzyme found in these *Bacillus* sp. was tryptophan synthase, with alpha and beta tryptophan synthase found in *B. paralicheniformis* (Table 4). In contrast, *Bacillus subtilis* only had tryptophanyl-tRNA synthetase, indole-3-glycerol phosphate synthase, and alpha and beta tryptophan synthase, as enzymatic functions involved in the production of indole (Table 4). Tryptophan synthase is an enzyme that catabolises indole (Miles, 2001). The last catalytic step in the synthesis of tryptophan is tryptophan synthase. It converts indoleglycerol phosphate into indole and glyceraldehyde 3-phosphate, which in turn produces L-tryptophan. Following additional enzyme activity, indole is produced as the end product (Xie et al., 2001).

Endophytes also promote plant growth through siderophore synthesis, which aids iron acquisition for plants particularly under iron deficient environments (Mohamad et al., 2018). By allowing microorganisms to sequester iron, siderophores further shield plants from pathogenic attacks (Y. Sun et al., 2022). Microbes' ability to obtain iron has been linked to a number of genes that are either involved in the acquisition or transportation of iron compounds. For example, the acquisition of iron (III)-hydroxidamate complexes is mediated by *fhuD* genes (Papik et al., 2020). *B. paralicheniformis* and *B. subtilis* yielded a number of siderophore production and transporting genes (*FhuC*, *FhuC*, *FhuG*, *Feu A*, *Feu B*, and *FeuC*) (Table 5). Microbes transport iron via the microbial outer membrane as ferrichrome- and ferric citrate siderophore complexes, which are catalysed by *FhuA* and *FeuA* genes, while transport across the cytoplasm membrane occurs by *FhuBCD* and *FeuBCDE*, respectively (Braun et al., 2004). *Bacillus paralicheniformis* and *Bacillus subtilis* also possessed bacillibactin-producing functions. *Bacillus* sp. secretes bacillibactin, an iron siderophore catecholic, which plays a significant role in iron acquisition, especially in iron deficient settings (Rabbee et al., 2019).

7.5.2. Genes functions associated with vital metabolites' production by endophytes

Endophytes have gene reservoirs relevant to the biosynthesis of metabolic compounds (Mengistu, 2020). *Bacillus paralicheniformis* and *Bacillus subtilis* endophytes had a number of gene activities related to the generation of metabolites that matched the compounds found in the NMR data. Table 6 and 7 show the presence of the corresponding genes and functions in the genomes for the biosynthesis regulation, transportation, and utilisation of metabolites obtained from *B. paralicheniformis* and *B. subtilis*, respectively. Similar results were obtained by Mülner et al. (2020), whereby metabolic profiles corresponded with the genomes of plant growth-promoting *Bacillus* species (Mülner et al., 2020). *Bacillus paralicheniformis* and *Bacillus subtilis* possessed a

number of genes involved in arginine biosynthesis, transportation, translocation, and utilisation, including *RocB* for arginine utilisation, Arginine pathway regulatory protein (*ArgR*), *TatAy*, *TatCy*, *TatAd*, and *TatCd* for twin-arginine translocation protein, and *ArtQ*, and *RocE* for other arginine processes (Tables 6 and 7). Arginine is one of the metabolically active amino acids, serving as a source of nitrogen, carbon, and energy in bacteria (Lu, 2006). Arginine is generated from L-glutamate through various pathways. The arginine deiminase route is one of the prevalent pathways, and a range of genes, such as *arcA*, *ArcB*, *arcC*, and *arcD* are among the most common, encoding the arginine/ornithine antiporter, arginine deiminase (Lu, 2006). Arginine is controlled by the arginine repressor *ArgR* gene, a transcription factor often preserved in bacterial genomes (Makarova et al., 2001). Conversely, a protein route known as the twin-arginine translocation system (Tat) transports two consecutive arginine residues (Biswas et al., 2009). Other bacteria, such as *B. subtilis*, have a functional Tat pathway that is driven by several genes, including *TatA*, *TatB*, and *TatC* (Biswas et al., 2009). Two genes on *B. subtilis*, *tatCd* and *tatCy*, have been implicated in the translocation of proteins with putative twin-arginine signal peptides.

The *SufS* gene responsible for cysteine desulfurase production was also present in both endophytes in Tables 6 and 7, respectively. Cysteine is a source of sulfur for the biosynthesis of many sulfur-containing compounds, including iron-sulfur clusters, thiamine, and biotin (Black & Dos Santos, 2015). The enzymes cysteine desulfurases assist in sulfur mobilization (Swindell & Dos Santos, 2024). Additionally, they catalyse the transformation of L-cysteine into L-alanine (Mihara & Esaki, 2002). The *SufS* genes encode the cysteine desulfurase. The *SuFS* substantially functions under unfavourable conditions such as iron deficiency and oxidative stress environment (Loiseau et al., 2003).

With regards to the ability of one of these strains to promote plant growth, and protect plants from abiotic factors, it contained trehalose-6-phosphate gene functions. One of the signalling elements of the trehalose pathway is trehalose-6-phosphate (Paul, 2007). At the gene level, nitrate and sugar molecules, as well as abscisic acid, cytokinin hormones influence trehalose- 6-phosphate (Paul, 2007). *Bacillus paralicheniformis* contains the trehalose-6-phosphate function, which is a component of the trehalose production pathway (Table 6). Trehalose-6-phosphate plays a crucial role in processes that synchronise metabolism with plant growth and development, particularly in embryogenesis, shoot branching and flowering, and adaptability to adverse environments (Lunn et al., 2014). For example, certain plants and bacteria may survive in a dehydrated state for a lengthy period and can be restored within a few hours after hydration (Iturriaga et al., 2009). Their ability to do so has been attributed to the presence of trehalose (Argüelles, 2000). In order to help cells adapt to abiotic stressors, to grow and develop, trehalose integrates external signals, and regulates

pathways (Iturriaga et al., 2009). Because microorganisms that possess trehalose can endure a wide range of harsh conditions, including drought, high and low temperatures, and nutrient limitation, they are the ideal choice for promoting plant growth (Argüelles, 2000).

Bacillus paralicheniformis and *Bacillus subtilis* contained gene activities for sugar compounds (xylose, fructose, sucrose, glucose, and ribose) (Tables 6 and 7). The presence of these gene activities for sugar compounds could be linked to the ability of the strains to produce sugar compounds, as more sugar compounds were recovered from the NMR results shown in Table 8 and Figure 9. These sugars are playing a significant role in plants and microbes. One of the most common disaccharides in nature is sucrose and is governed by multiple enzymes and genes. Numerous bacterial groups, including *Bacillus* species, repress sucrose structural genes through the action of regulatory proteins linked to sucrose utilisation genes, such as the LacI–GalR family of transcriptional regulators. *Bacillus subtilis* has been suggested as containing the sucrose utilisation systems (Deutscher et al., 2001). *Bacillus paralicheniformis* and *Bacillus subtilis* also possessed several sugar transport genes, including *MsmGE*, *MsmGF*, and *MsmG*, as well as the PTS system, fructose-specific IIA, IIB, IIC, and IID (Table 6 and 7). Transport across biological membranes is one of the important activities in life and is mediated by a group of transporters and translocators (Ferreira & Sá-Nogueira, 2010). ABC transporters are one of the predominant translocation facilitators. They play an important part in various activities, including nutrition absorption and assimilation, cell division, immunological response, and sporulation (Ferreira & Sá-Nogueira, 2010). The PTS is one of the prevalent methods for transporting sugar. It contains the enzyme HPr, as well as a variety of sugar-specific enzyme II proteins, particularly EIIA, EIIB, and EIIC. These sugar-specific enzyme II proteins vary between bacteria (Reid & Abratt, 2005).

7.5.3. Production of metabolites by endophytes

Extraction of good quality and quantity of metabolites is not assured, as most medicinal plants, if not all of them, contain complex mixtures (Li & Vederas, 2009). At times, the complex mixture may contain a multitude of structurally analogous compounds with similar therapeutic actions and the essential ones might be unstable in the mixture (Bhardwaj et al., 2018; Cardoso et al., 2019). Another challenge with the extraction of metabolites from medicinal plants is that two or more compounds can exist synergistically and dissolve when separated (Li & Vederas, 2009). Furthermore, the use of medicinal plants as sources of metabolites has some limitations, such as seasonality (Liu et al., 2009) Also, re-extracting important metabolites from medicinal plants can be difficult because medicinal plants metabolite production is both abiotic and biotic stress dependent (David et al., 2015). It is thus possible to get a good yield once, whereas the following time it might

fail due to the plants having been exposed to inconsistent biotic and abiotic stressors. Endophytes produced more varied metabolites compared to *A. afra* plants in the present study as seen in Table 8 and 9 respectively. In comparison to medicinal plants, microorganisms have a larger potential for the development of novel compounds because they use a range of biosynthetic pathways to produce metabolites, such as polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NROSs) (Baucom, 2015; Li & Vederas, 2009). Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NROSs) are able to resemble linkers for making metabolites by chain elongation and functional transformation resulting in possible alterations which might favour production of a new compound (Li & Vederas, 2009). However, there are limitations with regards to maximizing their potential ability in the laboratory setup and this has resulted in the under exploration of endophytic microbes for discovery of new novel drugs. Microbes, including endophytes, can exist in the environment as unicellular, multicellular, or cell clusters, while some gene clusters can be silent (Nortjie et al., 2022). Endophytes are a great source of metabolites. The reason for not having more different metabolites from endophytes under co-culture, as seen in Table 8, could be that being able to fight for available resources, endophytes might be spending more energy to survival than to producing metabolites. The mode of application and concentration can be looked into in order to increase the production of metabolites by endophytes in a co-culture system (Laffon et al., 2024).

7.5.4. Influence of endophytes on *Artemisia afra* plant growth

There is a dearth of research on the use of bacterial endophytes as bio-inoculants for medicinal plant growth promotion. Quality-certified medicinal plant products are required for the utilisation of medicinal plant material in product development (Albrecht et al., 2012). Medicinal plant cultivation in South Africa is still in its infancy, despite the huge demand for medicinal plants in the pharmaceutical industry (Albrecht et al., 2012). The present study evaluated the effect of native bacterial endophytes (*B. paralicheniformis* and *B. subtilis*) on *A. afra* growth and production of metabolites in the pot trial experiment. Findings revealed that inoculating *A. afra* with *B. paralicheniformis* and *B. subtilis* significantly increased *A. afra* growth as evidenced by increased plant height, total wet and dry biomass, shoot wet and dry biomass and root wet and dry biomass (Figure 2, 3, 4, 5 and 6). This appears to be the first study reporting on the influence of *B. paralicheniformis* and *B. subtilis* endophytes on *A. afra* growth and metabolites production. However, one of these endophytes has been utilised in a different plant including medicinal plants for plant development. For instance, *B. subtilis* increased fresh total biomass in *Arabidopsis* plants and showed a strong inhibitory effect on fungal infections (Lu et al., 2021). Inoculating tomato plants with *B. subtilis* considerably reduced tomato disease severity and index by more than 70%, along with an increase in plant roots, height length, and fresh weight (Samaras et al., 2016). The

strain was found to be able to inhibit fungal diseases by producing a range of antifungal qualities such as fengycins, iturins, and surfactins, and its capacity to stimulate growth was linked to its IAA synthesis. IAA has also been observed to increase the number of apical and lateral roots, resulting in increased nutrient and water intake (Luo et al., 2022).

In the present study, inoculation of *A. afra* with *B. subtilis* and *B. paralicheniformis* both individually and in consortium, significantly improved *A. afra* wet and dry root biomass (Figure 6). Endophytes colonise plants through roots, hence their first effect is more pronounced on the root system (Taulé et al., 2021). Inoculating plants with endophytes from the same host species has a significantly favourable effect on root biomass (Mayerhofer et al., 2013). Another advantage of inoculating plants with endophytes from the same host species is that these endophytic inoculants will adapt to the plant environment without compromising the plant's native microbial balance (X. Liu et al., 2016). Phytohormones, such as IAA, due to their ability to promote cell initiation and division, have been reported to play a role in improving endophyte colonisation efficiency and can lead to plant morphological and architectural modifications (Hardoim et al., 2015).

Endophytes also promote plant growth by increasing photosynthetic rate, carbon dioxide assimilation, and water use efficiency (Hardoim et al., 2015). Since roots are responsible for taking water from the soil and essentially supporting the entire plant, it has been suggested that improved root growth promotes plant survival (Adeleke et al., 2021). Inoculation with *B. paralicheniformis* (Figure 3B) and *B. subtilis* (Figure 3C) and their combined treatment (Figure 3D) protected the plants from water stress, which resulted in scotching of leaves in control as seen in Figure 3A. Plants subjected to biotic and abiotic challenges experience reduced and/or stoppage of photosynthesis, resulting in lower growth rates and yields, and eventually death (Yu et al., 2018). *Bacillus paralicheniformis* is one of the bacteria that have a high resistance to heat stress caused by elevated temperatures. Due to its thermophilic nature, it has been reported to produce hot temperature-resistant enzymes (Cuellar-Gaviria et al., 2023).

7.5.5. Influence of endophytes on *Artemisia afra* macronutrients composition

Plants contain a variety of components and bioactive chemicals, some of which are needed for plant growth and survival, while others have a vital role in the control of human metabolism in ethnomedicine (Dos Santos et al., 2022). Variations in plant element, macro- and micro-nutrients, were observed in the present study as shown in Figure 7 and 8, respectively. One possible explanation for this difference is that certain mineral elements are more efficiently absorbed than others, which causes plants to have larger concentrations of certain compounds in their cell saps than others (Rafiri et al., 2024). Differences in macro- and micronutrient nutrients from *A. afra*

leaves were also observed by (Rafiri et al., 2024). It has been underlined that critical nutrients play an important part in medicinal plants; numerous research studies have documented astounding data on the effects of these components on plant growth, specifically plant height, wet and dry biomass (Hossain et al., 2007; Kumar et al., 2002; Vetayasuporn, 2006). In the present investigation, *B. paralicheniformis* (Treat-AR) positively influenced macronutrients by increasing the level of most macronutrients (N, P, Mg, Ca, and S) (Figure 7). The importance of macronutrients on medicinal plants has been reported by researchers. For instance, N availability significantly increased *A. vera* height in a study conducted by (Hossain et al., 2007). Plants require a large amount of nitrogen which is a component of amino acids and nucleic acids. (Mu & Chen, 2021). *Bacillus paralicheniformis* (Treat-AR) influence on N could be related to its ability to fix biological nitrogen, and low N content was due to Treat-control (Figure 7). The low concentration of nitrogen in *A. afra* leaves was also observed by Rafiri et al., (2024) and attributed to the possibility of its preservation as plant energy. Inoculation of *A. afra* with *B. paralicheniformis* (Treat-AR) enhanced accumulation of phosphorus in *A. afra* leaves (Figure 7). Phosphorus has been shown to have an effect on some medicinal plants; for example, (Vetayasuporn, 2006) found that P availability significantly increased *Allium cepa* height. In addition, (Kumar et al., 2002) found that P significantly increased *Phaseolus trilobus* height. The ability of *B. paralicheniformis* (Treat-AR) to enhance P-content *A. afra* leaves (Figure 7) can be attributed to the ability of this endophyte to solubilise insoluble phosphate. Phosphorus fixation is prevalent in soil, and it can be chelated with molecules such as Fe to form Fe-P, which is not available for plant usage (Yuan et al., 2017). Inoculation with phosphate-solubilising microbes has been shown to not only increase growth, but also enhance plant phosphate uptake, resulting in high levels of P on plant vegetative (Bekkar & Zaim, 2023). Soil sometimes has strong buffering capacity, and ordinary phosphate solubilising microorganisms may not be as effective in solubilising organic bound P from Fe-P (Marschner et al., 2011). Therefore, substances that can chelate iron are required (Marschner, 2011). Endophytes and other microbes that produce siderophores have been identified as the best candidates for phosphate solubilisation of Fe-P, which would make both P and Fe available for plant usage (Marschner et al., 2011). *Bacillus paralicheniformis* (Treat-AR) also positively influenced availability of magnesium, calcium, and sulfur (Figure 7) in *A. afra* leaves. Magnesium plays a role in chlorophyll content, hence its deficiencies result in interveinal chlorosis of plants (Gerendás & Führes, 2013). It has an impact on plant primary (energy) metabolism, which may lead to a decrease in quality and quantity of plant metabolites (Grzebisz, 2013). Sulfur is also one of the essential elements for the growth and development of plants. It protects plants from oxidative damage under stressful situations through the synthesis of glutathione and its derivatives (Samanta et al., 2020). Certain vital elements, including iron, zinc, potassium, calcium, and magnesium, are chelated by

oxalic acid (like di-carboxylic acid) and phytic acids found in soil (Misra et al., 2024). Nevertheless, certain microorganisms, such as endophytes, break down these oxalic and phytic acids, releasing the chelated elements, and making them available to plants (Misra et al., 2024). They mobilize, hydrolyse, solubilise, or transform them into compounds that plants can utilise.

7.5.6. Influence of endophytes on accumulation of micronutrients in *Artemisia afra* leaves

Despite being required in trace amounts, micronutrients are essential for plants including medicinal plants, and their absence can significantly impact both plant growth and functions. For instance, iron deficiency can cause interveinal chlorosis in young leaves, which causes them to turn yellow while the veins remain green; if severe, the leaves will have brown blotches. Extreme Fe deficiency is characterised by the entire plant turning light yellow or white. However, endophytes' ability to produce siderophores allows them to chelate iron and make it available for plant usage (Zhang et al., 2019). This results in improved plant development as they alleviate Fe deficiency (Sultana et al., 2021). The capacity of *B. paralicheniformis* (Treat-AR) to enhance iron build-up in *Artemisia afra* leaves (Figure 8) could be due to this endophyte's ability to produce siderophores. The use of siderophore-producing endophytes, such as *B. paralicheniformis*, to promote plant development in various plants and crops could have a significant impact on crop production. In the present investigation, Treat-AB and Treat control had the same effect on iron accumulation, being the lowest. Competition among endophytes when combined under Treat-AB could explain why low quantities of iron and other elements were recovered from this treatment. For example, high siderophore-producing bacteria have been shown to have a competitive advantage in colonising plant tissues (Loaces et al., 2011). Other important micronutrients include copper. Copper is required for plant respiration, photosynthesis, and cell growth, and it modulates plant stress response by reducing oxidative stress. Copper is an electron carrier that plays a critical role in plant enzyme activity. *Bacillus paralicheniformis* (Treat-AR) and *B. subtilis* (Treat-EL2) enhanced accumulation of copper, and Treat-Control had the lowest content (Figure 8). The potential of these endophytes to alter the accumulation of elements, such as copper, could be one of the reasons why inoculated *A. afra* performed better in the current investigation. For example, low levels of Cu have been shown to result in reduced plant performance and photosynthesis (Koehorst, 2013). Boron is also a signalling molecule for plant bacterial interaction (Goldbach et al., 2002), and the positive influence of *B. paralicheniformis* and *B. subtilis* on Boron content may play a role in plant activities. For instance, Boron plays an important function in cell elongation and differentiation, and is intimately associated with leaf photosynthesis and nitrogen metabolism (Redondo-Nieto et al., 2008). Additionally, despite the fact that roots are essential for the transfer of water and nutrients throughout plants (Hajiboland, 2012). However, B deficiency in plants reduces the length and

number of root tips, which impacts the plants' ability to absorb water (Goldbach et al., 2002). Treat-AR also enhanced accumulation of Zinc in *A. afra* leaves, and Treat-Control had the lowest Zn content (Figure 8). Leaf chlorosis and necrotic was observed in control plants as shown in Figure 3, with some ultimately drying. Zinc deficiency in leaves can induce high levels of necrosis and chlorosis, as well as a significant accumulation of sucrose and starch, which can cause damage to chloroplast components. Zinc has a role in the metabolism of reactive oxygen species (ROS) and is critical in shielding cell structural elements from ROS (Hajiboland, 2012). Thus, Zn deficiency induces photo oxidative damage in plants by inhibiting photosynthetic carbon dioxide fixation, and a decrease in photosynthesis may cause a decline in plant development. In comparison to the other treatments, the control had the lowest manganese level, with Treat-AR having the highest Mn content, followed by Treat-EL2 and Treat-ABR (Figure 8). Necrotic patches and intravascular chlorosis are symptoms of manganese deficiency in leaves (Hajiboland, 2012). Leaf chlorosis and necrotic patches were observed in control plants as shown in Figure 3, with some ultimately drying. Minimal manganese deficiency, even in the absence of obvious leaf symptoms, can result in severe photosystem II (PSII), a multiple subunit, pigment protein complex found in thylakoid membranes of oxygenic photosynthetic organisms in plants. Even in the absence of overt leaf symptoms, a minimal manganese deficit can cause severe photosystem II (PSII), a multiple subunit, pigment protein complex present in the thylakoid membranes of oxygenic photosynthetic in plants (Hajiboland, 2012).

7.5.7. Influence of endophytes on medicinal plants' metabolites production

Cultivation has been recognised as one of the ways to enhance metabolites in medicinal plants (Albrecht et al., 2012). Some of those metabolites fulfil a critical function in promoting plant growth. For example, N-based substances such as allantoin, which were present in *A.* leaves from Treat-EL, have been demonstrated as some of the sole N sources for plant growth (Brychkova et al., 2008). Cysteine, whose accumulation was altered by endophyte inoculation (*B. paralicheniformis* and *B. subtilis*) (Table 9), is a byproduct of the photosynthetic sulphate assimilation pathway (Gotor et al., 2015). It is recognised to contribute to the production of organic sulfur and supply plants with a donor of metabolic sulfur (Hesse et al., 2004). The biosynthesis of this molecule in plants is extremely important since it also contains elements of nitrogen (Hesse et al., 2004). Additionally, it functions as a defensive molecule and is involved in redox signalling (Romero et al., 2014). This metabolite's presence in Treat-AR (*B. paralicheniformis*) may have contributed to *A. afra*'s build-up of sulfur. It was evident from the present study that endophyte inoculation affected *A. afra* metabolites synthesis (Figure 9). *Bacillus paralicheniformis* (Treat-AR) had the highest influence on metabolite accumulation, specifically sugar and aliphatic compounds (Figure

9B). *Bacillus subtilis* (Treat-EL) enhanced *A. afra* sugar accumulation more than aliphatic compounds (Figure 9C). The combination of *B. paralicheniformis* and *B. subtilis*, however, also had an effect on sugar build-up and aliphatic metabolites (Figure 9 D). Even though the effect was slight, the combination of treatments (Treat-ABR) showed promising effects on aromatic compounds (Figure 9 D). The beneficial effects of endophytes on medicinal plants were also documented by (Pandey et al., 2016). They found that inoculating *Catharanthus roseus* with *Choanephora infundibulifera* and *Curvularia* sp. considerably increased the production of metabolites, such as terpenoid indole alkaloid metabolites increase of more than 200% to 400%.

On the other hand, Treat-control had the least influence on *A. afra* metabolites production as shown by ¹H NMR spectra (Figure 2A). *Artemisia afra* plants from the control group may have experienced water stress, as previously indicated and illustrated in Figure 3. Water is essential for plant growth because it serves a number of functions, including photosynthetic activity and nutrient transport throughout the plant. Water has been recognised as one of the factors that influence metabolite synthesis (Mahajan et al., 2020). The metabolites produced under Treat-Control contained more sugar compounds (Table 9). Sugars serve as intermediates in plant metabolism and signalling-compounds. Sugars help plants tolerate desiccation (Zhang et al., 2016). When plants experience water stress, they accumulate sugar, which protects their membranes against ROS (Zhang et al., 2016). However, high amounts of ROS are known to be hazardous to plants, causing plant death. High ROS may have been a contributing factor to the wilting and death of some *A. afra* plants under Treat control. One of the sugars that build up more when plants are dehydrated is sucrose (Salerno & Curatti, 2003), and sucrose was high in Treat-control as shown in Table 9. Under unfavourable conditions, sucrose stabilises cellular structural elements and modifies cellular osmotic potential (Egert et al., 2015; Salerno & Curatti, 2003). During dehydration, the hydroxyl group of sugars replaces water molecules and proteins, which is why there is a significant build-up of sugars (Zhang et al., 2016). Furthermore, during stressful events, energy is redistributed and reconstructed, altering the related metabolic pathways and influencing the total production of metabolites in plants (Zhang et al., 2016). Thus, when plants are exposed to stressful situations, growth is usually minimal, and this was observed under control since the lowest plant height was obtained from Treat-control (Figure 2). Growth induction diverts resources for manufacture of metabolites for plant defence in stressful conditions (Bastías et al., 2021). Although stress is known to promote metabolites production, excessive occurrence directly impairs plant performance by interfering with photosynthesis, which is a determinant of metabolite production in plants. Plant stress has the potential to harm plant cell walls and membranes, as well as cellular enzymes and nucleic acids, leading to plant death (Rahman et al., 2023). However, endophytic bacteria have been

shown to upregulate numerous plant genes and enzymes involved in plant defense (Kohli et al., 2022). Because of their ability to protect the plant against stresses, endophytes contribute to the production and stability of plant metabolites (Kumari et al., 2023). Citrulline, for example, is an aliphatic molecule that shields plants against hydroxyl radicals and cellular enzymes from oxidative damage. Its accumulation and concentration in *A. afra* leaves may have been influenced by inoculation with *B. paralicheniformis* (treatment-AR) (Table 9). In addition, the presence of trehalose and suberate on plants inoculated with endophytes, particularly *B. subtilis* (treatment-EL), could be the reason why the inoculated plants were able to tolerate water stress compared to the control, as these metabolites were produced by *A. afra* under treatment-EL (Table 9). There is a substantial correlation between plants' capacity to manufacture trehalose in their intracellular tissues and their capacity to withstand water stress (Elbein et al., 2003). Trehalose contributes to stomatal conductance in plants and improves their water use efficiency (Lunn et al., 2014). It helps plants recover their activity as soon as they are rehydrated, in addition to promoting plant survival during dehydration (Patist & Zoerb, 2005).

Trehalose possesses antioxidant activity and protects plant cells from oxygen radical damage (Elbein et al., 2003). Trehalose has antimicrobial properties (Lunn et al., 2014). Conversely, suberate has been shown to act as a physical barrier that stops plants from losing water and possessing antimicrobial properties. Furthermore, ferulate, which was also present in inoculated plants, is known to strengthen plant cell walls, protecting plants from unfavourable conditions, and possesses pharmacological properties such as antioxidant, anti-inflammatory, and antimicrobial (Harris & Trethewey, 2010). Some of the compounds generated by *A. afra* have pharmaceutical applications. For example, chlorogenate (chlorogenic acid) has antioxidant, antidiabetic, and anti-inflammatory properties in addition to its capacity to protect against cardiovascular ailments, and lower the incidence of diseases such as cancer (Naveed et al., 2018). Furthermore, the AR-enhanced nonsteroidal, anti-inflammatory drug (NSAID), ibuprofen, even though also present in control in minute quantities, was surpassed by Treat-AR which had the highest level (Table 9). Its low toxicity and status as an over-the-counter medication account for its widespread use (Rainsford, 2009). At doses ranging from 800-1200mg daily, it is frequently used as a painkiller and antipyretic drug to treat and reduce fever, muscle aches, headaches, toothaches, backaches, and inflammation (Marsik et al., 2017; Mazaleuskaya et al., 2015). Ibuprofen and other compounds found in *A. afra* are possibly responsible for its anti-inflammatory qualities.

Endophytes use a variety of mechanisms to affect metabolites production in medicinal plants, and their effects begin at the colonisation site. For example, endophytes cause a response in the plant membrane, triggering receptors in the plasma membrane to convey signals to the plant (Shasmita et

al., 2018). Endophyte colonisation of plants alters the behaviours of both endophytes and plants, such as the detection of quorum-sensing signals and the expression of microbe and plant genes (Taulé et al., 2021). Plants identify endophytes as intruders and produce metabolites to combat their invasion (Taulé et al., 2021). However, endophytes form aggregates at colonisation sites to increase their resistance to harmful compounds produced by plants to prevent their entrance (Compant et al., 2005). Furthermore, endophytes secrete hydrolytic extracellular enzymes that facilitate plant colonisation by hydrolysing the surface of plant cells (Toghueo & Boyom, 2021). After being introduced into the plant, precursors activate cell signal generators, indicating a shift in cells that changes the plant's metabolite pathway (Ahmed et al., 2020). Also, the capacity of microbe-based elicitations to replicate a disease response in cells is one of their noted advantages. Typically, plants engage their defensive mechanisms, which encourage the production of metabolites (Shasmita et al., 2018). In addition to enhancing production of metabolites from medicinal plants, endophytes can aid in the conversion of their hosts' metabolites (Wei et al., 2021). For instance, co-culturing endophytes have been shown to increase synthesis of metabolites through conversions and substrate metabolism, and this depends on the strain's combined metabolic activity (Andriani et al., 2020). This was not the case in the current investigation as the combination of *B. paralicheniformis* and *B. subtilis* had less impact on metabolites compared to when each strain was used individually. Nonetheless, the combination had a beneficial effect, since it led to the build-up of sugars and aliphatic molecules (Figure 9 and Table 9). It has frequently been anticipated that co-culturing will increase the production of metabolites as it has been demonstrated to stimulate and activate dormant gene clusters. This would then lead to an increase in the yield and richness of metabolites, as well as their biological activities, yet co-culturing is a complex interaction (J. Li et al., 2022). Microbe-to-microbe interaction may cause stress and reduced activity when two or more endophytes are combined (Laffon et al., 2024). Furthermore, co-culturing microorganisms that produce multilytic enzymes may lead to cell wall degradation of other microbes (Ghosh et al., 2016). The ability of the *Bacillus* strains utilised in this investigation to produce a variety of hydrolytic enzymes, including protease, lipase, amylase, D-nase, and gelatinase, may have affected their capacity to cooperate in the promotion of metabolites in *A. afra*. To acquire better results from co-culturing, it is vital to properly understand the biological interaction that governs and shapes the byproducts of the co-culture system beyond determining their compatibility in the solid media agar (Kapoor et al., 2022). This could lead to an improved ability to predict and manage cell to cell interactions (Kapoor et al., 2022). Despite the fact that co-culture did not exhibit the substantial metabolites accumulation that was anticipated, it may have triggered the transmission of metabolites from endophytes to plants. The cis cis muconate metabolite, for instance, is one of the metabolites found in bacteria, particularly *Bacillus* species. Its presence in the inoculated plants (Treat-AB) (Table 9)

indicates the potential of endophytes to transfer some of the metabolites they possess. This may enhance the plant's therapeutic benefits, and it has been suggested that co-culturing increases the likelihood of producing novel bioactive chemicals (Tan et al., 2019).

7.6. CONCLUSION

Bacterial endophytes (*B. paralicheniformis* and *B. subtilis*) increased *A. afra* growth and metabolite production under greenhouse conditions. *Bacillus paralicheniformis* and *Bacillus subtilis* and their consortium significantly affected *A. afra* plant growth as evidenced by increased plant height, total wet and dry biomass, shoot wet and dry biomass, as well as root wet and dry biomass. The nutrient composition of *A. afra* was similarly impacted by endophytes, with *B. paralicheniformis* (Treat-AR) having the greatest impact due to its higher accumulation of macronutrients (N, P, Mg, Ca, and S) and micronutrients (Fe, Cu, Zn, and Mn). In comparison to control, inoculating *A. afra* with *B. paralicheniformis* and *B. subtilis* increased metabolites production by *A. afra*. The presence of metabolites such as trehalose and suberate in inoculated *A. afra* plants may be the reason why they were able to survive water stress, which caused un-inoculated plants to wither and some eventually to die. With the ability of these strains to protect plants from water stress, they can be perfect in mitigating climate change's negative effect on agricultural productivity. Additionally, a variety of genes and enzymes linked to metabolite production and plant growth promotion were present in *B. paralicheniformis* and *B. subtilis*. Due to their capacity to produce multiple metabolites, their genes, and their capacity to stimulate plant growth, *B. paralicheniformis* and *B. subtilis* are the most promising candidates for biocontrol and biofertilization.

8. CHAPTER EIGHT : GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1. GENERAL DISCUSSION

Plants are immobile, and under extreme conditions will produce different bioactive compounds that allow them to survive (Mannino et al., 2021). Medicinal plants, especially, adopt a variety of strategies, such as metabolites synthesis, to cope with changing unfavourable conditions, maintaining the physiological processes required for growth without being damaged by these fluctuations (Isah et al., 2018). *Artemisia afra* and *Lessertia frutescens* are two of the most popular medicinal plants of Southern Africa and are excellent sources of metabolites of pharmaceutical importance. Chapter 3 of this study aimed at evaluating phytochemistry and chemical variation of *A. afra* and *L. frutescens* metabolites and determining if plant microbiome has an impact on production of metabolites by these plants. Results from Chapter 3 revealed that, variations occurred in metabolites from *A. afra* samples collected from different regions, as well as *Lessertia frutescens* inter variations between plant parts samples from the same location. Medicinal plants produce mainly metabolites to fight against abiotic and biotic factors. For example, the excessive production of proline compounds may be attributed to the high abundance and diversity of opportunistic pathogenic endophytes in these medicinal plants. The build-up of proline in cells has been intimately associated to plant response to stressors such as salt, drought, and resistance to pathogens (Anwar et al., 2018). Several opportunistic pathogens endophytes were abundant, including *Proteus*, *Providencia*, *Kocuria*, *Sphingomonas*, and *Staphylococcus* from both *A. Afra* and *L. frutescenes*, all of which are believed to have played a huge role in these plants' microbial community structure and diversity. The plant microbiome has been suggested to have a direct impact on plant metabolites production, and vice versa. ATP, for example, which counted among the most abundant metabolites generated by these plants, is employed by them as an energy source to release bioactive compounds in the rhizosphere, attracting diverse endophytes (Pang et al., 2021). Among the intermediary molecules of the purine biosynthesis pathway are inosine monophosphate (IMP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), and guanosine triphosphate (GTP) (Kamatani et al., 2021). These compounds were prevalent in this current investigation. Purine metabolites serve a variety of physiological purposes in cells and can be a source of nitrogen (Jiménez & Puig, 2012). They play a huge role in intercellular and extracellular signalling, as well as energy transfer (Jiménez & Puig, 2012). On the other hand, fucose was one of the most prominent monosaccharides produced by both plants in the current investigation. Fucose is

a methyl pentose sugar present in gangliosides, glycolipids, and glycoproteins (PHILIPPART, 1987). Fucose production and availability are heavily influenced by proteases. It has been reported that during cellular turnover, protease eliminates the protein moiety of glycoprotein, leaving oligosaccharide chains with fucose assumed to be connected to the margins of those chains (PHILIPPART, 1987). The enzyme α -fucosidase then breaks down molecules to release fucose (PHILIPPART, 1987). Fucose is recognised as the backbone unit of polysaccharides (Meng et al., 2023). This observation can support the speculation that the types of metabolites produced by these plants can be associated with endophytes present in the plants. Some of the compounds that were produced by these medicinal plants are linked to plant growth promotion that were obtained from this study which included tryptophan. It is not unexpected that more than 80% of endophytes isolated from both plants were able to synthesize IAA, as shown in Table 1 of Chapters 4 and 5. Tryptophan (Trp) serves as the precursor in the Trp-dependent IAA biosynthesis pathway for production of IAA. Tryptophan promotes plant growth and helps plants withstand both biotic and abiotic stresses. It is an essential aromatic acid, and indole serves as a functional group in it. Approximately 90% of tryptophan is metabolised through kynurenine pathway and lesser fraction via methoxyindole pathway (using 1-2% of L-tryptophan) and serotonin (Sadok et al., 2017). It has been indicated that, in the absence of nicotinamide or nicotinic acid, tryptophan is the only source of substrate for NAD⁺ and NADP⁺ production (Moffett & Namboodiri, 2003). A variety of enzymes and various intermediates, such as kynurenines, are involved in the production of NAD and NADP from tryptophan (Moroni, 1999).

In the present study, a number of hydrolytic enzymes such gelatinase, protease, lipase, D-nase, and amylase, were produced to varying degrees by endophytes isolated from *A. afra* and *L. frutescens*. Endophytes produce these enzymes to colonise plants, subsequently impacting on plant growth and development. Endophytes affect plants through a variety of interdependent mechanisms, including phytostimulation (through the production of auxins like IAA), biofertilisation (through N-fixation, P-solubilisation, and Fe-chelation), and biocontrol (through the production of hydrolytic enzymes, metabolites, and phytopathogenic agents like siderophore) (Aydi Ben Abdallah et al., 2019). As seen in Chapters 4 and 5, endophytes that were isolated from these plants showed plant growth-promoting activities with *L. frutescens* endophytes and were tested for their *L. frutescens* seedling growth promotion. *Bacillus licheniformis* and *Bacillus velezensis* significantly increased *L. frutescens* seedling growth over a period of two months. With Sustainable Development Goals (SDGs) intent on achieving all the goals by 2030, the use of quick, economic and reproducible solutions is of the utmost importance. Endophytes have been identified as one of the natural resources promoting green processes (Choi & Shin, 2023). Endophytes from these plants have

shown potential for promoting plant growth and can therefore be recommended for use in a variety of crops and vegetables, including large-scale medicinal plant production. Medicinal plant regeneration can result in higher plant biomass production, and so overcome challenges associated with overharvesting of medicinal plants from the wild (Isah et al., 2018). This was evident in the present study as *L. frutescens* native strains (*B. paralicheniformis* and *B. velezensis*) significantly promoted *L. frutescens* growth through the promotion of height, total wet and dry biomass, root wet and dry biomass, as well as shoot wet and dry biomass, as shown in Chapter 6. The consortium gave the highest agronomic parameters compared to all other treatments while control had the lowest. Also, *A. afra* growth was significantly increased by inoculation with *B. paralicheniformis* and *B. subtilis*, and their consortium. This was demonstrated by increase in height, total wet and dry biomass, root wet and dry biomass, and shoot wet and dry biomass as shown in Chapter 7. Root growth is one of the important features of plant physiology that influence plant yield and its quality (Liu et al., 2023). Native strains *B. paralicheniformis* and *B. subtilis* of *A. afra* demonstrated the ability to protect *A. afra* against potential water stress in the greenhouse, as the plants were initially watered every other day and then daily as signs of water stress appeared. This was demonstrated by the fact that every inoculated plant survived, whereas some of the plants for control died. The ability of the plant to resist water stress is one of the key elements in plant growth performance and crop productivity, especially in this era of climate change (Hussein et al., 2019). The use of these endophytes could be beneficial in crop production, as it has been indicated that, in order to meet global food demand, it is imperative to increase food production by 50% in 2030 (Zhou et al., 2016). Endophytes are the greatest bio-inoculants due to their coexistence and mutualist relationship with their hosts (Priya et al., 2021). Furthermore, due to the prevailing environmental changes, plants, including medicinal plants, are submitting to a variety of stresses such as drought and salinity, adversely impacting on agricultural productivity (Hussein et al., 2019). This needs development of strategies that stimulate growth, while mitigating stress and protecting the plant cell from consequential damages, and later to increase productivity of plants by influencing plant growth parameters, such as height, root length (for accessing water from deeper levels), root biomass, shoot length, and number of leaves) (Hussein & El-Anssary, 2019).

A multitude of endophytes have remained unexploited for their ability to produce bioactive compounds of pharmaceutical and agricultural importance (Asmita & Sashirekha, 2016). Only about 10% of the proximity-known one million terrestrial endophytes have been exploited (Asmita & Sashirekha, 2016). As previously stated, *Bacillus* species were investigated for their ability to promote *L. frutescens* and *A. afra* growth in a greenhouse pot trial. Their genes related to plant growth capacity and metabolite synthesis were evaluated prior to their inclusion in the pot

trial. Microorganisms, primarily bacteria, play a significant role in the maintenance of the nitrogen cycle. Genes involved in N-fixation processes obtained from *Bacillus* sp. employed in this study's greenhouse pot trial include *NorQ*, *NorD*, *NarT*, *NasA*, *NifU*, *NtrC*, and *NarT*, as indicated in Table 3 and 2 of chapters 6 and 7, respectively. These genes have an essential role in N availability; for example, nitrogen regulatory protein C (NtrC) regulates the transcription of genes necessary for nitrogen metabolism in certain bacteria (Douceff et al., 2005). The *Nor* genes, on the other hand, are functional indicators for assessing nitrification and denitrification processes (Sun et al., 2021). Endophytes also promote plant growth by solubilizing insoluble phosphate and making it available to the plant. *Bacillus* sp. from this study contained genes responsible for phosphate solubilization, regulation, and transportation, such as *PpaX*, *RapK*, *YcsE*, and *PstA*, *PstB*, *PstC*, *PstS*, *PhoA*, *PhoC*, *PhoH*, *PhoP*, and *PhoR*, as indicated in Tables 4 of Chapters 6 and 7. The presence of these genes in microorganisms is vital because through nutrient cycling, they can considerably add to the strains' agricultural significance (Wan et al., 2020). Microbes stimulate plant growth by producing siderophores, which help plants acquire iron under iron-deficient conditions (Mohamad et al., 2018). The ability of microorganisms to acquire iron has been linked to a variety their genes involved in either binding or transportation of iron complexes. In this study, whole genome sequencing revealed numerous genes (*Fur*, *Feu A*, *FeuB*, *FeuC*, *FeoA*, *FeoB*, and *FhuA*, *FhuB*, *FhuC*, *FhuG*) involved in the production and transportation of siderophores by these *Bacillus* sp. (Table 5, chapters 6 and 7). It has also been shown that naturally occurring siderophore-antibiotic conjugates encoded in siderophore genes are more effective in targeting human diseases. Thus, the prominence of siderophore-producing endophytes in the medical sector provides a significant possibility in the manufacture of effective medications and illness management (Swayambhu et al., 2021). Whole genome can also assist in gene identification of endophyte bioactive compounds, which can aid in the elucidation of ways to improve endophyte metabolite production (Atanasov et al., 2015). Biosynthetic gene clusters have been found to encode a variety of compounds present in microorganisms, including endophytes (Dinesh et al., 2017). This was demonstrated in the current investigation, because *Bacillus* sp. used in chapter 6 and 7 pot trial experiments exhibited gene functions necessary for metabolite biosynthesis and several gene activities connected to the production of metabolites that matched the metabolites observed in the NMR data. *TatAy* and *TatCy* were among the genes implicated in arginine synthesis, transport, translocation, and utilization. Other common genes functions for biosynthesis of various metabolites included *TatAd*, *TatCD*, *ArtP*, *ArtQ*, *RocB*, and *RocR*, as illustrated in Table 6 and 7 of Chapter 6 and 7. The *SufS* gene, which produces cysteine desulfurase, was also present, along with gene activity for sugar molecules (xylose, fructose, sucrose, glucose, and ribose). The presence of the relevant genes in the genomes of these *Bacillus* sp. for the production of metabolites is of paramount relevance. This represents the

possibility of creating novel metabolic pathways powered by a plethora of genes as intermediary stages, because the development of metabolic pathways is primarily controlled by genes and enzymes (Kliebenstein & Osbourn, 2012). Endophytes have gene reservoirs associated to the biosynthesis of metabolic compounds, providing plants with additional metabolic capacity (Mengistu, 2020).

All endophytes used for *A. afra* and *L. frutescens* propagation produced more metabolites compared to the inoculated plants (Table 1 and 2 of Chapters 6 and 7 respectively). It has been observed that metabolites produced by endophytes, isolated from medicinal plants, and dispose the diverse and unique structural growth of their host (Egamberdieva et al., 2017). This is the indication that endophytes possess host independent mechanisms for the production of metabolites (Egamberdieva et al., 2017). On the other hand, the crude extracts of these bacterial endophytes included several similar compounds obtained from inoculated *L. frutescens* and *A. afra* leaves, indicating their possible influence on promoting metabolites production by these plants. Because endophytes can enhance plants' ability to produce metabolites through gene exchange, it is typical to see endophytes and plants generating similar metabolites (Z. Li et al., 2022). The ability of endophytes, isolated from these medicinal plants to enhance growth and metabolites production, is an indication for a better future in the agricultural and pharmaceutical industries. It has been estimated that the global market value of biofertilizer will by 2030 be approximately \$12 billion (Luo et al., 2022). The use of microbes as biocontrol agents for plant protection against pathogens and as biofertilizer for plant growth promotion has been recognised as the best alternative green strategy for sustainable crop production, as opposed to pesticides and chemical fertiliser usage, being not only eco-friendly, but also cost effective (Luo et al., 2022).

8.2. GENERAL CONCLUSION

Medicinal plants contamination, re-discovery of previously identified metabolites from medicinal plants with known functions, low metabolite production by medicinal plants, and irregular supply of medicinal plants due to extinction, seasonality, and environmental variations are some of the main factors influencing the pharmaceutical industries' derailed continued use of medicinal plants in the search for novel pharmaceutical compounds. However, using endophytes as plant growth regulators to stimulate metabolite production in medicinal plants has been demonstrated to be one of the most successful ways to increase metabolites and that can assist in sustainable production of medicinal plants for medical research purposes. Endophytes can transfer new genes to medicinal plants via horizontal gene transfer, assisting in the activation of existing genes while promoting the

formation of new metabolites, as genes and enzymes are intermediates in the production of metabolites. Overall, associate endophytes can influence the generation of medicinal plant metabolites. The findings of this study demonstrated that, *Artemisia afra* plant organ type and medicinal plant propagation method (seed cultivation and vegetative cutting cultivation method) had an influence on metabolite production as well as the endophytic bacterial community. Metabolite production varied between *Artemisia afra* samples collected in Pretoria (vegetative cutting cultivation method) and Klerksdorp (seed cultivation method). These differences were found in plant parts (stems and leaves), as well as in the cultivation methods used in two locations (Pretoria and Klerksdorp). More metabolites were obtained from leaf samples than from stem samples, and from Pretoria, where the vegetative cutting cultivation method was utilized, than from Klerksdorp, where the seed cultivation method was used, with sugars such as xylose and fucose being highly synthesized. *Artemisia afra* harbours a variety of beneficial endophytes as well as opportunistic endophytes which might also play a role in the functionality of the plant. Correlation analysis demonstrated a positive and negative correlation between endophytes and metabolites from *A. afra*. On the other hand, the endophytic bacterial community of *L. frutescens* medicinal plants and metabolite synthesis varied by plant parts. The findings of the multivariate analysis showed that the metabolite synthesis of *L. frutescens* varied between samples of leaves and stems and Chenomx-assisted annotation revealed that there were more metabolites from leaves compared to the stems. Bacterial endophytes have a significant correlation with *L. frutescens* metabolites. Redundancy analysis (RDA) revealed a positive association between bacterial endophytes and metabolites in *L. frutescens* leaves, but a negative correlation for stem samples. Endophytes are a sustainable source of nutrients, and this study revealed that native *Lessertia frutescens* and *Artemisia afra* endophytes can be employed as a better alternative to promote plant growth. *Lessertia frutescens* and *Artemisia afra* contain plant growth-promoting and enzyme-producing endophytes. About 64% of endophytes from *A. afra* fixed biological N, 79% solubilised insoluble triphosphate, 86% produced IAA, and 79% produced siderophore. On the other hand, of endophytes isolated from *L. frutescens*, 86% possessed nitrogen-fixing, phosphate solubilising, and IAA-producing abilities, while approximately 71% were able to exhibit siderophore-producing capabilities. Conversely, 86% of the endophytes isolated from *L. frutescens* could fix biological nitrogen, solubilising phosphate, and synthesising IAA, while approximately 71% were able to exhibit siderophore-producing capabilities. While, compared to *A. afra*, which was the only plant to exceed *L. frutescens* in having more proteolytic-producing endophytes, *L. frutescens* now possessed more D-nase, amylolytic-, gelatinolytic-, and lipolytic-producing endophytes. Furthermore, *B. licheniformis* and *B. velezensis* from *L. frutescens* significantly increased *L. frutescens* seedling growth over a period of two months. Furthermore, in the five-month pot trial experiment, inoculating *L. frutescens* with

B. paralicheniformis and *B. velezensis* caused variations in metabolite synthesis by *L. frutescens*. In addition to altering the accumulation of metabolites, *B. paralicheniformis* enhanced their quantity; the highest concentration of most metabolites was found in *L. frutescens* inoculated with *B. paralicheniformis* (treatment-CL). On the other hand, *B. paralicheniformis* and *B. subtilis* increased *Artemisia afra* growth and metabolite production in the five-month pot trial experiment. Endophytes appear to be a best choice for metabolite production due to their high turnover rate for synthesis by *L. frutescens* and *Artemisia afra*.

8.3. RECOMMENDATIONS

In the present study, more metabolites were recovered from *Artemisia afra* leaves compared to stem samples, and more from Pretoria, where the vegetative cutting culture method was used, compared to Klerksdorp, where seed propagation method was used. For *Artemisia afra*, the vegetative cutting cultivation approach is therefore suggested as one of the most effective propagation techniques. Compared to the stem, more metabolites and endophytes were found in the leaves of *L. frutescens*. Given that leaves are the primary plant parts used for medicinal purposes, it is advised that study attention be directed onto leaves rather than stems. The direct impact of endophytes on medicinal plants metabolite production in a field environment is still challenging. The molecular processes by which endophytes impact the presence of metabolites in plants are currently unclear. To better understand the impact of endophytes on plant metabolite production, it is vital to study the genomes of key microbiota that influence production. In order to aid in the reconstruction of plant and endophyte metabolic networks, it is crucial to design a system in which a subset of endophytes are assessed and utilized in a controlled environment such as greenhouse following the evaluation of their entire genomes. The majority of *Artemisia afra* and *L. frutescens* strains' ability to exhibit a broad range of hydrolytic activities and plant growth-promoting properties in this study suggests their potential long-term agricultural benefits. Plant nutrition is the major factor influencing the growth and development of plants. Inadequate supply of plant nutrients is detrimental to plant growth and has a negative effect on the yield. Utilising bacterial endophytes such as *B. paralicheniformis* KSW7, *B. velezensis*, *Bacillus subtilis* KSW6, and *B. paralicheniformis* KSW1 can help enhance nutritional composition of plants and reduce the dependence on chemical fertiliser. Thus, it is vital to select microorganisms whose roles include securing plant nutrients and ensuring increased plant health and development. This study provides insights into the functional roles of endophytic bacteria of *L. frutescens* and *A. afra* and their potential plant growth-enhancement, while highlighting their potential for sustainable agriculture and ecosystem management. Considering the importance of endophytes, it is strongly recommended that future studies should focus on the way endophytes react with the plant host in order to ascertain the best

way to make them effective for continuous crop production. Targeting the genes that endophytes and medicinal plants use to produce metabolites, as well as tracking gene transfer from endophytes to medicinal plants, would be ideal in order to take advantage of the effects of endophytes on promoting the production of metabolites by medicinal plants. This would allow for the acquisition of additional knowledge regarding the cellular mechanisms and pathway that lead to the production and accumulation of metabolites in plant cells. The many chemical elements of medicinal plants and endophytes have biological properties that can benefit human health. Thus, it is recommended that metabolites from the propagated medicinal plants and endophytes should be examined for their ability to control different ailments in an in vitro investigation.

8.4. LIMITATIONS OF THE STUDY

Even though it has been noted that the flowering stage of medicinal plants is crucial and significantly affects the synthesis of metabolites and the medicinal plants in this study were collected during their flowering time because it is anticipated that metabolite production will be at its peak. Medicinal plant metabolite synthesis might vary by season, and this study only focused on the flowering season of these plants. A comparison of the seasonal variations in the metabolite of *A. afra* and *L. frutescens* would be perfect for future research. On the other hand, even though this study's third chapter demonstrated the correlation between endophytes and medicinal plants. Identification of genetic materials between endophytes and plants would be important to reveal methods used by both plants and endophytes to promote plant metabolic production throughout their interaction.

Even though NMR is excellent for in-depth structural analyses of unidentified molecules, as this work demonstrated. One of the limitations of this study was the analysis of individual compounds, which may be accomplished with the use of GC and LC-MS. While NMR is non-destructive, provides richer structural information, and requires less energy, LC-MS and GC-MS have superior sensitivity for detecting low concentrations and are best at separating complex mixtures with high throughput. They are also suitable for the study of volatile and non-volatile compounds, providing a robust analytical platform for such compounds. It can also be noted that, optimal metabolic analysis should include (i) metabolic profiling, which identifies related groups of compounds both qualitatively and quantitatively; (ii) metabolic fingerprinting, which concentrates on sample analysis; (iii) metabolomic analysis, which entails both qualitative and quantitative analysis of all metabolites present in the samples; and, finally, (iv) targeted compound analysis, which focuses on quantifying particular metabolites present in the samples. The combined usage of NMR, GC and LC-MS would be ideal.

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APPENDICES

ANNEXURE A:

SECTION CHAPTER 4

1. Hogland solution

Solution 1

Table 1: Complete Hoagland nutrient solution (1L)

Chemical Compound	Concentration (M)	Weight(g)/L	mL/L for working stock	Final Concentration (mM)
KNO ₃	2	202.2	2.5	5
Ca (NO ₃) ₂ .4H ₂ O	2	472.3	2.5	5
KH ₂ PO ₄ (Ph=6)	1	136.09	1	1
MgSO ₄ .7H ₂ O	2	492.94	1	2
Fe-EDTA*	0.05	2.8	1	50
Micro-nutrients			1	
Total			9 mL/L	
*0.05M Fe-EDTA (200ml): 3.72g DETA in 70 mL double distilled water mixed with 2.78 g FeSO ₄ .7H ₂ O in 70 mL double distilled water and fill it up to 200 mL				

Solution 2

Table 2: Hoagland -Nitrogen nutrient solution (1L)

Chemical Compound	Concentration	Weight(g)/L	mL/L for working stock	Final Concentration (mM)
KSO ₄	0.5	87	10	5

CaCl ₂ .2H ₂ O	2	294.02	2.5	5
KH ₂ PO ₄ (Ph=6)	1	136.09	1	1
MgSO ₄ .7H ₂ O	2	492.94	1	2
Fe-EDTA*	0.05	2.8	1	0.1
Micro-nutrients			1	
Total			16.5 mL/L	
*0.05M Fe-EDTA (200ml): 3.72g DETA in 70 mL double distilled water mixed with 2.78 g FeSO ₄ .7H ₂ O in 70 mL double distilled water and fill it up to 200 mL				

Solution 3

Table 3: Hoagland -Phosphorus nutrient solution (1L)

Chemical Compound	Concentration (M)	Weight(g)/L	mL/L for working stock	Final Concentration (mM)
KNO ₃	2	202.2	2.5	5
Ca(NO ₃) ₂ .4H ₂ O	2	472.3	2.5	5
KSO ₄	1	87	10	10
MgSO ₄ .7H ₂ O	2	492.94	1	20
Fe-EDTA*	0.05	2.8	1	0.1
Micro-nutrients			1	
Total			18 mL/L	
*0.05M Fe-EDTA (200ml): 3.72g DETA in 70 mL double distilled water mixed with 2.78 g FeSO ₄ .7H ₂ O in 70 mL double distilled water and fill it up to 200 mL				

Solution 4

Table 4: Micro-nutrients (1L)

Chemical	Weight(g)/L	Final
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Compound		Concentration (M)
Boric Acid (H ₃ BO ₃)	2.86	0.046
Manganese Chloride (MnCl ₂ .4H ₂ O)	1.81	0.009
Zinc Sulphate (ZnSO ₄ .7H ₂ O)	0.22	7.5X10 ⁻⁴
Copper Sulphate(CuSO ₄)	0.08	3.2X10 ⁻⁴
Molybdic Acid (H ₂ MoO ₄ .4H ₂ O)	0.02	1.11X10 ⁻⁴

2. ANOVA table for effect of endophytes on *Lessertia frutescens* seedling growth after two months

Table 5: ANOVA on the effect of endophytes on *Lessertia frutescens* seedling growth after two months

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	381,0113	127,0038	147,6073	8,24E-14
Error (within groups)	20	17,2083	0,8604		
Total	23	398,2196	17,3139		

Significant level =0.05

3. Section chapter six: ANOVA Tables for the significant effect of endophytes on *Lessertia frutescens* growth in the pot trial experiment

Table 1: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant height

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
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Groups (between groups)	3	1541,7084	513,9028	75,1595	4,59E-11
Error (within groups)	20	136,75	6,8375		
Total	23	1678,4584	72,9765		

Significant level =0.05

Table 2: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant total wet biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	2297,8298	765,9433	764,5689	1,11E-16
Error (within groups)	20	20,036	1,0018		
Total	23	2317,8657	100,7768		

Significant level =0.05

Table 3: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant root wet biomass.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	47,2438	15,7479	105,2131	2,03E-12
Error (within groups)	20	2,9935	0,1497		
Total	23	50,2373	2,1842		

Significant level =0.05

Table 4: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant shoot wet biomass.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	1691,2087	563,7362	774,8925	3,33E-16

groups)			
Error (within groups)	20	14,5501	0,7275
Total	23	1705,7587	74,1634
Significant level =0.05			

Table 5: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant total dry biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	89,8666	29,9555	246,7082	7,77E-16
Error (within groups)	20	2,4284	0,1214		
Total	23	92,295	4,0128		
Significant level =0.05					

Table 6: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant root dry biomass.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	24,6127	8,2042	240,729	4,44E-16
Error (within groups)	20	0,6816	0,03408		
Total	23	25,2944	1,0998		
Significant level =0.05					

Table 7: ANOVA table for the significant effect of endophytes on *Lessertia frutescens* medicinal plant shoot dry biomass.

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	21,2202	7,0734	120,6552	5,59E-13
Error (within groups)	20	1,1725	0,05862		
Total	23	22,3927	0,9736		

Significant level =0.05

4. Section chapter seven: ANOVA tables for effect of endophytes on *Artemisia afra* medicinal plants growth in the pot trial experiment

Table 6: ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant height

Source	DF	Sum of Square	Mean Square	F-Statistic	P-value
Groups (between groups)	3	3501,7484	1167,2495	303,6681	2,22E-16
Error (within groups)	20	76,8767	3,8438		
Total	23	3578,6251	155,5924		

Significant level =0.05

Table 7: ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant total wet biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	1780,2389	593,413	317,0112	0
Error (within groups)	20	37,438	1,8719		
Total	23	1817,6768	79,0294		

Significant level =0.05

Table 8 ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant root wet biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	67,5464	22,5155	230,5576	1,11E-15
Error (within groups)	20	1,9531	0,09766		
Total	23	69,4995	3,0217		

Significant level =0.05

Table 9 ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant shoot wet biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	1180,3512	393,4504	213,2356	2,11E-15
Error (within groups)	20	36,9029	1,8451		
Total	23	1217,2541	52,9241		

Significant level =0.05

Table 10 ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant total dry biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	116,8572	38,9524	450,169	3,33E-16
Error (within groups)	20	1,7306	0,08653		
Total	23	118,5877	5,156		

Significant level =0.05

Table 11: ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant root dry biomass

Source	DF	Sum of Mean	F Statistic	P-value
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		Square	Square		
Groups (between groups)	3	26,0358	8,6786	138,8132	1,48E-13
Error (within groups)	20	1,2504	0,06252		
Total	23	27,2862	1,1864		
Significant level =0.05					

Table 12: ANOVA table for effect of endophytes on *Artemisia afra* medicinal plant shoot dry biomass

Source	DF	Sum of Square	Mean Square	F Statistic	P-value
Groups (between groups)	3	32,933	10,9777	93,0824	6,38E-12
Error (within groups)	20	2,3587	0,1179		
Total	23	35,2917	1,5344		
Significant level =0.05					