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Unravelling the Role of PGPR *"Pseudomonas fluorescens"* in Semi- Arid Soils of the Rio Grande Valley

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UNRAVELLING THE ROLE OF PGPR "*PSEUDOMONAS FLUORESCENS*" IN SEMI-ARID SOILS OF THE RIO GRANDE VALLEY

A Thesis

by

MANDIP TAMANG

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ABSTRACT

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Chapter 1: In this chapter, we have provided a review on the components of rhizosphere engineering and the potential use of PGPR, and its challenges to serve as an efficient component for sustainable agriculture.

Chapter 2: In this chapter, we isolated 35 different strains of a PGPR, *Pseudomonas fluorescens* and characterized various plant growth promoting traits such as production of ammonia, protease, Indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylate deaminase (ACC) deaminase, ammonia, hydrogen cyanide (HCN), and solubilization of zinc and phosphate.

Chapter 3: In this chapter, we tested the influence of plant beneficial soil microbe, *P. fluorescens*, on the growth and development of sunn hemp (*Crotalaria juncea* L., Fabaceae) and sorghum (*Sorghum bicolor* L., Moench).

Chapter 4: In this chapter, we went through the major challenges in application of PGPR and importance of advanced technologies such as meta-proteomics, nanotechnology, and rhizosphere engineering to produce an effective and eco-friendly PGPR.

In summary, our results showed that *P. fluorescens* isolated from sunn hemp can help in plant growth of sorghum under growth chamber, but further research are needed in field conditions.

DEDICATION

I want to dedicate my master's degree to my family. The completion of my degree wouldn't have been possible without their love and affection. This degree's journey had its rough time, but all the support and care from my mom, dad, and brother gave me a strong sense of motivation and enthusiasm to overcome all the difficulties. Thank you for your presence in my normal and academic life, even from a distance of 8300 miles away.

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CHAPTER I

USING PLANT GROWTH-PROMOTING RHIZOBACTERIA AS RHIZOSPHERE ENGINEERING TOOLS: OPPORTUNITIES AND CHALLENGES

Abstract

Recent issues of global warming and rapid population growth have severely affected food production. In addition, excessive use of chemical fertilizers in agriculture has further degraded soil health and fertility. Therefore, various approaches have been implemented to achieve sustainable and eco-friendly agricultural production. Some of the approaches are no-tillage, plantation of cover crops, and application of plant growth-promoting rhizobacteria (PGPR). PGPR are free living soil bacteria known to facilitate and stimulate plant growth either through direct or indirect mechanisms. The direct mechanism involves biological nitrogen fixation, phytohormone production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production, solubilization of phosphate, while the indirect mechanism involves the siderophore and hydrogen cyanide (HCN) production. With the use of modern technologies, there has been significant progress in the understanding of beneficial soil microbes and their mechanism of plant growth promotion, but the functioning of the soil microbes is complex and heavily influenced by the soil environment and the interactions of these microbes with their biotic and abiotic environment remains largely unexplored. In this review, I present the current understanding of the growth and promotion through the direct and indirect mechanism of

plant growth promotion - the beneficial soil microbes, PGPR their potential in rhizosphere engineering and the challenges in doing so.

Introduction

The increase in global demand for food has led to excessive use of chemical fertilizers and pesticides. However, plants utilize only one-fifth of the total fertilizers applied (Hakim et al., 2021) and half of the applied fertilizers are wasted and retained in the environment (Fageria, 2014; Hakim et al., 2021). While the use of agrochemicals has caused an increase in crop yield, it has resulted in a significant decline in soil health, particularly the soil microbial community. Intensive farming practices such as tillage and use of synthetic fertilizers and pesticides have shifted the composition as well as functioning of the soil microbes, particularly the bacteria in the rhizosphere which promote plant growth and defense against biotic and abiotic stress along with nutrient cycling (Verdenelli et al., 2019).

With the increasing concern over the excessive use of agrochemicals and sustainability of agroecosystems, sustainable agriculture practices have garnered considerable interest. Several strategies such as rhizosphere management (Zhang et al., 2010), fertilizer management (Panhwar et al., 2018), and integrated nutrient management (Rakshit et al., 2015) have been adopted to ensure the nutrient use efficiency (NUE) in crops. A new strategy in agriculture is a 4R strategy; the right source of nutrients at the right time, the right rate, and the right place (Gao et al., 2018; Hakim et al., 2021). The 4R strategy improves crop production enhancing crop nitrogen use efficiency (NUE: gain of nitrogen by crops per unit of available nitrogen in the soil) in the intensive small farming system (Li et al., 2019).

This R4 approach could also be applied in rhizosphere engineering using biofertilizers, which have gained considerable interest among scientists and growers. There are some

challenging aspects in managing the rhizosphere's microbiome but commercial biofertilizers have the potential to alter the soil microbiome in eco-friendly and sustainable way (Bhattacharyya et al., 2020; Hakim et al., 2021). Inoculating crops with the right microbe strain at the right time, right place, and the right amount (4R) can provide the plant's defense against biotic and abiotic stress and improve crop growth and yield (Hakim et al., 2021). Biofertilizers consist of living microorganisms that interact with the plant's rhizosphere or endosphere and promote plant growth through improving soil fertility and stimulating nutrient uptake to increase yield (Mącik et al., 2020). Nitrogen-fixing bacteria, arbuscular mycorrhizal fungi (AMF), and plant growth-promoting rhizobacteria (PGPR) are considered important in promoting plant growth and stress tolerance (Fasusi et al., 2021). This review will focus on the plant-soil feedback, particularly the plant growth-promoting rhizobacteria (PGPR) and the potential of using PGPR as a rhizosphere engineering (RE) tool and its some challenges.

Plant-soil feedback

Soil is an essential environmental medium shared by both plants and microbes; they constantly interact with each other (Hernandez et al., 2021). Plant and microbes interact in three different ways: positive (mutualistic), negative (antagonist, pathogens/parasites), and neutral interactions (Ali et al., 2017). Microbes interact positively with plants through facilitation in nutrient uptake, biological nitrogen fixation, phosphorus solubilization, siderophore production, induction of tolerance against biotic and abiotic factors, antagonism against different plant pathogens, and priming of plant defense (Ali et al., 2017; Jung et al., 2012). Microbes such as plant-parasitic fungi and nematodes have a detrimental effect on plants (Verbeek et al., 2016).

Although very rare and less in number, soil fungal and bacterial pathogens cause various diseases in humans (Ali et al., 2017). The soil microbial community is complex and heavily

influenced by the soil biotic and abiotic factors such as aboveground plant cover, soil structure, pH, nutrient concentration, soil organic matter, moisture, temperature, etc. These microbes are also reported to interact with each other resulting in complex soil food webs (Singh et al., 2009). The soil microbial community changes rapidly with the changes in the environment. Proper management of the soil in agricultural systems can assist in promoting soil microbial community and enhancing their benefits to plants. One of the important components of soil microbial community are plant growth promoting rhizobacteria (PGPR). PGPR are the group of bacteria found in rhizosphere of the plant which helps in better growth and promotion of plant (Nelson, 2004). While the technological advances have transformed our understanding of soil microbes, the mechanisms of these interactions and the processes driving the interactions of these microbes remain largely unknown. Furthermore, most of our understanding of these microbes is based on greenhouse which do not match field conditions and tend to exaggerate or underestimate the plant-microbe interactions. Further site-specific studies are necessary to determine the influence of environmental factors in the structure and functioning of PGPR and their influence on plant growth.

Mechanism of plant growth promoting rhizobacteria (PGPR) on plant growth and promotion

PGPR promote plant growth through direct and indirect mechanisms (Glick, 1995). Direct mechanisms include employment of bacterial traits that lead to direct plant growth by either assisting in resource acquisition (nitrogen, phosphorus, and essential minerals) or modulating plant hormone levels such as the production of auxin, ACC deaminase, cytokinin, gibberellin (Glick et al., 1999). The indirect mechanisms include the bacterial traits that inhibit the functioning of plant pests and pathogens through the production of antibiotics, Iron chelating siderophores, plant pathogens enzymes, cyanide, induced systemic resistance, and quorum

quenching (Bhattacharyya & Jha, 2012; Jha & Saraf, 2015; Olanrewaju et al., 2017). Not all PGPR have all these traits, however different strains of PGPR can possess more than one trait. These traits in PGPR are heavily influenced by environmental factors.

Direct mechanism

Indole-3-acetic acid

Auxin, often interchanged by IAA, is an important phytohormone that helps in the overall growth and promotion of plants (Yousef, 2018). Around 80% of rhizosphere microbes are known to produce and excrete IAA as a secondary metabolite (Patten & Glick, 1996). IAA production helps in the development of root system, which improves the water uptake of the plant, ultimately leading to better plant growth and promotion (Tsavkelova et al., 2007). IAA includes the cell elongation with increasing osmotic content of cell, synthesis of protein, and cellular components (Mohite, 2013). Furthermore, IAA are involved on promotion of seedling growth, inhibition or delay on abscission of leaves and stimulation of flowering, and fruiting (Zhao, 2010).

Pseudomonas fluorescens and *Pseudomonas putida* isolated from *Triticum* spp. rhizoplane and rhizosphere from Africa-Algeria produced 89µg/ml and 116 µg/ml IAA respectively in invitro and both were also able to solubilize phosphate (Meliani, 2017). Furthermore, inoculation of Lentil (*Lens culinaris*) and Barley (*Hordeum vulgare*) seeds with *P. fluorescens* and *P. putida* increased the rate of germination, seedling growth, and vigor index under the same gnobiotics conditions in comparison to control (Meliani, 2017). *P. fluorescens* and *Azospirillum brasilense* isolated from bright eyes or Cape periwinkle (*Catharanthus roseus*) from India were able to produce IAA either individually or in combination (Karthikeyan et al., 2009). Seed treatment of ‘rosea’ and ‘alba’ variety of *Catharanthus roseus with P.*

fluorescens and Azospirillum brasilene individually and with the combine treatment of *P. fluorescens and Azospirillum brasilene* exhibited positive effects on plant height and root length over control. Similarly, the combined inoculation exhibited maximum ajmalicine content in 'rosea' variety on 90 DAP (Karthikeyan et al., 2009).

Nitrogen fixation

Nitrogen is the most essential nutrient required for the proper growth and development of plants (Bano et al., 2016). It is required for the production of amino acids which are essential for the photosynthesis (Bano et al., 2016). Even though earth has 78 % nitrogen content, it is not easily available to the plants (Raymond et al., 2004). Plants can only utilize nitrogen in the form of ammonium (NH_4^+) and nitrate (NO_3^-) (Hakim et al., 2021). Plants get the nitrogen from the atmosphere through natural nitrogen fixation (Raymond et al., 2004). Meanwhile, plants also acquire nitrogen from microbes through the biological nitrogen fixation (BNF) process, where the microbes fix the inert nitrogen (N_2) gas and make it available to plant in its useable form (Dixon & Kahn, 2004).

P. fluorescens is known to produce nitrogen in the form of ammonia. All 12 strains of *P. fluorescens* isolated from the rhizosphere of fava bean (*Vicia faba* L.) exhibited production of siderophore, IAA, HCN, and ammonia (Alemu & Alemu, 2015). Seed bacterization with *P. fluorescens* increased fava bean leaves number, branches number, height, root length, lateral roots, and the number of nodules per plant (Alemu & Alemu, 2015). Bacterization of white radish (*Raphanus sativus*, cv. L) with *Bacillus subtilis* and *P. fluorescens*, obtained from the microbial culture collection at faculty of Agriculture, Ain Shams University, Egypt resulted significant increase in fresh and dry masses of root and leaves, photosynthetic pigments, proline, total free amino acids, and crude protein contents as in comparison to non-inoculated seeds under

saline conditions (Mohamed & Gomaa, 2012). Both strains of bacteria also produced IAA and GA3 contents along with increment in the content of N, P, K^{+}, Ca^{+2} and Mg^{+2} but the level of ABA and Na^{+} and Cl^{-} were reduced (Mohamed & Gomaa, 2012).

ACC-deaminase production

Ethylene, a gaseous hormone produces in the plant by its precursor, ACC is involved in various development and physiological process in plants like fruit ripening, tissue differentiation, breaking seed dormancy, synthesis of anthocyanin, leaf and flower senescence, root hair formation, and volatile compounds production (Abeles, 1992; Bleecker & Kende, 2000; Frankenberger & Arshad, 2020; Spaink, 1997). Under normal condition, the plant produces a minimum level of ethylene required for the development of plants, but during abiotic and biotic stress condition plant produces more ethylene from its precursor ACC, and higher accumulation of ethylene can inhibit root growth and its associates' metabolism and may cause senescence in crop plants (Sheehy et al., 1991).

Various PGPR strains have an enzyme known as ACC deaminase (Glick et al., 1998) that can degrade ACC into alpha ketobutryate and ammonia and therefore, decreases the level of ethylene in seedling and stressed plants (Mayak et al., 2004). PGPR having ACC deaminase enzymes enhance the survival of developing seedling by facilitating the formation of the longer root cells under biotic and abiotic stress (Grichko & Glick, 2001). Hence, PGPR having ACC deaminase property cleave ACC and reduce the level of ethylene under abiotic and biotic stress, and lowers the damage to the plant (Saravanakumar & Samiyappan, 2007).

P. fluorescens, isolated from the plant rhizosphere, exhibiting ACC deaminase activity have increased the plant growth and development in stress conditions. Application of ACC deaminase producing strains of *P. fluorescens* and *P. putida* isolated from soil samples from Iran

improved the germination and seedling growth of canola (*Brassica napus* L.) under saline conditions (Jalili et al., 2009). Meanwhile, the treatment of genetically modified ACC deaminase producing *P. fluorescens* with the canola (*Brassica campestris* cv. Express) improved the plant's growth under drought conditions (Wang et al., 2000). Whereas the treatment of *P. Syringae* and *P. fluorescens* strains known for producing ACC deaminase, obtained from Soil Microbiology and Biochemistry Section, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan, significantly improved the yield of maize (*Zea Mays* L.) when applied without mineral fertilizer and promising results were obtained when they both were applied with the mineral fertilizers (Zafar-ul-Hye et al., 2014).

Phosphate solubilization

Phosphorous is the second limiting nutrient for the plant after nitrogen (Browne et al., 2009). Phosphorus (P) is a major macronutrient required for plant growth and development, but because of its low solubility and fixation in the soil, P is not readily available to the plant (Fernández et al., 2007; Vyas & Gulati, 2009). Phosphorous is frequently immobilized and is found in other impure forms such as aluminum and iron/oxides/hydroxides, insoluble Ca-P, Fe-P and Al-P (Richardson, 2001). Plants cannot utilize these insoluble phosphate forms, and therefore the total yield of crops is usually low (Browne et al., 2009).

P. fluorescens has been reported to solubilize the phosphate and help plant in growth and development. Bacterial strains *P. fluorescens* and *Bacillus megaterium* isolated from soil samples of India were found to solubilize phosphate and combine inoculation of these 2 PSB increased radical and plumule length of chickpea (*Cicer arietinum*) as in comparisons to individual PSB bacterial strain (Sharma et al., 2007). (Schoebitz et al., 2013) performed quartz sand potted experiments where the combined treatment of immobilized *P. fluorescens* C139,

originally isolated from the rhizosphere of wild blackberry (*Rubus ulmifolius*), and 3.25 ppm of inorganic phosphate increased P uptake up to 62% by the wheat (*Triticum aestivum*) plant after 60 days of treatment.

Zinc solubilization

Zinc (Zn) is a micronutrient required for plants in a minimal amount for its proper growth and development (Mousavi, 2011). Zinc plays important role in nitrogen uptake and metabolism, synthesis of protein and chlorophyll components, and gene expression (Mousavi, 2011). Mainly, zinc is found in various insoluble forms in the soil in the form of zinc sulphate and oxides (Hakim et al., 2021; Shaikh & Saraf, 2017). Zinc deficiency leads to yellowing of plants, small leaves, and retarded shoot growth (Mousavi et al., 2011). Soil contains minimal amount of Zn^{+2} cation form, which can be utilized by plants (Zlobin, 2021).

PGPR have potential to solubilize the insoluble form of zinc and made it available to the plant in plant available form. (Sirohi et al., 2015) reported the isolation of zinc solubilizing strains of *P. fluorescens* from the rhizosphere of black gram (*Vigna mungo).* In this study, the inoculation of zinc solubilizing, *P. fluorescens* facilitated the increase in the growth and productivity of wheat (*Triticum aestivum* var. HD2851) crop in a zinc-deficient soil.

Indirect mechanism

Hydrogen cyanide (HCN)

HCN is a secondary volatile metabolite which is produced by rhizobacteria (Abd El-Rahman et al., 2019). HCN is produced by bacteria when a membrane-bound flavoenzyme HCN synthase oxidizes glycine (Castric, 1977). HCN accounts for selective advantages, biocontrol of pathogens, to its producers through the inhibition of the plant bacterial disease (Lanteigne et al., 2012). *P. fluorescens* isolated from the plant rhizosphere are known to produce HCN and

exhibit biocontrol activity against various plant pathogens. *P. fluorescens* strains isolated from the rhizosphere of sunflower (*Helianthus annus* L.) from India produced HCN in both qualitative and quantitative assays as a secondary metabolite (Reetha et al., 2014).

Siderophore production

Iron (Fe) is essential in a living organism for cellular mechanisms such as transportation, storage and activation of molecular oxygen, ribonucleotides and dinitrogen reduction, peroxide activation and decomposition, transportation of electron with the use of electron carriers (Katiyar & Goel, 2004). Iron (Fe) is generally found in soil in the crystalline and amorphous iron oxides and Fe minerals form (Alexander & Zuberer, 1991b). Solubilization/dissolvement of these Fe minerals and oxides forms give rise to Fe^{+2} and Fe^{+3}, which can be assimilated/utilized by the plant (Alexander & Zuberer, 1991b). But Fe present in earth crust is largely unavailable for microbial and plant assimilation (Gull, 2012) as Fe is mostly present in an insoluble Fe (III) mineral form under the aerobic conditions at neutral and alkaline pH and plants which are primarily dependent on Fe from the soil are susceptible to the Fe-deficiency (Alexander & Zuberer, 1991b).

Microorganisms have developed strategies to acquire iron under low iron availability conditions, one of the strategy involved is the production of non-ribosomal peptides having low molecular weight compounds called siderophore, which have a high affinity for Fe and acquires the iron through the chelation of ferric iron by siderophore under Fe deficiency conditions (Alexander & Zuberer, 1991a). Siderophore produce by rhizobacteria can enhance plant growth either by providing Fe as a nutrient source to the plant or inhibiting the colonization or attack of plant pathogens (Crowley, 2006).

P. fluorescens collected from biofertilizer resource center (BIRCEN) culture collection, Pakistan was able to produce siderophore (Gull, 2012). *P. fluorescens* was very effective against *Rhizoctonia Solani* root rot disease as it resulted in a 70% reduction in disease in the wheat (*Triticum aestivum* cv. INQ-91) plant. Among many other antifungal agent productions, siderophore production was the key factor for root rot disease suppression (Gull, 2012). Siderophore producing *P. fluorescens* are also known to have bio fertilizing potential. *P. fluorescens* strain, a cold-resistant mutant was developed, and under in-vitro conditions, this strain produced almost 17-fold more siderophore than its wild type (Katiyar & Goel, 2004). Seeds treated with this strain increased 28% shoot length and 35 % root length on mung bean (*Vigna Radiata* L. Wilzeck var.PM-4).

PGPR and its role in agriculture and ecosystem sustainability

PGPR play a significant role in agriculture as it has been applied as biofertilizer, phytostimulator, biopesticides, and bioremidators (Prasad et al., 2019). PGPR facilitate the growth and development of the plant as they biologically fix nitrogen (Hayat et al., 2012), produce siderophore (Gupta & Gopal, 2008), and solubilize phosphate and zinc (Gontia-Mishra et al., 2017). Meanwhile, PGPR also produces phytohormones such as IAA, cytokinin, and gibberellin and function as a plant growth stimulator (Maheshwari et al., 2015). In addition, PGPR produce hydrogen cyanide (HCN), antibiosis, and enzymes and help in plant protection from several plant pathogens (Siddiqui, 2006). PGPR are also known to remove the heavy metal contamination from soil though the production of siderophores (Prasad et al., 2019). Hence, PGPR exhibits biofertilizing and biocontrol properties and promote plant growth and development. Its application has increased beneficial microbes around the roots, which have improved the soil health and fertility and resulting in less use of chemical fertilizers (Prasad et

al., 2015). Overall, there are an increasing number of studies reporting that the application of PGPR has helped in achieving eco-friendly and sustainable agriculture.

Current challenges and future perspective

While there is huge potential for PGPR application in agriculture, there are several challenges such as microbial screening, PGPR's efficacy, marketing, and commercialization (Kumari et al., 2019). Moreover, research on PGPR is limited mostly to laboratory and greenhouse, but the field soil environment conditions cause a significant impact on the structure and functioning of these microbes. Hence, PGPR research needs to be performed in field conditions to test its true efficacy. Nevertheless, PGPR will play a crucial role in higher agriculture production in the near future. With the recent development in the field of genetics, molecular biology, nanotechnology, meta-proteomics, and rhizosphere engineering, there is huge potential for developing eco-friendly and efficient PGPR (Hakim et al., 2021). Particularly nanotechnology has enormous potential to be used as nano-fertilizers and biocontrol agents. However, nano-product still needs to be cost-effective and of acceptable quality (Prasad et al., 2019). Therefore, further studies are needed to overcome the current challenges and develop eco-friendly and efficient PGPR.

Figure

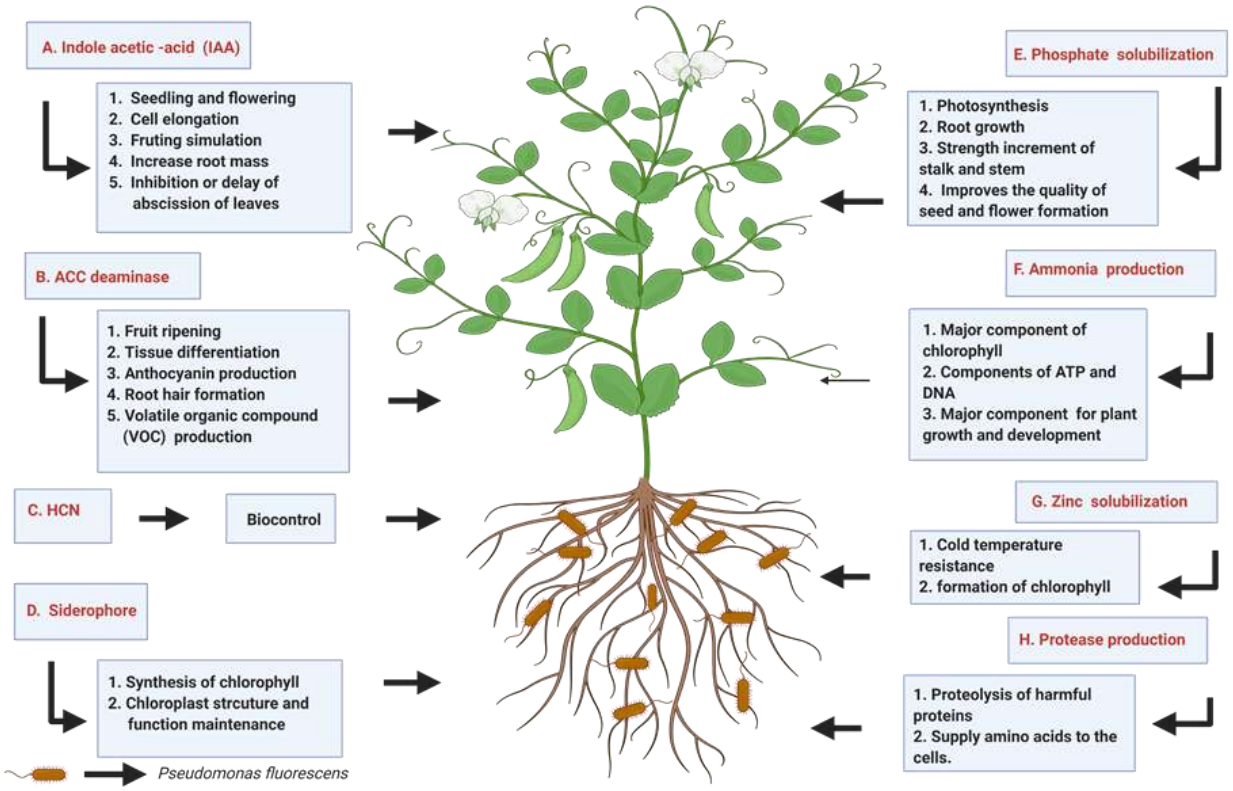

Figure 1: Phytohormones production by *Pseudomonas fluorescens* and its role in plant growth and development.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF A PGPR, *PSEUDOMONAS FLUORESCENS,* IN SEMI-ARID SOILS OF THE RIO GRANDE VALLEY

Abstract

Pseudomonas fluorescens is a gram-negative, plant growth-promoting rhizobacteria (PGPR) that colonizes the rhizosphere of many plants and facilitates plant growth and promotion through symbiotic interactions with plants. In the last decade, there has been a growing interest in the application of *P. fluorescens* in agriculture across US (United States). However, little work has been done on the microbial functional diversity of the semi-arid soils of South Texas. In this study, we characterized *P. fluorescens* from the rhizosphere of plant sunn hemp (*Crotalaria juncea* L., *Fabaceae*), a warm-season annual legume grown in semi-arid soil of sub-tropical south Texas (Rio Grande Valley). The study site was divided into 2 regions based on the high and low canopy coverage (CC) of sunn hemp. A total of 35 isolates, 17 from high and 18 from low, of *P. fluorescens* were isolated and screened for potential biofertilizer and biocontrol traits such as the production of indole acetic acid (IAA), hydrogen cyanide (HCN), siderophore, ammonia, protease, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and solubilization of phosphate and zinc. Our result showed that out of 35 strains of *P. fluorescens,* 28 strains (80%) were positive for ammonia production, 25 (77%) strains were positive for HCN production,

strains (37%) were able to produce ACC deaminase, 21 strains (60%) were positive for protease and 22 strains (62%) were positive for phosphate solubilization. All 35 strains produced siderophore and IAA, while none of the strains were able to solubilize zinc. This study will provide an important step towards understanding the functional fingerprinting of *P. fluorescens* in plant growth and promotion in Rio Grande Valley.

Introduction

Agriculture production is highly dependent on climate. Climatic variables such as temperature and rainfall (timing and amount) are major risk factors in agriculture and food production systems, especially in the arid and semi-arid regions (Mongi et al., 2010). Crops have an optimal temperature, salinity, and moisture requirements for growth and productivity and have limited adaptability against abrupt changes (Shinde, 2019). Any changes in solar radiation, temperature, saline conditions, and precipitation will give rise to various environmental stress and causes a significant negative impact on crop yield (Mongi et al., 2010; Salinger et al., 2005).

Farmers have been adopting multiple sustainable practices to improve resilience against climate stress (Salinger et al., 2005). The most commonly adopted practices include reduced use of agrochemicals (Bhandari, 2014), crop rotation, cover cropping (Fageria et al., 2005), reduced and no-till (Madari et al., 2005), and improving soil health to promote beneficial soil microbes (Hobbs et al., 2008). Among standard practices, the application of plant growth-promoting rhizobacteria (PGPR) for gaining maximum yield has been widely studied (Beneduzi et al., 2008; Gururani et al., 2013; Jeon et al., 2003; Ramamoorthy et al., 2001; Vacheron et al., 2013). Plant growth-promoting rhizobacteria (PGPR) are plant beneficial bacteria found in the rhizosphere of the plant which helps in plant growth and development through root colonization (Bhattacharyya & Jha, 2012). PGPR are known to be environmentally friendly and are a better

alternative than synthetic chemicals as PGPR have no detrimental effects on the environment and have the potential to achieve sustainable agriculture in arid and semi-arid regions (Gouda et al., 2018; Grobelak et al., 2018; Nagargade et al., 2018; Shrivastava & Kumar, 2015). Common PGPR genera exhibiting plant growth-promoting activity are *Pseudomonas, Rhizobium, Erwinia, Mycobacterium, Azospirillum, Burkholdaria, Enterobacter, Azotobacter, Bacillus, Mesorhizobium, and Flavobacterium* (Singh, 2013).

Of the different PGPRs, *P. fluorescens* is a well-known and extensively studied PGPR due to its unique bio-fertilizing properties (Åström & Gerhardson, 1988; Bhattacharyya & Jha, 2012; Fouzia et al., 2015; Jain et al., 2013; Shrivastava & Kumar, 2015). Several plant growth traits of *P. fluorescens,* isolated from different plant rhizosphere from different parts of the world, have been well described before (Fouzia et al., 2015; Hafeez et al., 2006; Hakim et al., 2021; Niu et al., 2018; Reetha et al., 2014). *P. fluorescens* improve plant growth and development through various mechanisms such as enhancement of the nutrient status of host plants by biological nitrogen fixation, increasing the availability of nutrients in rhizosphere, production of volatile growth stimulants, and production of various biocontrol agents and plant hormones (Banchio et al., 2008).

P. fluorescens can be reintroduced/promoted in agricultural fields in different ways such as the use of commercial biofertilizers (Hien et al., 2014), farm management practices such as reduced tillage (Guerrieri et al., 2020), and adding different soil amendments to promote native populations of *P. fluorescens* (Kokalis-Burelle et al., 2006). In addition, incorporating green manure and cover cropping is also reported to promote *P. fluorescence* in soil (Guerrieri et al., 2020). The goal of this study was to evaluate the *P. fluorescence* in the rhizosphere of a widely used summer cover crop, sunn hemp (*Crotalaria juncea* L., Fabaceae), from a certified organic

grain farm in subtropical south Texas, the Lower Rio Grande Valley (LRGV). LRGV is one of the largest vegetables growing regions in Texas. In addition, grain sorghum, citrus, pecan, alfalfa, and cotton are also grown in this region (Samani et al., 2009). The LRGV experiences a warm and hot climate during the summer and occasionally freezing climate during winter (Adhikari & White, 2014). Soils here have high pH and salinity, and poor nitrogen and soil organic matter. Due to extensive agricultural practices, soil health in this region is reported to be highly degraded with poor soil microbial community. Specific objectives of this study were to isolate different *P. fluorescence strains* and characterize different plant growth promoting traits.

Materials and methods

Sampling site

Study site and soil sample collection

Plants and rhizospheric soil samples of sunn hemp were collected from a certified organic dryland grain system field in semiarid subtropical, Texas (26.3685° N, 97.9177° W) in October 2019 (Figure 1A). The LRGV has a semi-arid subtropical climate with long and hot summer with average high temperatures of 35°C, whereas winters are mild with only occasional frost or freezing (Adhikari & White, 2014). The rainfall occurs mostly in September and October, with an average precipitation of 68.2 cm (Eddy & Judd, 2003). This region is in subtropics and are prone to lesser precipitation and higher temperatures in the future (Hernandez & Uddameri, 2014). Besides, soils have high pH and salinity, and low nitrogen content.

Sample collection and Isolation of *P. fluorescens*

Rhizospheric soil samples were collected from healthy sunn hemp based on their canopy coverage percentage of high (30-60%) and low (5-29%) in October 2019. A suspension of rhizosphere sunn hemp soil was obtained by shaking/rinsing a small piece of root in 2 ml of

Luria broth (LB). After a series of serial dilution, 40 µl of the suspension was spread into the King's B media (KB) agar plates (King et al., 1954), and incubated at 28^0C for 48 hours (Figure 1B). Bacterial colonies were observed under the UV light (366 nm). Green fluorescence-producing colonies were picked and streaked on KB agar plates for obtaining pure cultures. Thirty-five isolates/strains of *P. fluorescens* were isolated on (KB) media and purified on KB agar plates by repeated sub-culturing. All the strains were stored in Luria-Bertani (LB) broth containing 80% (w/v) glycerol and stored at -80°C until further use.

Functional fingerprinting of *P. fluorescens*

Ammonia detection

Production of ammonia by *P. fluorescens* was detected in peptone water broth (peptone–10g/L; NaCl–5g/L) with the addition of Nessler's reagent. Bacterial cultures were inoculated in 5 ml peptone water followed by incubation period of 72 h at 28^0C. The development of yellow to brown color after the addition of 0.25 ml Nessler's reagent indicated ammonia production (Cappuccino & Sherman, 1992; Gupta & Pandey, 2019).

HCN production

Production of HCN was studied on KB medium amended with 0.4 % (W/V) glycine. A Whatman filter paper was placed on the upper lids of the petri plates after it was treated with an alkaline picric acid solution (2.5 % picric acid in 2% Na_2CO_3) followed by incubation of the plates for 4 days at 28^0C. A color changed of the filter paper from yellow to red-brown was considered as HCN producing bacteria (Castric, 1975).

Siderophore production

Previously, siderophore production was determined either using the $FeCl_3$ test or the chrome azurol S agar (CAS) assays (Naik et al., 2008). However, in our present study, all the bacterial strains were screened for the siderophore production on *P. fluorescens* by using a cetrimide agar medium (Rachid & Ahmed, 2005). Bacterial colonies producing the green fluorescent pigment under UV light were considered as siderophore-positive strains.

Phosphate solubilization test

Solubilization of phosphate determined by single strain was streaked on the Pikovaskya's agar medium supplemented with 2 % (w/v) tricalcium phosphate (TCP) and incubated at 28^0C for 4 days (Gupta & Pandey, 2019). Strains that developed a clear zone around the colonies were considered positive (Gupta & Pandey, 2019; Katznelson & Bose, 2011).

Protease production

The determination of casein hydrolysis activity was estimated from clearing zones in skim milk agar (5.0 g pancreatic digest of casein, 2.5 g yeast extract, 1.0 g glucose, 7% skim milk solution, and 15 g agar per liter of distilled water). Bacterial strains were spot inoculated on skimmed milk agar and incubated at 28^0C. After 2 days, proteolytic activity was confirmed positive with a clear zone formation around the bacterial colony (Naik et al., 2008; O'Sullivan et al., 1991).

Zinc Solubilization

Zinc solubilizing capacity of *P.fluorescens* was determined using Tris-minimal medium (Tris–HCl 6.06 g; NaCl 4.68 g; KCl 1.49 g; NH_4Cl 1.07 g; Na_2SO_4 0.43 g; $MgCl_2.2H_2O$ 0.2 g; $CaCl_2.2H_2O$, 30 mg, pH 7.0; amended with 1.5% agar and 0.1% (w/v) insoluble zinc sulfate

($ZnSO_4$) per liter of distilled water) (Gupta & Pandey, 2019). The plates were incubated for 14 days at 28^0C. Halo zones formation around the bacterial colonies indicated the solubilization of zinc.

IAA production

For the determination of IAA production, bacterial isolated were inoculated in LB broth amended with 5 mM tryptophan and incubated in an orbital shaker at 200 rpm and 28°C for 7 days. Development of red color confirmed the IAA production with the addition of Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution) and cell-free supernatant (in the ratio of 4:1 and measured by UV-vis spectrophotometer at 530 nm (Chrastil, 1976; Gupta & Pandey, 2019).

ACC deaminase production

Quantification of ACC deaminase was evaluated by reliable colorimetric ninhydrin assay using heat-resistant polypropylene chimney-top 96-well PCR plates (Li et al., 2011). A single bacterial colony was inoculated into 1.5 ml of liquid LB medium and incubated at 28^0C for 24 hours on a shaker at 200 rev min^{-1}. 1.5 ml of each culture was transferred into a new 1.5 ml microcentrifuge tube by centrifugation at 8000 g for 5 min. The cell pellet was washed with 0.5 ml of liquid Dworkin and Foster (DF) medium and then suspended in 1 ml of DF-ACC medium in a 1.5 ml microcentrifuge tube and incubated for 24 hours at 28^0C on a shaker at 200 rev min^{-1}. A 1.5 ml DF-ACC medium without inoculation was also incubated in parallel and later was used as control. One ml of each culture was centrifuged in a 1.5 ml microcentrifuge tube at 8000 g for 5 min which was followed by dilution of 100 µl supernatant in 1 ml of liquid DF medium in a 1.5 ml microcentrifuge tube. After this, 60 µl of supernatant obtained from dilution was used for

the ninhydrin-ACC assay in 96 well plates. Then 100 µl of the remaining reaction solution was transferred and the absorbance of microplate wells was measured at 570 nm with the Bio-Rad model 680 microplate reader. A bacterial isolate having a lower absorbance of supernatant compared with that of the DF-ACC medium without bacterial inoculation was regarded as an ACC-utilizing bacterial isolate (Li et al., 2011).

Results

P. fluorescens isolation

A total of 35 strains of *P. fluorescens* were isolated from sunn hemp rhizosphere soil samples, collected from a field (15 acres) located at Hilltop Gardens, Lower Rio Grande Valley, Texas. Based on the canopy coverage of sunn hemp, we divided the field into the high and low regions (Fig. 2A). Out of 35 isolates, 17 were obtained from high and 18 from low canopy coverage regions of sunn hemp. All 35 strains were identified by their intrinsic fluorescence under the UV light and were streaked on KB agar plates for obtaining pure cultures (Figure 2B).

Functional diversity of biofertilizing *P. fluorescens*

Ammonia production

Ammonia is a macronutrient and directly helps in plant growth and development (Bhattacharyya & Jha, 2012). Ammonia is a major component of chlorophyll and improves fruit and seed production (Markou et al., 2016). In order to evaluate whether 35 strains of *P. fluorescens* from high and low regions produced ammonia, we performed an ammonia production assay. The development of yellow to the brown color in the presence of Nessler's reagent indicates ammonia positive strain (Figure 3). A total of 28 (80%) strains were positive for ammonia production. Based on the sunn hemp canopy coverage (CC), 14 (82%) strains from high CC and 14 (77%) from low CC were tested positive for ammonia production (Table 1).

HCN production

To determine *P. fluorescens* ability to produce HCN, we performed a HCN production assay in KB medium supplemented with 0.4 % glycine. The HCN production by strains was confirmed after observing a change in the color of the filter paper from yellow to red brown (Figure 4). A total of 27 (77%) strains of *P. fluorescens* were able to produce HCN. Among them, 12 (70%) strains from high CC, and 15 (83%) from low CC were tested positive for HCN production (Table 1).

Siderophore production

Siderophore-producing potential of 35 strains of *P. fluorescens* was carried out in a cetrimide agar medium. All strains (100%) were positive for the production of siderophore on the basis of the production of green fluorescent pigment under UV light (302 nm and 366 nm) (Figure 5). Among them, 17 (100%) strains from high CC and 18 (100%) CC from low were tested positive for siderophore production (Table 1).

Phosphate solubilization

Phosphorus (P) is an essential macronutrient necessary for proper development of plant (Schoebitz et al., 2013). P helps in plant root development, maturity and improves growth in cold temperature. To determine whether 35 strains of *P. fluorescens* were capable of inducing phosphate solubilization, we conducted phosphate solubilization assay in Pikovskaya's medium supplemented with 2% TCP. A total of 22 (62%) strains solubilized phosphate after a clear zone of phosphate solubilization was formed around the bacterial colonies (Figure 6). Among them, 12 (70%) strains from high CC and 10 (55%) CC from low were tested positive for phosphate solubilization (Table 1).

Protease production

To evaluate *P. fluorescens* ability to produce protease, we performed protease production assays in a skimmed milk agar plate. A total of 21 (60%) strains were able to produce protease, which was confirmed by the formation of a clear zone around the bacterial colony (Figure 7). Among them, 11 (64%) strains from high CC and 10 (55%) from low CC were tested positive for protease production (Table 1).

Zinc solubilization

The zinc solubilizing potential of 35 strains were assessed in the Tris-minimal medium. Interestingly, all the 35 strains were unable to solubilize the zinc sulfate ($ZnSO_4$), which was confirmed by the lack of halo zone formation around the bacterial colonies (Figure 8). Based on the sunn hemp's canopy coverage (CC), none of the strains tested positive for zinc solubilization (Table 1).

IAA production

IAA is an important phytohormone for plants, and its production by PGPR play a vital role in the facilitation and stimulation of plant growth and promotion (Kochar et al., 2011; Mohite, 2013). IAA production promotes the length of plant roots with the increased number of root hairs and helps in better uptake of nutrients from the soil (Ashrafuzzaman et al., 2009). To evaluate the IAA producing potential by 35 strains of *P. fluorescens*, we performed IAA production assay. Production of IAA was identified in all 35 (100%) strains by observing the higher absorbance of Salkowski reagent and cell-free supernatant (4:1) than the control (Figure 9). Among them, 17 (100%) strains from high CC and 18 (100%) from low CC were tested positive for IAA production (Table 1).

ACC deaminase production

We used an ACC deaminase production assay to determine the potential of 35 strains of *P. fluorescens* to produce ACC deaminase. The ACC deaminase production was observed in 13 (37%) strains, as shown by the lower absorbance than the control on Dworkin and Foster (DF) minimal salt medium (Figure 10). Among them, five (29%) strains from high CC and eight (44%) from low CC were tested positive for ammonia production (Table 1).

Discussion

In this study, we isolated 35 strains of a particular PGPR, *P. fluorescens,* from the rhizospheric soil of sunn hemp from Hilltop garden, Texas. This is the first report of isolation and PGP traits characterization of *P. fluorescens* from sunn hemp in RGV. Sunn hemp is a cover crop commonly grown in arid and semi-arid regions for better yield of plants, suppression of weeds, and biocontrol of several plant pathogens (Collins et al., 2008). More importantly, it improves the beneficial plant microbes (PGPR) population in its soil, subsequently helping in plant growth and development via the production of various plant growth-promoting (PGP) traits (Mendonça & Schiavinato, 2005). In this work, we characterized eight different plant growth-promoting traits such as the production of ammonia, protease, HCN, IAA, ACC deaminase, and siderophore, and solubilization of zinc and phosphate.

Nitrogen is one of the critical components required for plant growth and development (Dixon & Khan, 2004). The nitrogen-fixing potential is an essential attribute of *P. fluorescens*. Among 35 isolates, all of the strains were able to produce nitrogen in the form of ammonia. Our study was in line with other study (Nehra et al., 2014), which reported the production of ammonia and other several PGPR traits by a single strain of *P. fluorescens* isolated from the rhizosphere of cotton.

Solubilization of phosphate is another crucial trait for plant growth and promotion (Browne et al., 2009). *P. fluorescens* have the potential to solubilize insoluble phosphate and make it readily available for plants. Our study showed a total of 22 (62%) strains of *P. fluorescens* were able to solubilize phosphate in the form of TCP. A similar observation was reported by (Yadav et al., 2016), which showed phosphate solubilization by several *P. fluorescens* (PSM1, PSM2, PSM3, PSM4, and PSM5) strains isolated from the wheat rhizosphere. Likewise, *P. fluorescens* strains (IISR-6, IISR-8, IISR-11, IISR-13, and IISR-51) obtained from root bits of black pepper were able to solubilize phosphate (Diby et al., 2005). Furthermore, these strains increased root biomass, root length, total root area, feeder roots, and nitrogen and phosphorus uptake. Another study by (Yu et al., 2011) showed, *P. fluorescens* W12 along with TCP addition significantly increased plant height, shoot and root dry weight, and nitrogen and phosphorus uptake of walnut seedlings. The co-inoculation of the *P. fluorescens* W12 strain and the other two PSB (*P. chlororaphis*, W24 and *Bacillus cereus*, W9) with TCP addition exhibited the most significant positive effect on the growth of walnut plants (Yu et al., 2011).

Our study revealed all strains (100 %) of *P. fluorescens*, produced IAA suggesting that the rhizosphere of the sunn hemp plant provides a suitable environment for root growth. Meanwhile, sorghum seed inoculated with *P. fluorescens* Psd enhanced the sorghum root growth, which correlated with IAA level produced by *P. fluorescens* Psd (Kochar et al., 2011). ACC deaminase is one of the key enzymes produced by PGPR that facilitates plant growth and development by degrading ACC into alpha ketobutryate and ammonia, which decreases ethylene levels in plants and increases plant tolerance against biotic and abiotic stresses (Raghuwanshi & Prasad, 2018). In the present work, a total of 13 (37%) strains were able to produce ACC

deaminase enzyme. Studies by (Safari et al., 2018) showed that better germination and better growth of wheat under saline conditions with the treatment of four ACC deaminase producing *P. fluorescens* strains (PGU 2-70, WBO-3, WKZ1-93, and WB17a). Several studies also reported the application of ACC deaminase producing *P. fluorescens* TDK1, *P. fluorescens* (B10, B2-10, B2-11, and B4-6), *P. fluorescens* S20, and *P. fluorescens* (KACC10070) improved growth of groundnut (Saravanakumar & Samiyappan, 2007), Barley (Azadikhah et al., 2019), Maize (Nadeem et al., 2009), and Chinese cabbage (Soh et al., 2014) respectively under saline conditions.

Protease production is another essential trait of PGPR as protease plays a crucial role in seed germination, helps in nutrient remobilization, prevents protein misfolding and degradation, and protects plants against various pathogens via triggering of immune response (Martinez et al., 2019). In our present study, a total of 20 (57%) strains were able to produce protease. Likewise, (Timm et al., 2015) reported 11 *P. fluorescens* strains out of 19 strains were positive for protease production. Similarly, *P. fluorescens* strains (UM16, UM240, UM256 and UM270) were able to produce protease along with phenazines, cyanogen, ACC deaminase, IAA, and siderophore (Hernández-León et al., 2015).

Zinc is an essential micronutrient for plant growth as it controls several plant physiological processes (Mousavi, 2011). Zinc solubilizing bacteria provides zinc to the plant and helps in achieving sustainability (Kamran et al., 2017). In our study, zinc solubilizing ability of *P. fluorescens* was determined on the Tris-minimal plates supplemented with insoluble zinc complex, $ZnSO_4$, but none of the 35 strains were able to solubilize the zinc, suggesting that either the sunn hemp plant was having zinc deficiency or, zinc was supplied by other PGPRs.

Siderophore production helps in acquisition of Fe under iron limiting condition as they have high affinity towards the Fe (Mirleau et al., 2000). Our study revealed that all 35 (100%) strains produced siderophore. Our observation was in line with studies by (Naik et al., 2008) which showed all strains (100%) produced siderophore. A study by (Lim et al., 2002) reported that the production of hydroxamate siderophore under iron-limited conditions *by P. fluorescens* GL20. Under iron limiting conditions, GL20 inhibited spore germination and hyphal growth of *Fusarium solani* and enhanced growth of bean plant (Lim et al., 2002).

HCN is a volatile antibody that exhibits the biocontrol property against various plant pathogens (Lanteigne et al., 2012). Our study demonstrates that a total of 25 (71%) strains of *P. fluorescens* produced HCN. Likewise, (Nagarajkumar et al., 2005) reported the production of HCN by 14 strains (100%) of *P. fluorescens* from the rhizosphere of rice. Among them, strain, *P. fluorescens* (PfMDU2) produced the highest HCN and inhibited the mycelial growth of *Rhizoctonia solani*. HCN produced by *P. fluorescens* is also known to have both biocontrol and bio fertilizing properties. Moreover, under the *in vitro* conditions, a specific strain *P. fluorescens* P13 produced HCN and inhibited the mycelial growth of *Sclerotina sclerotiorum*, causal agent of sclerotina stem root (SSR) by 88.45% and also inhibited sclerotium formation by 95-100% (Li et al., 2011). However, HCN production by *P. fluorescens* is also reported to have detrimental effects on the plant. A study done by (Kremer & Souissi, 2001) reported inoculation of three *P. fluorescens* strains (297, 126 and 672) reduced the seedling root length of both lettuce and barnyard grass up to 77.67% and 78.33%, respectively. However, the positive impact on plant growth outweighs the detrimental effect of HCN production.

Taken together, our results suggest that *P. fluorescens* can induce the production of IAA, HCN, ammonia, protease, ACC deaminase, and siderophore and solubilization of zinc and

phosphate, thereby improving growth of plants. The application of *P. fluorescens* as inoculants biofertilizers may serve as an efficient approach to replace chemical fertilizers and pesticides for sustainable agriculture production in semi-arid regions of RGV. However, further investigations, including efficiency tests of *P. fluorescens* under greenhouse and field conditions, are needed to better understand the role of *P. fluorescens* as a biofertilizer and biocontrol agent for overall plant growth and development.

Table

Table 1. Biofertilizing and biocontrol traits results based on sunn hemp's canopy coverage, high and low.

Traits	High	Low	Total
Siderophore	17 (100%)	18 (100%)	35 (100%)
IAA	17 (100%)	18 (100%)	35 (100%)
Ammonia	14 (82%)	14 (77%)	28 (80%)
HCN	12 (70%)	15 (83%)	27 (77%)
Phosphate	12 (70%)	10 (55%)	22 (62%)
Protease	11 (64%)	10 (55%)	21 (60%)
ACC deaminase	5 (29%)	8 (44%)	13 (37%)

Figures

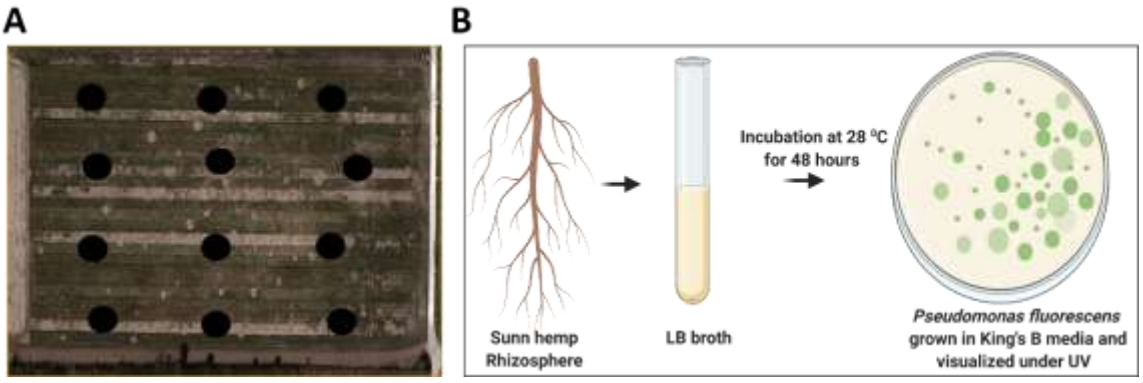

Figure 2: Aerial image of the sampling site. Rhizosphere samples were collected based on the canopy coverage of sunn hemp (A). Isolation of *P. fluorescens* from the rhizosphere of sunn hemp (B).

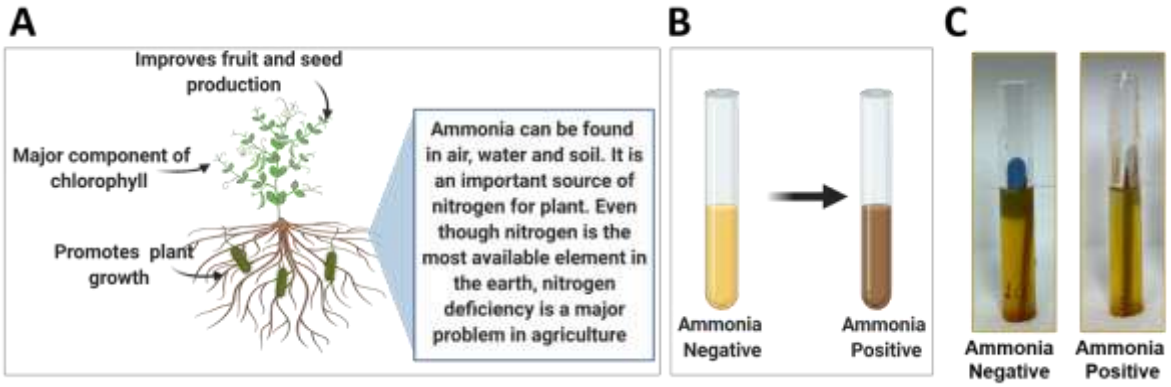

Figure 3: Role of ammonia in plants, its production by PGPR, *P. fluorescens*. Schematic illustration of ammonia production by *P. fluorescens* and its biofertilizer trait on plant (A). Laboratory method (B) and the real image (C) of detection of ammonia by *P. fluorescens* and was confirmed by a change of initial color from yellow to light green.

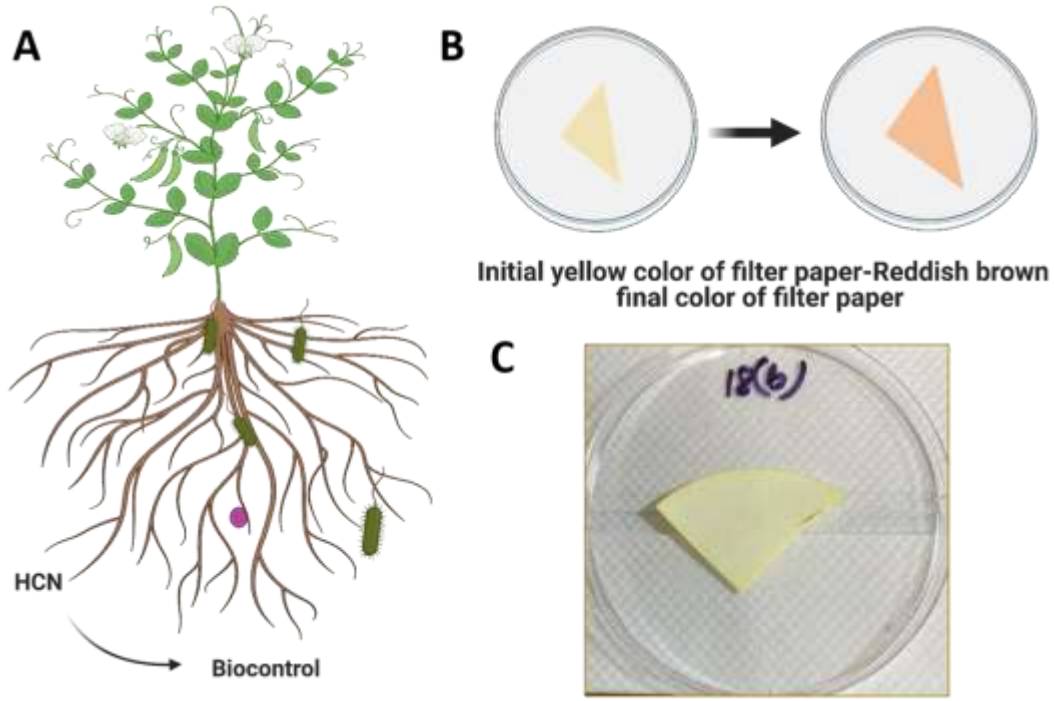

Figure 4: Role of HCN in plants, its production by PGPR, *P. fluorescens*. Schematic representation of HCN production (A) and detection in the laboratory (B). HCN production by *P. fluorescens* is confirmed with a color change of filter paper from yellow to reddish-brown (c).

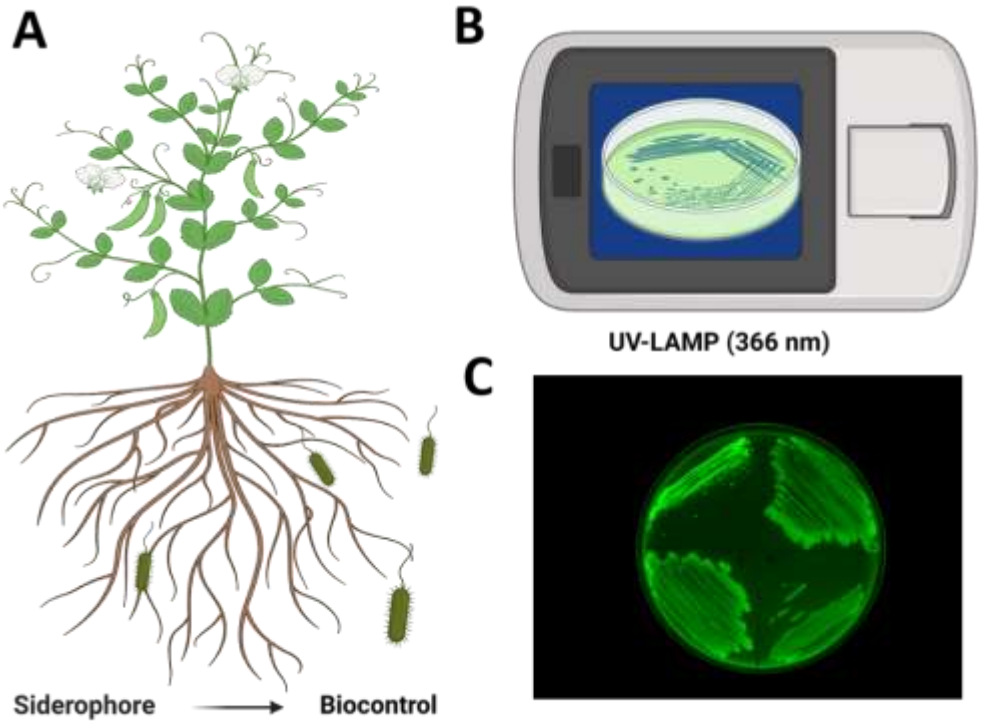

Figure 5: Role of siderophore in plants, its production by PGPR, *P. fluorescens*. Schematic illustration of siderophore production (A) and its detection in the laboratory (B). Siderophore production by *P. fluorescens*, is confirmed with a green-fluorescence pigment formation under UV light (366 nm) (C).

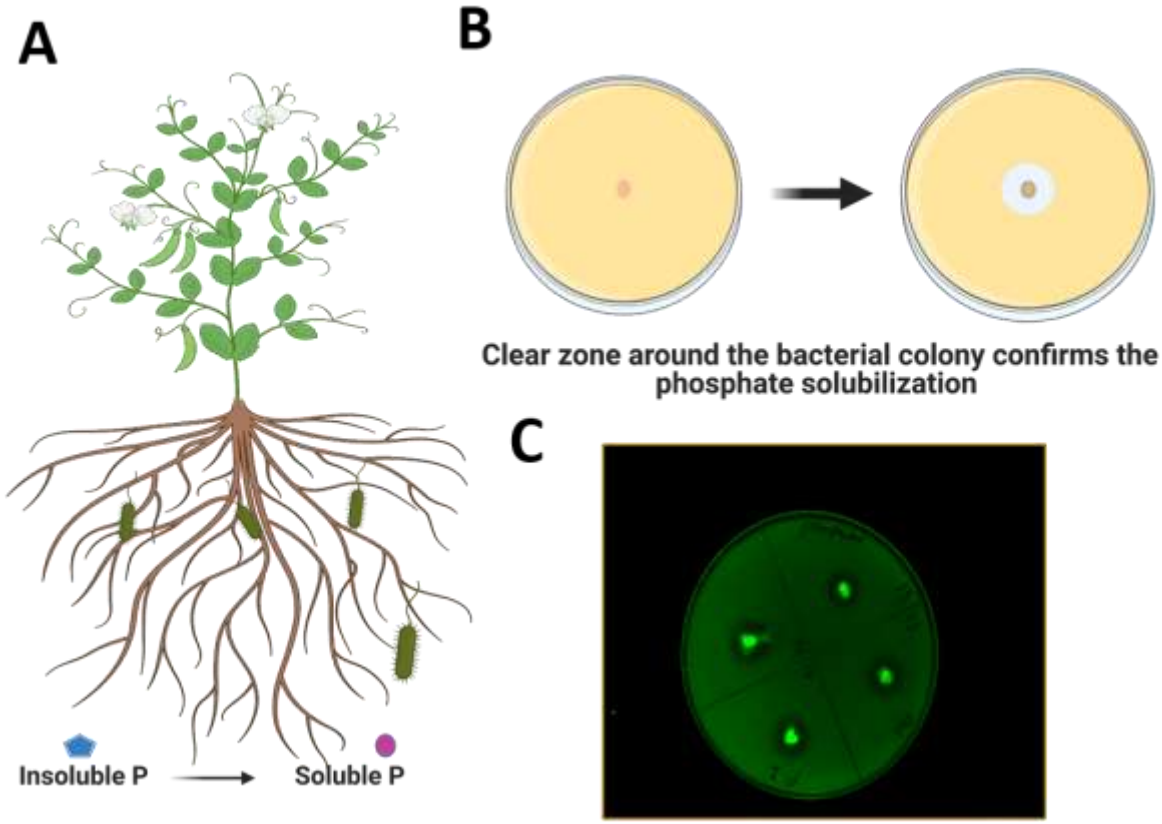

Figure 6: Phosphate solubilization by PGPR, *P. fluorescens*. Schematic illustration of phosphate solubilization (A) and its detection in the laboratory (B). Phosphate solubilization by *P. fluorescens*, is confirmed with a clear zone formation around the bacterial colonies (C).

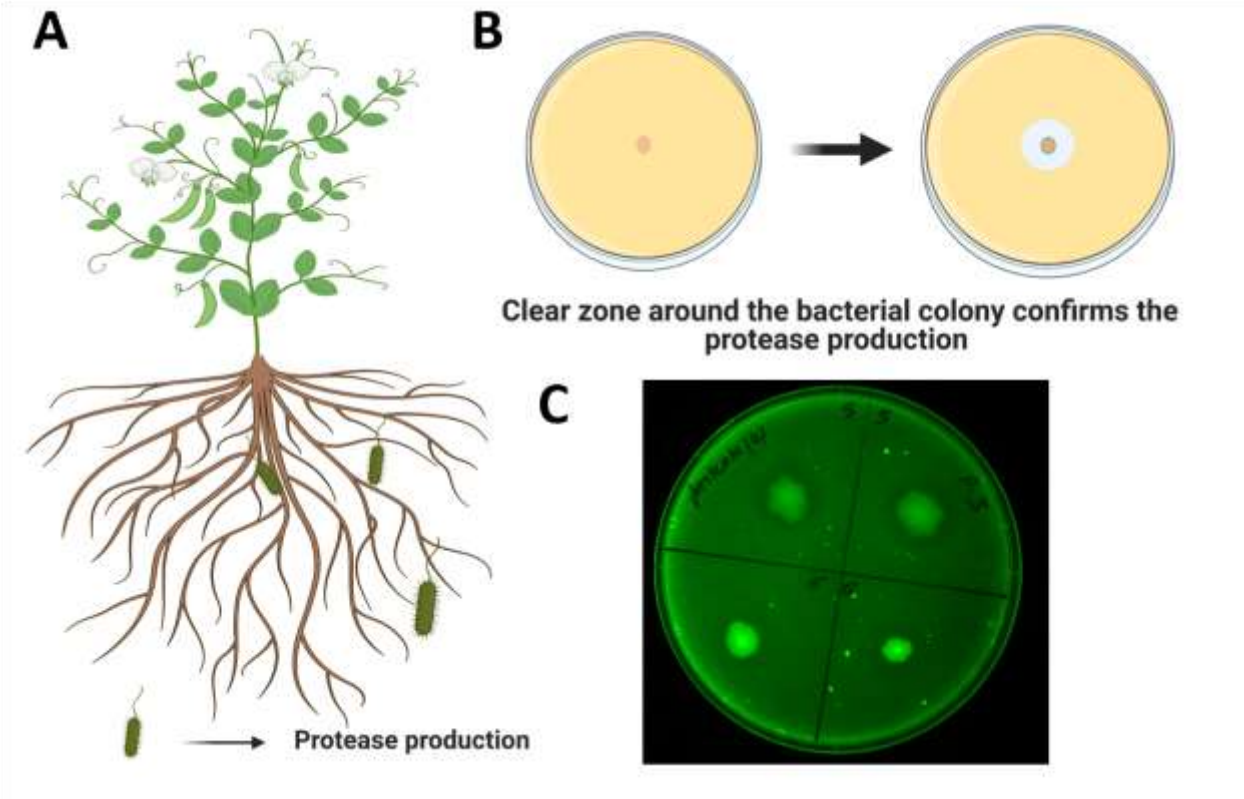

Figure 7: Protease production by PGPR, *P. fluorescens*. Schematic illustration of protease production (A) and its detection in the laboratory (B). Protease production by *P. fluorescens*, is confirmed with a clear zone formation around the bacterial colonies (C).

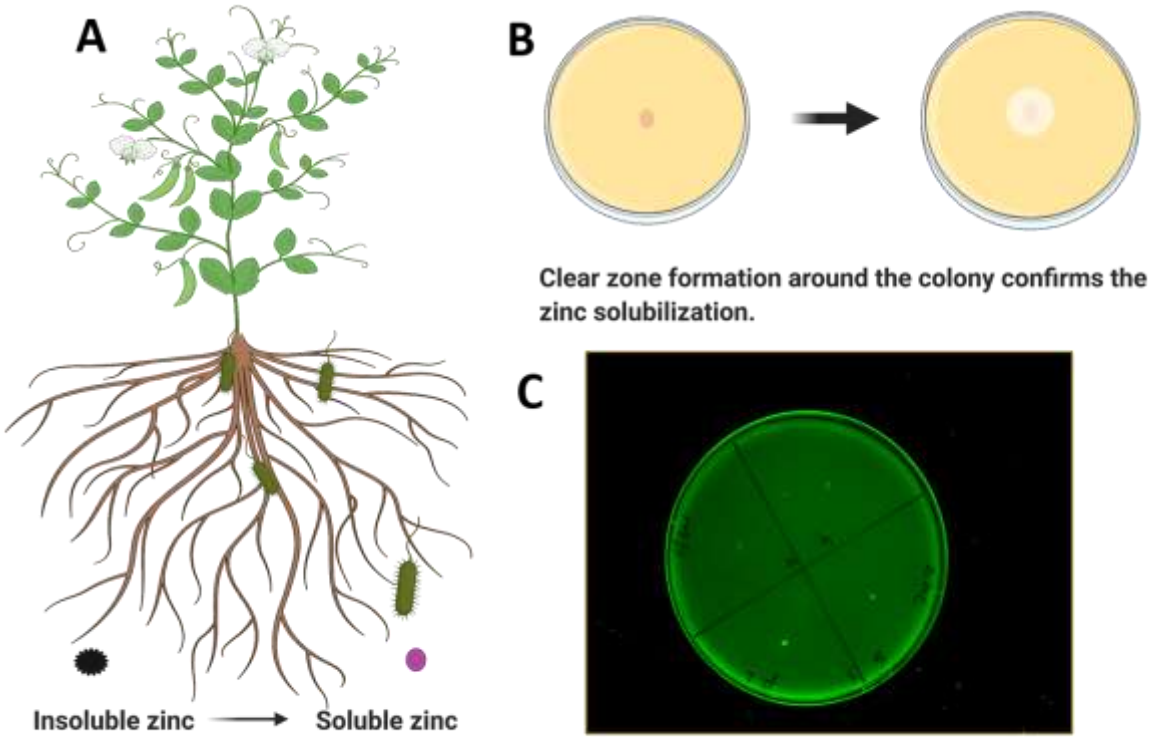

Figure 8: Zinc solubilization by PGPR, *P. fluorescens.* Schematic illustration of zinc solubilization (A) and its detection in the laboratory (B). Zinc solubilization by *P. fluorescens*, is confirmed with clear zone around the bacterial colonies (C).

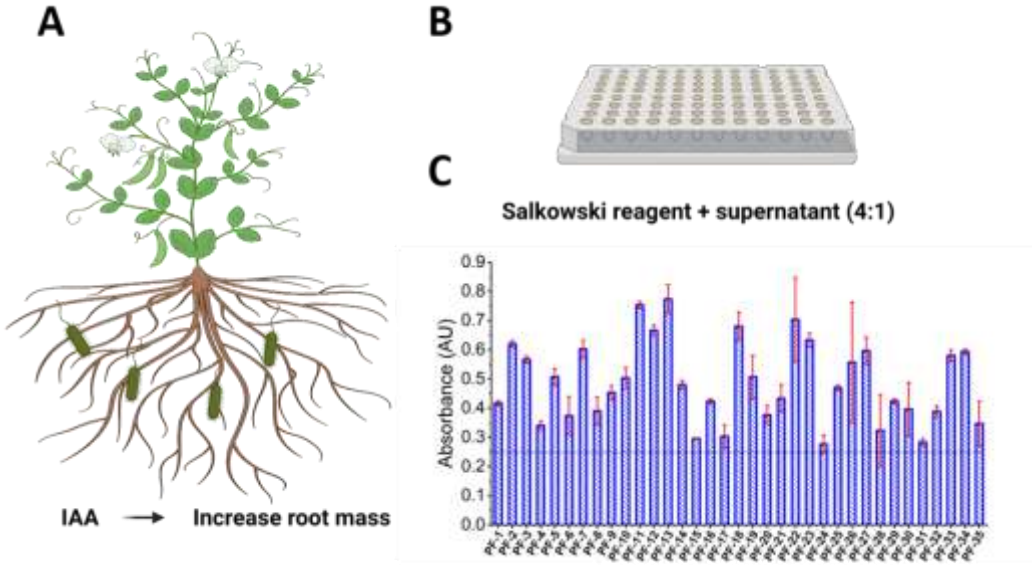

Figure 9: IAA production by PGPR, *P. fluorescens.* Schematic illustration of IAA production (A) and its detection in the laboratory (B). IAA production by *P. fluorescens* is confirmed by higher absorbance of Salkowski reagent and cell free supernatant (4:1) than the control (C). Black line indicates cut off value from control (C).

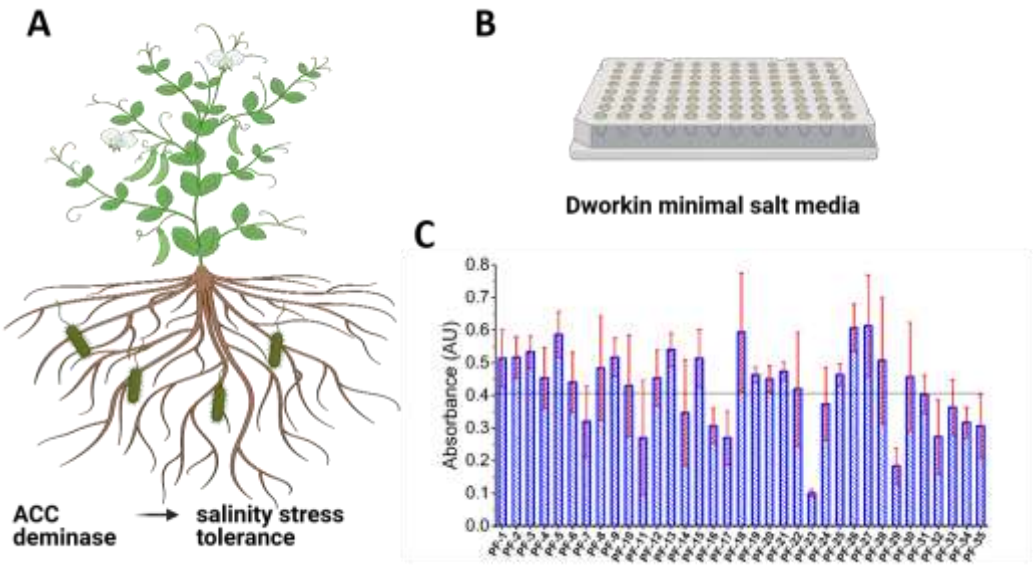

Figure 10: Role of ACC deaminase in plants, its production by PGPR, *P. fluorescens*. Schematic illustration of ACC deaminase (A) and its detection in the laboratory (B). Absorbance lower than the control on Dworkin and Foster (DF) minimal salt medium confirmed ACC deaminase production (C). Black line indicates cut off value from control (C).

CHAPTER III

INFLUENCE OF *PSEUDOMONAS FLUORESCENS* ON SORGHUM AND SUNN HEMP GROWTH UNDER GROWTH CHAMBER

Abstract

The ability of *Pseudomonas fluorescens* to promote plant growth is well documented, but knowledge of the impact of *P. fluorescens*, isolated from a cover crop on the growth of its subsequent crop, is scanty. In this study, the growth and development of sunn hemp and sorghum inoculated with five different strains of *P. fluorescens* (*Pf* 6, *Pf* 9, *Pf* 10, *Pf* 17, and *Pf* 34) were evaluated by measuring germination rate, total biomass, carbon: nitrogen (C: N) concentration, physiological, allocational, and morphological determinants of growth. The selected strains were positive for three different plant growth-promoting (PGP) traits, such as the production of ammonia (*Pf* 6), ACC-deaminase production (*Pf* 10), and solubilization of phosphate (*Pf* 17). Strain, *Pf* 34 was positive for all PGPR traits, while strain, *Pf* 6, was negative for all three PGP traits. The application of *P. fluorescens* on sorghum (*Sorghum bicolor* L., Moench) and sunn hemp (*Crotalaria juncea* L., Fabaceae*)* seeds had different species-specific impacts on plant growth and biomass allocation. In sorghum, the highest germination rate (80%) was recorded with the application of strain *Pf* 10, while in sunn hemp strain, *Pf* 34 resulted in the highest germination rate (82%). Strains *Pf* 6 and *Pf* 34 resulted in a significant increase in total

biomass in sorghum, 1.12g and 0.96g, respectively. In sunn hemp, strain *Pf* 34 resulted in significantly higher total biomass, 1.08g. Similarly, stomatal conductance was significantly higher in sorghum treated with strain *Pf* 10 (0.02 mmol $m^{-2}s^{-1}$). Interestingly, application of strain *Pf* 9, which tested positive for ammonia resulted in significantly lower root surface area in sorghum, while there was a significant difference across different treatments in sunn hemp. In addition, the C:N ratio was significantly higher with the application of strain *Pf* 17 (P = 0.02) in the sunn hemp plant. Besides, application of strain *Pf* 6 in sorghum resulted in significantly lower specific root length (SRL) (P = 0.0091) as in comparison to control. These results support the potential application of *P. fluorescens*, isolated from sunn hemp, to have better plant growth and development in its subsequent crop, i.e., sorghum.

Introduction

Soil health is an overall sum of soil functionality that plays a vital role in living ecosystem (USDA-NRCS). However, with extensive use of agrochemicals and tillage, there has been a significant decline in soil health which is critical in improving plant growth and increased crop yield (Arias et al., 2005). With the increase in awareness among growers of the declining soil health and its impact on crop yield and the environment, they have gradually started to adopt several farm management practices that promote soil health. Some of the management practices widely adopted by growers include the application of biochar (Yuan et al., 2017), crop rotation (Paungfoo-Lonhienne et al., 2017), reduced or no tillage (Nunes et al., 2019), increased input of organic matter (Sihi et al., 2017), and minimal use of synthetic fertilizers and pesticides (Prashar & Shah, 2016). Among these approaches, incorporating crop residues, and cover cropping are becoming increasingly popular (Mitchell et al., 2017). (Wallander, 2021) reported that between 2012 and 2017, the use of cover corps increased by 50% on US croplands.

Farmers interest in growing cover crops directly relate to the reported agroecological benefits such as the increase in soil productivity (Fageria et al., 2005), enhancing water use efficiency by crops (Kahimba et al., 2008), reducing weed pressure (Soti & Racelis, 2020), suppresses disease and insect pests (Bowers et al., 2020), and other ecosystem services (Mitchell et al., 2017). Cover crops are also known to promote soil health by increasing the population of plant beneficial microbes (Soti et al., 2016) and reducing the parasitic and pathogenic ones (DuPont et al., 2009). A recent metanalysis reported that cover corps significantly increased soil microbial abundance, activity, and diversity by 27%, 22%, and 2.5%, respectively (Kim et al., 2020).

However, the adoption of cover crops is precluded by several challenges such as cost and availability of seeds, cost of labor, timing and technique for planting and harvest, and cover crop species selection (Clay et al., 2020). In addition, cover crops performance and their benefits vary significantly based on the local environmental conditions such as temperature, moisture, soil nutrient status etc., reducing the perceived benefits. For example, (Kasper et al., 2019) reported a failure to form nodules in leguminous cover crops treated with recommended inoculants. In addition, the soil health benefits of cover crops are negated by extensive soil disturbance during termination and do not provide the perceived benefits to the subsequent cash crops.

Standing cover crops influence the soil microbial community through the root exudates, litter input, and change in the soil microclimate (Carrera et al., 2007). Most of our understanding of the influence of cover crops on soil microbes comes from soil analysis after termination (Finney et al., 2017). However, the microbial community composition can shift after termination due to the change in soil abiotic conditions. Understanding the relationship between plant and soil microbial communities is crucial in farm/need specific species selection and cover crop

management to maximize soil health benefits of cover crops to the subsequent cash crops. However, there is limited information on the cover crop and their influence on soil microbial community before termination and their influence on the cash crop growth and yield.

The objective of this study was to determine the impact of beneficial soil microbe, *P. fluorescens,* isolated from the rhizosphere of standing sunn hemp (*Crotalaria juncea* L., Fabaceae) plants on the germination and growth of sorghum (*Sorghum bicolor* L., Moench). We hypothesized that the *P. fluorescens,* isolated from the rhizosphere of sunn hemp will have positive impact on the germination and growth of both sunn hemp and sorghum, a cash crop.

Materials and methods

Bacterial strains and seed inoculation

In this study, we used five different strains of *P. fluorescens* isolated from the rhizosphere of sunn hemp growing in a certified organic grain farm in south Texas and cultured in the Cell Biology lab at the University of Texas Rio Grande Valley (See Chapter 2). The selected strains were positive for the production of ammonia, ACC deaminase, protease, HCN, IAA, and siderophore and solubilization of phosphate. Strain specific traits for each of the selected traits are listed in Table 1. We tested the influence of these selected strains on the growth and biomass allocation of sunn hemp and sorghum.

In this study, 150 seeds each both sorghum and sunn hemp were surface sterilized in 70% (v/v) ethanol for 1 min and was fully immersed in 1% (v/v) sodium hypochlorite solution (NaClO) for ten minutes and finally washed with sterile deionized water for 6–7 times (Gupta & Pandey, 2019). The seeds were then inoculated with the culture suspension ($10^8 – 10^9$ CFU mL^{-1}) of the selected strains for one hour and air dried aseptically under a laminar air flow hood. The seeds for control from both plant species were immersed in NB broth without the bacterial strains for 1 hour.

Experimental design

The inoculated of both plant species were planted in (180ml) pots filled with sterilized farm soil and perlite mix (1:1) at 2 cm depth. Each treatment was replicated 10 times. The pots were then randomly arranged in a temperature, light, and humidity-controlled growth chamber (Percival, Iowa, USA). The growth chamber was maintained under 80% relative humidity, 16:8 light: dark photoperiod at 25^0C for 26 days for both plants. Plants were watered daily with 15-20 ml of sterile DI water to maintain 15% soil moisture.

Measurements

Plants of both species in all microbial treatments were grown for 26 days and harvested destructively for growth measurements. Data on germination rate, and plant growth parameters were collected for this study. We measured plant height, physiological parameters of growth, stomatal conductance and fluorescens, allocational parameters of growth such as root mass ratio (RMR), shoot mass ratio (SMR), leaf mass ratio (LMR), and specific root length (SRL), and morphological parameter of growth such as specific leaf area (SLA) and root surface area (RSA).

Stomatal conductance and fluorescence were measured 30 minutes after watering using a Licor Li-600 porometer/fluorometer (Licor, Lincoln, Nebraska, USA). At the end of the study period, plants in each treatment were destructively harvested for the measurements. Leaf area was measured using a Li-3300C (Licor, Lincoln, Nebraska, USA) and root area was measured using a WinRhizo (Regent, Canada) after carefully washing the roots with tap water. Different plant parts were separated and dried in an oven for 72 hours at 65^0C. After drying the weights of each plant were recorded. The leaves from all treatments were ground finely and analyzed for total carbon and nitrogen (C:N) concentration. The calculations for each of the parameters were done as follows:

1. LMR: leaf mass/total biomass

2. RMR: Root mass/total biomass

3. SMR: Shoot mass/total biomass

4. SLA: Leaf area/leaf biomass

5. SRL: Root length/root biomass

Data analysis

Since the sample size was unequal, we conducted non-parametric tests on all the variables. We conducted a Wilcoxon each pair test to compare the difference in the germination rate and plant growth parameters in each plant species. To compare the species-specific impact of the different treatments on the two plant species, we conducted Friedman's test with plant and microbial strains as two fixed factors. Results were considered significant when ($P \leq 0.05$). The Friedman's test was conducted in R 3.6.1 (package). All other data were analyzed using JMP (SAS Institute Inc., North Carolina, USA).

Results

Plant germination, biomass yield, physiology, and morphology

After the bacterial inoculation, various growth parameters such as total biomass, plant height, germination rate, allocational, physiological, and morphological determinants, and carbon: nitrogen (C:N) ratio were measured (Table 3, 4). The different strains selected had a species-specific impact on the growth and biomass allocation in the two plant species ($P = 0.017$). (Table 5; Figure 11). The selected strains also had a significant impact on the germination rate of both plants (Table 3, 4). In sorghum, the germination rate ranged from (53.33 – 80%) highest with strain *Pf* 10 (80%) followed by strain *Pf* 17 (70%), while the lowest germination rate was with strain *Pf* 9 (53.33%) (Table 3). Germination rate in sunn hemp ranged from (48 - 82%) with the highest in strain *Pf* 34 (82 %) followed by *Pf* 17 (64%) and the lowest germination in strain

Pf 6 (Table 4). The bacterial treatment did not have any impact on the plant height in both plant species ($P > 0.05$) (Table 7).

While there was a strain-specific impact on the allocational determinants of growth such as root mass ratio (RMR), shoot mass ratio (SMR), and leaf mass ratio (LMR), these differences were not statistically significant for both the plant species (Table 8, 9, and 10). However, there was strain specific impact on the physiological parameters measured, stomatal conductance and fluorescence. In sorghum, stomatal conductance ranged from (0.01 - 0.02 mmol $m^{-2}s^{-1}$). Strain *Pf* 10, had significantly higher stomatal conductance than control ($P = 0.0026$) (Figure 12), with the highest in strain *Pf* 6 and *Pf* 10 (0.02 mmol $m^{-2}s^{-1}$), all other treatment had stomatal conductance of 0.01 mmol $m^{-2}s^{-1}$ (Table 3). In sunn hemp, stomatal conductance ranged from (0.02 - 0.17 mmol $m^{-2}s^{-1}$), with the highest in strain *Pf* 17 (0.17 mmol $m^{-2}s^{-1}$) followed by strain *Pf* 10 (0.09 mmol $m^{-2}s^{-1}$) (Table 4), and the lowest in strain *Pf* 6 (0.02 mmol $m^{-2}s^{-1}$) (Table 4). Similarly, fluorescence measured in sorghum leaves ranged from (87.85 - 80.94) with the highest in strain *Pf* 9 (87.85), followed by strain *Pf* 10 (86.99) (Table 3), lowest was measured in strain *Pf* 6 (80.94) (Table 3). In sunn hemp, fluorescence ranged from (91.65 - 115.66) highest with strain *Pf* 34 (115.66) followed by strain *Pf* 9 (111.87) (Table 4), and the lowest was strain *Pf* 10 (91.65) (Table 4).

Similarly, there was a strain specific impact on the morphological parameters such as root surface area (RSA), specific leaf area (SLA), and specific root length (SRL) in the two plant species. In sorghum, the RSA ranged from (16.06 - 19.62 cm^2) with the highest in strain *Pf* 34 (19.62 cm^2) followed by control (17.06 cm^2) (Table 3), while RSA was significantly lower in plants treated with strain *Pf* 9 (16.11 cm^2) ($P = 0.03$) (Figure 13). In sunn hemp, RSA ranged from (20.47 - 21.54 cm^2) with the highest in strain *Pf* 9 (21.54 cm^2) followed by strain *Pf* 10

(20.47 cm^2) (Table 4) and in strain *Pf* 17 (21.53 cm^2) (Table 4). However, this difference was not statistically significant. SLA ranged from (87.76 - 159.28 cm^2) in the sorghum, with the highest in strain *Pf* 9 (159.28 cm^2) followed by strain *Pf* 10 (134.93 cm^2) (Table 3), while the lowest was with strain *Pf* 6 (87.76 cm^2) (Table 3). But these difference in SLA was not statistically significant. In sunn hemp, SLA ranged from (59.58 - 134.57 cm^2) with the highest in strain *Pf* 10 (134.57 cm^2) followed by strain *Pf* 9 (114.36 cm^2) (Table 4), and lowest in control (59.98 cm^2). Strain *Pf* 10 resulted in significantly higher SLA than control in the sunn hemp plant (P = 0.034) (Table 4; Figure 14), while there was no significant difference among others. In addition, different selected strains had no species-specific impact on the specific root length (SRL) in the two plant species (P = 0.18) (Table 11). In sorghum, SRL ranged from (1152.89 - 2307.63 cm g^{-1}) with highest in control (2307.63 cm g^{-1}) followed by strain *Pf* 10 (1912.34 cm g^{-1}) (Table 3), and lowest in strain *Pf* 6 (1152.84 cm g^{-1}). Strain *Pf* 6 resulted in significantly lower specific root length (P = 0.0091) as in comparison to control (Figure 15). In sunn hemp, SRL ranged from (2889.67 - 8555.23 cm g^{-1}) with the highest in strain *Pf* 10 (8555.23 cm g^{-1}) followed by strain *Pf* 9 (4418.26 cm g^{-1}) (Table 3), and lowest in strain *Pf* 6 (2865.8 cm g^{-1}). But these difference in SRL was not statistically significant.

Carbon nitrogen ratio in sorghum leaf tissue ranged from (42.02 - 46.65) with the highest in strain *Pf* 34 (46.65) followed by control (45.92) (Table 3), while the lowest C:N ratio was with strain *Pf* 17 (42.02) (Table 3). In sunn hemp, C:N ratio ranged from (38.01 - 75.28), with the highest in *Pf* 17 (75.28), followed by control (58.04) (Table 3). Plants treated with strain *Pf* 17 were significantly higher compared to the control (P = 0.02), while plants treated with strain *Pf* 6 and strain *Pf* 9 had significantly lower C:N ratio (P = 0.01 and P = 0.03 respectively) (Figure 16).

Discussion

In this study, we assessed the potential benefits of *P. fluorescens* strains isolated from the rhizosphere of a cover crop, sunn hemp, on the growth and biomass allocation of both sunn hemp and grain sorghum. We used strains that were positive for different plant growth-promoting traits including production of ammonia, HCN, IAA, protease, siderophore, and ACC deaminase, and solubilization of phosphate. These traits have been reported to increase plant growth promotion through increase nutrient availability and biotic and abiotic stress tolerance (Goswami et al., 2016).

Similarly, we found mixed results in the two plants in plant growth parameters such as germination rate, total biomass, plant height, morphological, physiological, and allocational determinants. Germination rates of seeds treated with different strains of *P. fluorescens* were higher compared to the control in both sunn hemp and sorghum. In sorghum, strain producing ACC deaminase, IAA, and siderophore resulted in highest germination rate of 80%, while in sunn hemp, the highest germination rate (82%) was observed with *P. fluorescens* positive for production of ammonia, IAA, siderophore, ACC deaminase activity, HCN, and solubilization of phosphate. Similar results of these phytohormones increasing germination rates have been reported in previous studies (Gholami et al., 2009; Kaymak, 2011; Maheshwari et al., 2015). Similarly, (Moeinzadeh et al., 2010) reported 94 % and 87 % germination rate of sunflower (*Helianthus annuus*) seeds after its inoculation with two different strains of *P. fluorescens*, Meanwhile (Raj et al., 2004) reported a 90 % germination rate of pearl millet (*Pennisetum glaucum*) after its seeds were inoculated with two different strains of *P. fluorescens.* While we did not analyze for the production of gibberellin, this phytohormone is mainly responsible for breaking seed dormancy and increasing germination rate (Bottini et al., 2004). But IAA production is also known to help in seed germination, and from our study, all of our strains were

able to produce IAA, and it might have increased the seed germination rate (Marathe et al., 2017). A study by (Slavov et al., 2004) reported increase in germination of broomrapes (*Orobanche* spp.) which correlate with the production of IAA.

P. fluorescens is also reported to increase total biomass and plant height (Shaharoona et al., 2006). In our study, there was an increase in total biomass, but there was no significant difference in plant height across the different treatments. Sorghum seeds treated with *P. fluorescens* strains positive for production of IAA, protease, siderophore, HCN, and ACC deaminase and solubilization of phosphate had significantly higher biomass. While in sunn hemp, *P. fluorescens* strains positive for the production of IAA, siderophore, and HCN, phosphate solubilization, and acc deaminase activity resulted in significantly higher total plant biomass. Though there was a difference in the total biomass among different treatments, we did not see any significant difference in the biomass allocation pattern among the different treatments.

The different *P. fluorescens* treatments did have an impact on the plant morphology. Plants treated with *P. fluorescens* positive for the production of IAA, protease, and siderophore significantly lower specific root length (SRL) than the control. This was an expected result as the nutrient availability would be potentially higher for plants treated P. fluorescens, thus reducing the root growth. The two plant species treated with the different *P. fluorescens* strains had different results in RSA. While there was no significant difference in sunn hemp, in sorghum, RSA was significantly lower in plants treated with *P. fluorescens* that was positive for the production of ammonia, IAA, protease, and siderophore. This could be because of our experiments was carried out in controlled environmental conditions where all the plants received

equal amount of water and light. So, the plant did not utilize the extra IAA to increase their root structure as they weren't under any climatic or biotic stress.

Specific leaf area (SLA) was also different for the two plant species treated with the different *P. fluorescens* strains. While sorghum plants under different treatments did not have any significant difference in SLA, it was significantly higher in sunn hemp plants inoculated with *P. fluorescens* positive for ACC- deaminase activity, IAA, and siderophore. While we did not measure the lateral root numbers, SLA of sunn hemp might have been increased due to the production of IAA and ACC- deaminase activity, which might have increased the lateral root numbers of sunn hemp subsequently increasing the water uptake and iron availability of the plant through the siderophore production resulting in greater leaf area.

Physiological determinants such as conductance and fluorescence were also measured in sorghum and sunn hemp. In sorghum, treatment of *P. fluorescens* positive for ACC-deaminase activity, production of IAA, and siderophore significantly increased the stomatal conductance. Although there was no significant difference in stomatal conductance in the sunn hemp plant, *P. fluorescens* positive for solubilization of phosphate, production of protease, IAA, siderophore, and HCN resulted in 64 % more stomatal conductance than control. Interestingly, *P. fluorescens* positive for protease, IAA, and siderophore resulted in the lowest stomatal conductance in sunn hemp. Our results are in contrast to a study by (Ansari & Ahmad, 2019) which reported 53.8 % higher stomatal conductance in wheat treated with *P. fluorescens,* positive for ammonia production and phosphate solubilization. Similarly, a study by (Ahmad et al., 2013) reported improvement of stomatal conductance in mung bean (*Vigna radiata* L.) under salinity conditions after the inoculation or co-inoculation of PGPR such as *Pseudomonas syringae and Rhizobium phaseoli*. While the stomatal conductance measured in our study is

significantly low, the difference in our results could be explained by the growth conditions. All plants in this study received equal amount of water and were not drought-stressed. We did not see a significant difference in the fluorescence among different treatments in both plant species.

The different *P. fluorescens* did result in different nitrogen concentrations in the leaves of the two plant species. In sunn hemp leaves, application of *P. fluorescens* positive for solubilization of phosphate, production of protease, IAA, and siderophore had significantly higher C:N ratio than control, while treatment with two strains of *P. fluorescens* positive for the production of ammonia, protease, IAA, and siderophore resulted in significantly lower C:N ratio. The lower C:N ratio might be explained as plants treated with the ammonia producing bacteria might have absorbed the ammonia produced by the bacteria resulting in greater nitrogen content in sunn hemp leaves.

Conclusion

The present study describes the application of a PGPR, *P. fluorescens* isolated from the rhizosphere of sunn hemp in both sorghum and sunn hemp under normal condition in a growth chamber. Overall, our results show that the *P. fluorescens* did have a significant impact on some of the plant growth parameters. While there is a possibility that the results could be different in a field conditions, our results indicate that sunn hemp not only improved soil health by increasing soil organic matter (nitrogen concentration), it also promoted beneficial soil microbes. However, further research is needed to evaluate the efficiency of these strains under actual field conditions with various abiotic conditions such as salinity, high temperature, and flooding.

Tables

Table 2: Representation of different strains and their PGP characteristics

Strains	Ammonia	Phosphate	ACC deaminase	Protease	IAA	Siderophore	HCN
Pf 6	-	-	-	+	+	+	-
Pf 9	+	-	-	-	+	+	-
Pf 10	-	-	+	-	+	+	-
Pf 17	-	+	-	+	+	+	+
Pf 34	+	+	+	-	+	+	+

Table 3: Germination, and different plant growth parameters sorghum treated with *P. fluorescens.*

	Treatment					
Sorghum	Control	*Pf* 6	*Pf* 9	*Pf* 10	*Pf* 17	*Pf* 34
Germination rate (%)	60	60	53.33	80	70	66.67
Total length (cm)	433.04_{a}	423.27_{a}	380.38_{a}	425.46_{a}	375.96_{a}	411.21_{a}
Plant height (cm)	34.5_{a}	37.99_{a}	36.31_{a}	34.43_{b}	35.52_{a}	37.41_{a}
Total root surface area (cm^2)	17.06_{a}	16.68_{a}	16.11_{b}	16.36_{a}	16.06_{a}	19.62_{a}
SLA	134.93_{a}	87.76_{b}	159.28_{a}	134.51_{a}	122.08_{a}	99.79_{a}
Conductance (mmol $m^{-2}s^{-1}$)	0.01_{b}	0.02_{ab}	0.01_{b}	0.02_{a}	0.01_{b}	0.01_{b}
Fluorescens	84.13_{a}	80.94_{a}	87.85_{a}	86.99_{a}	83.79_{a}	83.3_{a}
Total biomass (g)	0.75_{b}	1.12_{a}	0.74_{b}	0.7_{b}	0.68_{b}	0.96_{a}
Root shoot ratio	0.61_{a}	0.52_{a}	0.66_{a}	0.57_{a}	0.48_{a}	0.48_{a}
LMR	0.38_{a}	0.41_{a}	0.37_{a}	0.37_{a}	0.42_{a}	0.41_{a}
RMR	0.34_{a}	0.33_{a}	0.37_{a}	0.34_{a}	0.31_{a}	0.32_{a}
SMR	0.26_{a}	0.24_{a}	0.25_{a}	0.28_{a}	0.26_{a}	0.26_{a}
Specific root length (cm g^{-1})	2307.6_{ab}	1152.8_{c}	1497.8_{ab}	1912.3_{ab}	1908.2_{b}	1425.3_{b}
C:N	45.92_{a}	44.37a	45.83_{a}	46.07_{a}	42.02_{a}	46.65_{a}

Table 4: Germination, and different plant growth parameters sunn hemp treated with *P. fluorescens.*

	Treatment					
Sunn hemp	Control	*Pf* 6	*Pf* 9	*Pf* 10	*Pf* 17	*Pf* 34
Germination rate (%)	56	48	56	58	64	82
Total length (cm)	300.51_{b}	324.46_{b}	309.34_{b}	308.18_{b}	341.24_{b}	325.41_{b}
Plant height (cm)	12.63_{a}	13.33_{a}	14.12_{a}	12.33_{a}	12.07_{a}	15.77_{a}
Total root surface area (cm^2)	21.33_{a}	21.01_{a}	21.54_{a}	20.47_{a}	21.53_{a}	21.35_{a}
SLA	59.58_{b}	65.7_{ab}	114.36_{ab}	134.57_{ab}	88.08_{ab}	59.75_{ab}
Conductance (mmol $m^{-2}s^{-1}$)	0.06_{a}	0.02_{a}	0.08_{a}	0.09_{a}	0.17_{a}	0.07_{a}
Fluorescens	101.51	111.47	111.87	91.65	105.97	115.66
Total biomass (g)	0.75_{b}	0.75_{ab}	0.78_{b}	0.57_{b}	0.72_{b}	1.08_{a}
Root shoot ratio	0.21_{a}	0.21_{a}	0.24_{a}	0.27_{a}	0.44_{a}	0.16_{a}
LMR	0.66_{a}	0.7_{a}	0.64_{a}	0.66_{a}	0.63_{a}	0.69_{a}
RMR	0.16_{a}	0.17_{a}	0.17_{a}	0.17_{a}	0.22_{a}	0.14_{a}
SMR	0.16_{a}	0.13_{a}	0.18_{a}	0.16_{a}	0.14_{a}	0.16_{a}
Specific root length (cm g^{-1})	3207.1_{a}	2865.8_{a}	4418.2_{a}	8555.2_{a}	3049.2_{a}	2889.6_{a}
C:N	58.04_{b}	40.42_{c}	38.01_{c}	42.3_{b}	75.28_{ab}	56.42_{ab}

Table 5: Nonparametric multiple comparison of total biomass between strain and sorghum plant using Wilcoxon each pair method. Strain *Pf* 6 (P=0.0028) and *Pf* 34 (P = 0.021) resulted significant difference in total biomass as in comparison to control.

Level	Level	p-Value
Pf 6	Control	0.0028
Pf 34	*Pf* 10	0.0028
Pf 34	*Pf* 17	0.0039
Pf 34	Control	0.021
Pf 34	*Pf* 9	0.0246
Pf 9	*Pf* 6	0.0025
Pf 17	*Pf* 6	0.0012
Pf 10	*Pf* 6	0.0003

Table 6: Nonparametric tests of each pair of total biomass between strain and sunn hemp plant using Wilcoxon method. Strain *Pf* 34 (P = 0.017) resulted significant difference in total biomass as in comparison to control.

Level	Level	p-Value
Pf 34	Control	0.017
Pf 34	*Pf* 9	0.043
Pf 34	*Pf* 10	0.013
Pf 34	*Pf* 17	0.023

Table 7: Two way-ANOVA, effect tests of plant height of sorghum and sunn hemp plant by strain. There was no significant effect of species-specific impact on plant height (P = 0.86).

Effect Tests					
Source	Nparm	Df	Sum of squares	F ratio	Prob > F
Plant	1	1	12910.32	880.22	<0.0001
Strain	5	5	150.65	2.05	0.0783
Plant * Strain	5	5	27.59	0.37	0.8638

Table 8: Two way-ANOVA, effect tests of root mass ratio (RMR) of sorghum and sunn hemp plant by strain. There was no significant effect of species-specific impact on RMR (P = 0.79).

Effect Tests					
Source	Nparm	Df	Sum of squares	F ratio	Prob > F
Plant	1	1	0.68	55.57	<.0001
Strain	5	5	0.02	0.35	0.8792
Plant * Strain	5	5	0.02	0.47	0.7937

Table 9: Two way-ANOVA, effect tests of leaf of shoot mass ratio (SMR) of sorghum and sunn hemp plant by strain. There was no significant effect of species-specific impact on SMR (P = 0.98).

Effect Tests

Source	Nparm	Df	Sum of squares	F ratio	Prob > F
Plant	1	1	0.28	24.03	<.0001
Strain	5	5	0.01	0.21	0.9551
Plant * Strain	5	5	0.08	0.13	0.9833

Table 10: Two way-ANOVA, effect tests of leaf mass ratio (LMR) of sorghum and sunn hemp plant by strain. There was no significant effect of species-specific impact on LMR (P=0.96)

Effect Tests					
Source	Nparm	Df	Sum of squares	F ratio	Prob > F
Plant	1	1	1.85	81.03	<.0001
Strain	5	5	0.02	0.24	0.9418
Plant * Strain	5	5	0.02	0.18	0.9690

Table 11: Two way-ANOVA, effect tests of specific root length (SRL) of sorghum and sunn hemp plant by strain. There was no significant effect of species-specific impact on LMR (P = 0.18).

Effect Tests					
Source	Nparm	DF	Sum of Squares	F ratio	Prob > F
Plants	1	1	152723190	12.77	0.0006*
Strain	5	5	107335709	1.79	0.12
Plants*Strain	5	5	92093104	1.54	0.18

FIGURES

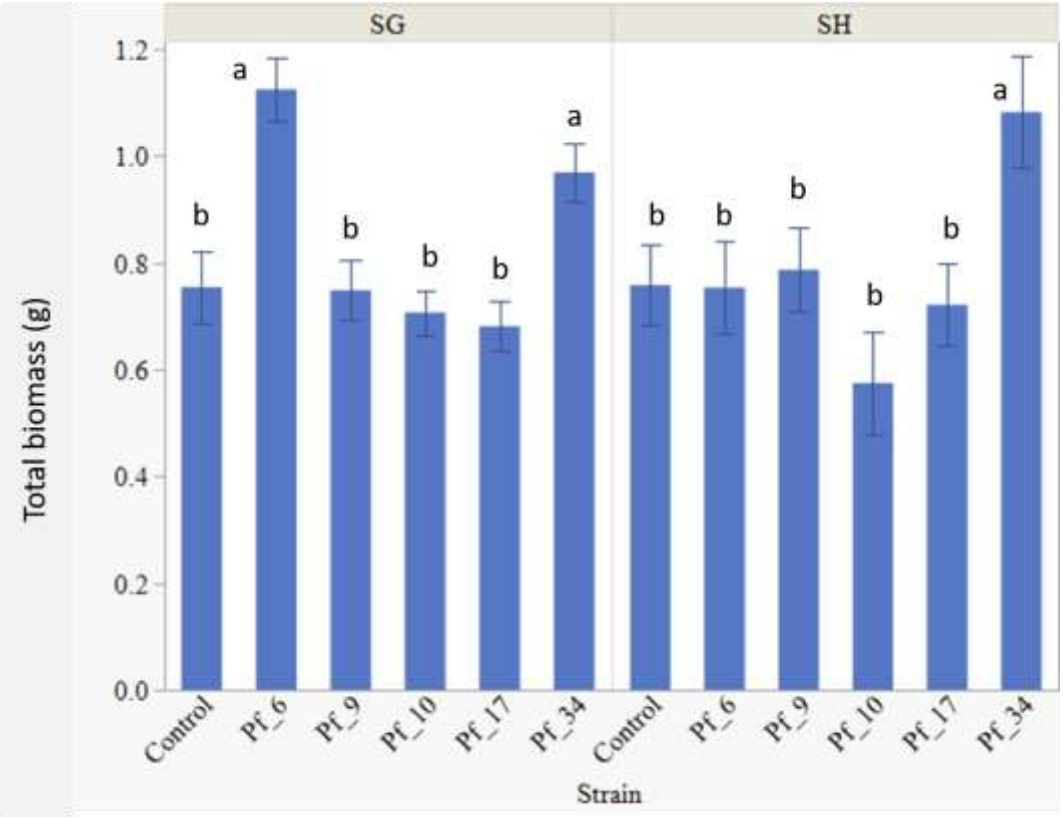

Figure 11: Total biomass (mean ± standard error) in the two plant species SG (sorghum) and SH (sunn hemp) treated with the different *P. fluorescens* strains.

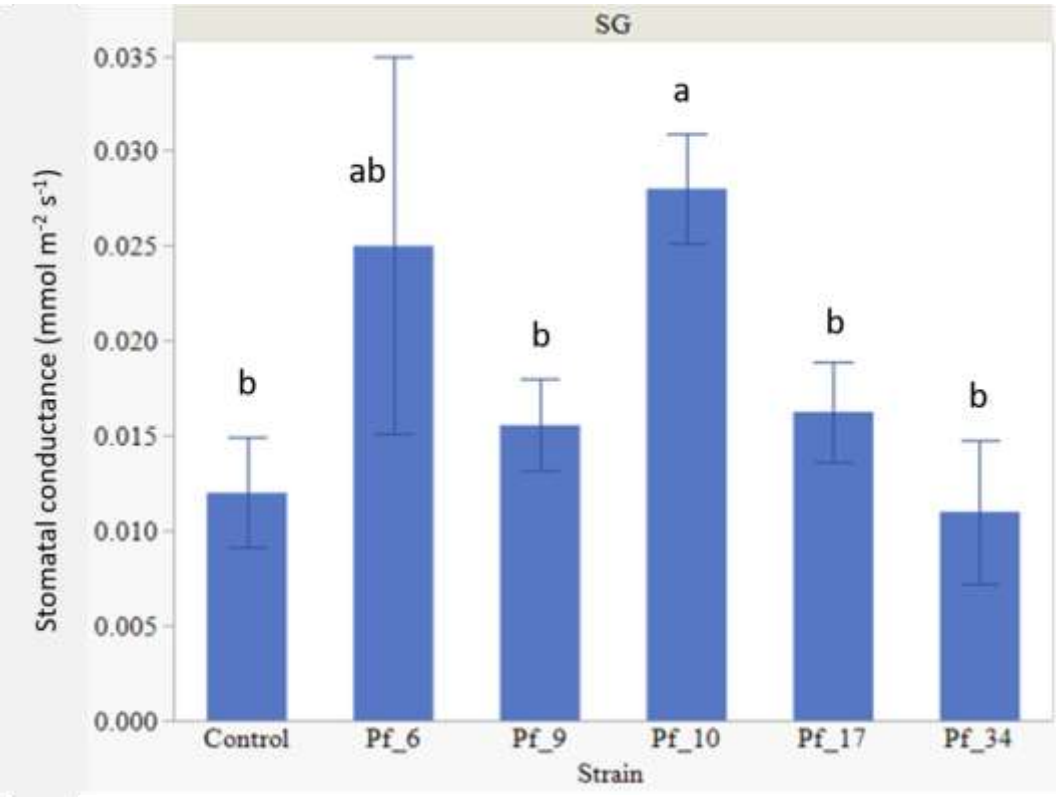

Figure 12: Impact of the different strains on the stomatal conductance of SG (sorghum). Strain number *Pf* 10 had significantly higher conductance than control ($P < 0.002$). Values are the means of ten replicates ± standard error of the mean.

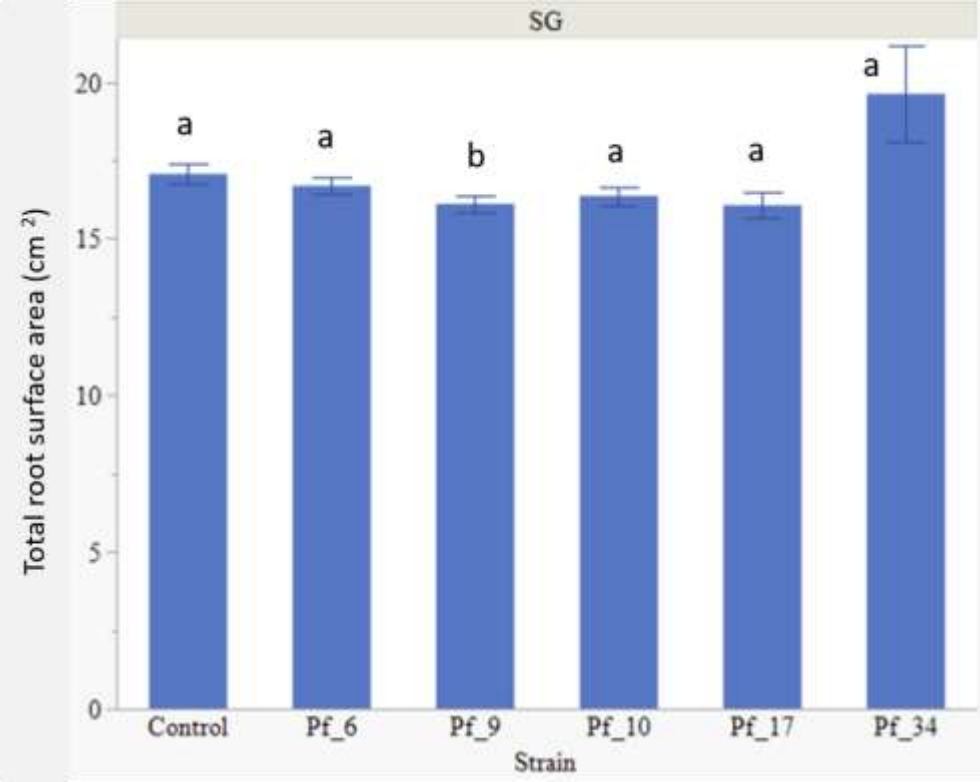

Figure 13: Impact of the different strains on the total root surface area (cm^2) of SG (sorghum). Strain number *Pf* 9 had significantly lower total root surface area than control ($P < 0.03$).Values are the means of ten replicates ± standard error of the mean.

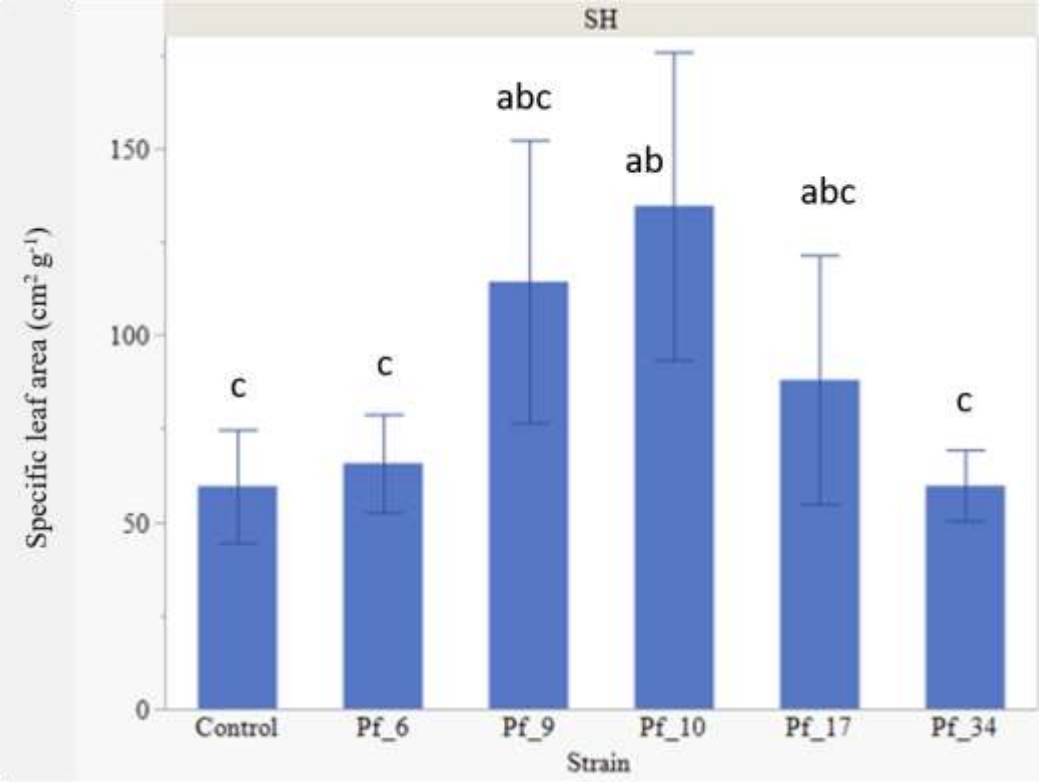

Figure 14: Impact of the different strains on the specific leaf area (SLA) of SH (sunn hemp). Strain number *Pf* 10 had significantly higher SLA than Control ($P < 0.034$).Values are the means of ten replicates ± standard error of the mean.

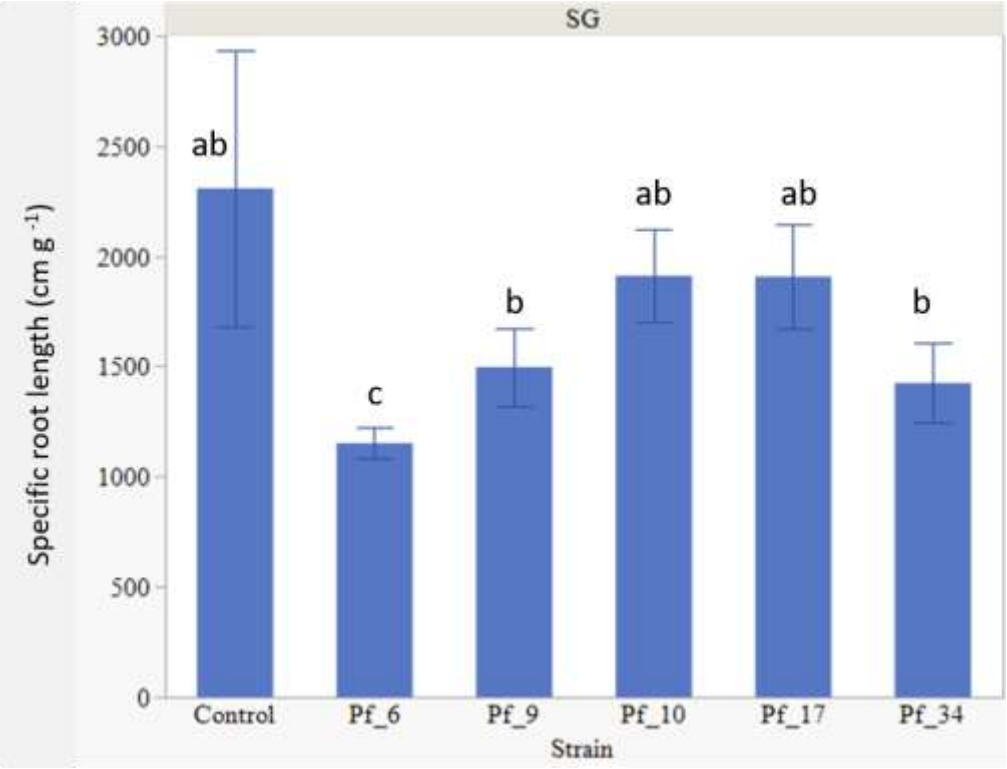

Figure 15: Impact of the different strains on the specific root length of SG (sorghum). Strain number *Pf* 6 resulted in significantly lower specific root length as in comparison to control (P = 0.0091).

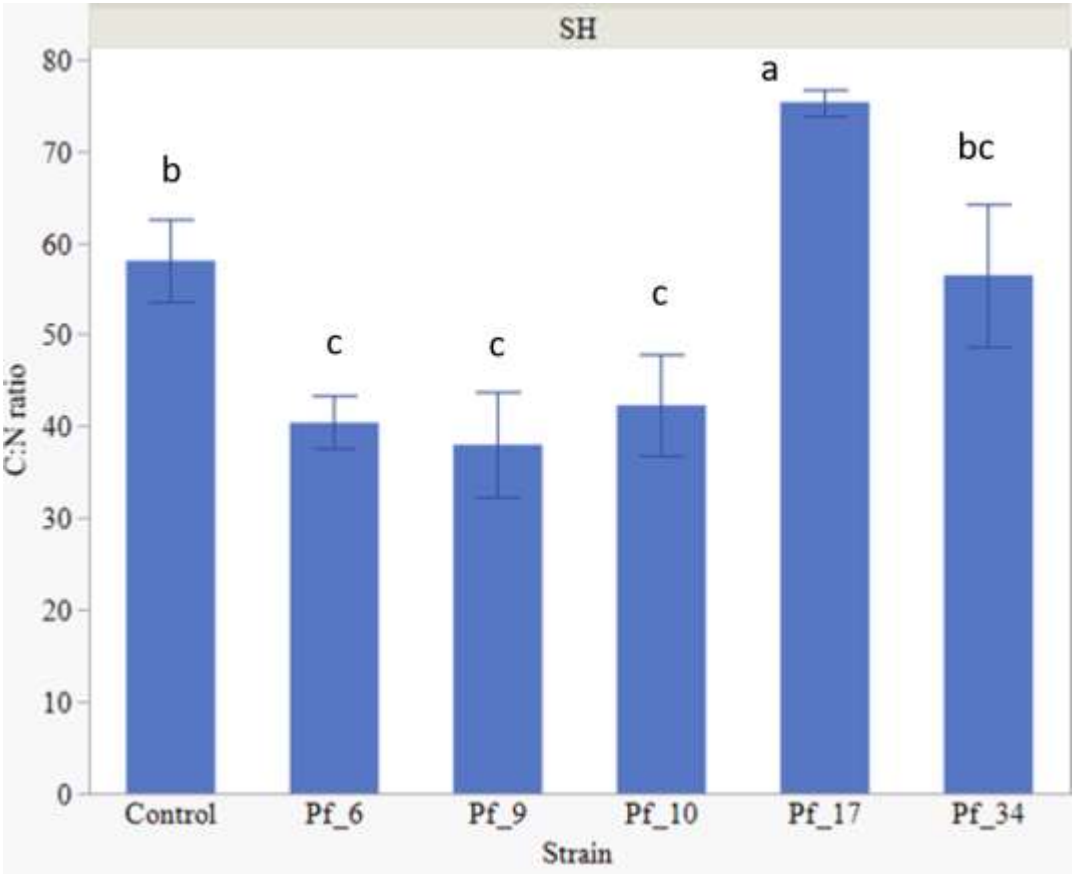

Figure 16: Impact of the different strains on the C:N ratio of SH (sunn hemp). Strain number *Pf* 17 had significantly higher C:N ratio than control ($P < 0.02$). While, strain *Pf* 6 ($P < 0.01$) and strain *Pf* 9 ($P < 0.03$) significantly reduced the C:N ratio. Values are the mean ± standard error of the mean.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Plant growth-promoting rhizobacteria (PGPR) are the soil bacteria, that have several biofertilizing and biocontrol properties and are known to colonize the plant's rhizosphere and improve plant growth and development (Beneduzi et al., 2012). Among various PGPR, *Pseudomonas fluorescens* is a common and prevalent PGPR (Jain et al., 2013). *P. fluorescens* has the potential to improve soil health and plant growth promotion through atmospheric nitrogen fixation, production of phytohormones, solubilization of zinc and phosphate, production of biocontrol agents such as HCN, and siderophore, and activation of induced systematic resistance (ISR) (Bhattacharyya & Jha, 2012). In this study, we isolated 35 strains of *P. fluorescens* from the rhizosphere of sunn hemp based on its canopy coverage (CC) percentage. We isolated 17 strains from high CC and 18 strains from low CC regions. These strains were screened for production of biofertilizing and biocontrol traits and various plant growth promoting traits were tested positive.

Furthermore, we performed plant growth-promoting experiments on sorghum and sunn hemp with five strains of *P. fluorescens*, isolated from the rhizosphere of sunn hemp. *S*train *Pf* 9, positive for ammonia production, strain *Pf* 10, positive for ACC deaminase activity, and strain *Pf* 17, positive for phosphate solubilization were selected . While strain *Pf* 6, negative for all three PGPR traits, and strain *Pf* 34 positive for all three PGP traits were also selected for plant

growth experiments. With the inoculation of five selected strains of *P. fluorescens* on sorghum and sunn hemp seeds, we measured physiological determinant of growth (stomatal conductance and fluorescens), allocational determinant growth such as root mass ratio (RMR), shoot mass ratio (SMR), and leaf mass ratio (LMR), and morphological determinant of growth such as root surface area (RSA) and specific leaf area (SLA). Furthermore, germination rate, total biomass, and total tissue carbon: nitrogen (C:N) concentration were also measured. Our results showed an increase in germination rate after the application of *P. fluorescens* on both sorghum and sunn hemp plant. Meanwhile, the different selected strains had a strain-specific impact on both plant species' growth and biomass allocation. Interestingly, with the inoculation of *P. fluorescens*, there was no significant difference in allocational determinants such as SMR, RMR, and LMR. Furthermore, in sorghum, strain *Pf* 10 resulted in higher stomatal conductance than control. While, C:N ratio was more with the inoculation of strain *Pf* 17 in sunn hemp.

From our study, we found that even though the *P. fluorescens* strains were able to produce PGP traits, they didn't significantly improve the overall plant growth determinants in both sorghum and sunn hemp. So, it proved that even though the PGPRs are known to produce several PGP traits, their effectiveness in plant growth traits may not be successful. Moreover, many PGPR studies are mostly conducted in the greenhouse and growth chamber. To better understand the microbe's interaction and their effectiveness against various environmental stress, PGPR studies need to be performed in natural field conditions. Therefore, further studies are needed to better understand the role and interaction of non-culturable bacteria with natural environmental conditions. Furthermore, in future, effective and eco-friendly PGPR should be produced using advanced technologies such as meta-proteomics, nanotechnology, and

rhizosphere engineering to gain more agricultural yield with minimal impact on the environment (Hakim et al., 2021).

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BIOGRAPHICAL SKETCH

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