TRANSCRIPTOMIC PROFILING OF *Cannabis sativa* SUPPLEMENTED WITH THE PLANT-GROWTH-PROMOTING BACTERIA: *Bacillus velezensis* S141

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ทรานสคริปโตมิกส์ของกัญชาเสริมด้วยแบคทีเรียที่ส่งเสริมการเจริญเติบโต: *Bacillus velezensis* S141

นายภิรมย์ อุ่นคำ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คำสำคัญ: กัญชา/*Bacillus velezensis* S141/plant-growth-promoting bacterium (PGPB)

Cannabis sativa L. เป็นพืชที่มีการใช้ประโยชน์หลากหลาย ทั้งในอุตสาหกรรมสิ่งทอ อาหาร น้ำมัน และยา เพื่อลดผลกระทบต่อสิ่งแวดล้อมจากการใช้ปุ๋ยเคมี งานวิจัยนี้ได้ศึกษาประสิทธิภาพของ *Bacillus velezensis* S141 ในฐานะแบคทีเรียส่งเสริมการเจริญเติบโตของพืช (PGPB) ในกัญชา โดยประเมินผลกระทบของ S141 ต่อการเจริญเติบโตของพืชและวิเคราะห์ทรานสคริปโตมเพื่อระบุกลไกชีวโมเลกุลที่เกี่ยวข้อง ผลการศึกษาพบว่าการให้หัวเชื้อ S141 สามารถกระตุ้นการเจริญเติบโตของกัญชาได้อย่างมีนัยสำคัญทั้งในสภาพห้องปฏิบัติการและโรงเรือน นอกจากนี้ การวิเคราะห์เชิงปริมาณด้วยเทคนิค qPCR พบว่าแบคทีเรียสะสมอยู่ในใบมากที่สุด รองลงมาคือลำต้นและราก การวิเคราะห์ทรานสคริปโตมระบุยีนที่มีการแสดงออกแตกต่างกัน (differentially expressed genes; DEGs) จำนวน 976 ยีน โดยยีนที่ถูกกระตุ้นมีความเกี่ยวข้องกับกระบวนการเมแทบอลิซึม กระบวนการระดับเซลล์ และกิจกรรมของเอนไซม์เร่งปฏิกิริยา โดยเฉพาะในกลไกลการสังเคราะห์ของฟีนิลโพรพานอยด์ การปฏิสัมพันธ์ระหว่างพืชกับเชื้อโรค และกลไลการส่งสัญญาณของฮอร์โมนพืช นอกจากนี้ การทดลองใช้ S141 สายพันธุ์กลายที่สูญเสียความสามารถในการสังเคราะห์ออกซินและไซโตไคนิน พบว่าความสามารถในการส่งเสริมการเจริญเติบโตของพืชลดลง ซึ่งยืนยันบทบาทสำคัญของฮอร์โมนเหล่านี้ในพัฒนาการของกัญชา ผลการศึกษานี้ชี้ให้เห็นถึงศักยภาพของ S141 ในการส่งเสริมการเจริญเติบโตของกัญชาอย่างยั่งยืน และให้ข้อมูลเชิงลึกเกี่ยวกับกลไกที่เกี่ยวข้อง ซึ่งสามารถนำไปประยุกต์ใช้ในระบบการเกษตรที่เป็นมิตรต่อสิ่งแวดล้อมได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา _____

PHIROM AUNKAM : TRANSCRIPTOMIC PROFILING OF *Cannabis sativa* SUPPLEMENTED WITH THE PLANT-GROWTH-PROMOTING BACTERIA: *Bacillus velezensis* S141 THESIS ADVISOR: ASSIST. PROF. Pakpoom Boonchuen, Ph.D., 74 PP.

Keywords: *Cannabis sativa* L./*Bacillus velezensis* S141/plant-growth-promoting bacterium (PGPB)

Cannabis sativa L. has a variety of uses, including textiles, food, oil, and medicine. In response to environmental concerns regarding chemical fertilizers, *Bacillus velezensis* S141 was examined as a plant-growth-promoting bacterium (PGPB) for cannabis. This study evaluated the effects of S141 on cannabis growth and utilized transcriptomic analysis to identify the responsive pathways. Cannabis inoculation with S141 significantly increased growth in laboratory and field environments, with most of the bacteria residing in the leaves, followed by the stems and roots, as determined by quantitative polymerase chain reaction (qPCR). Transcriptomic analysis revealed 976 differentially expressed genes. Upregulated genes were associated with metabolism, cellular processes, and catalytic activities, especially in the biosynthesis of phenylpropanoid, plant–pathogen interactions, and hormone signaling pathways. S141 mutants deficient in the production of auxin and cytokinin displayed reduced growth enhancement, which affirmed the roles of these hormones in cannabis development. These findings emphasize the potential of S141 as a sustainable growth promoter for cannabis and provide insights into the underlying pathways it influences.

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LIST OF ABBREVIATIONS

°C	=	Degree celsius
µ	=	Micro
cDNA	=	Complementary DNA
CFU	=	Colony forming unit
DEPC	=	Diethyl pyrocarbonate
DNA	=	Deoxyribonucleic acid
DAI	=	Days post-inoculation
gDNA	=	Genomic DNA
g	=	Gram
h	=	Hour
GO	=	Gene Ontology
KEGG	=	Kyoto encyclopedia of genes and genomes
l	=	Liter
m	=	Milli
min	=	Minute
ml	=	Milliliter
n	=	Nano
NaCl	=	Normal saline
pH	=	Potential of hydrogen ion
q	=	Quantitative
RNA	=	Ribonucleic acid
SD	=	Standard deviation
Seq	=	Sequencing
v/v	=	Volume per volume
w/v	=	Weight per volume
%	=	Percent

CHAPTER I
INTRODUCTION

1.1 Background

Cannabis sativa L., more commonly known as marijuana, belongs to the Cannabaceae family and is widely cultivated around the globe (Kanabus et al., 2021). Cannabis has served diverse purposes since ancient times; it has acted as a folk medicine, a psychoactive drug, and a material in the production of textiles and rope (Andre et al., 2016; Dariš et al., 2019; Rupasinghe et al., 2020). With the global interest in cannabis cultivation on the rise, optimizing plant health, productivity, and cultivation methods has become critically important. Among several options, organic cannabis farming has gained popularity due to concerns connected to the health, sustainability, and quality of the produce (Bruce et al., 2022). One promising approach for enhancing the growth and overall health of cannabis plants involves the use of plant-growth-promoting bacteria (PGPBs). In contemporary agriculture, addressing the dual challenges of increasing food demand and environmental sustainability has become imperative. PGPBs have emerged as sustainable bioresources capable of enhancing crop productivity and resilience in various agroecosystems. These bacteria not only improve nutrient availability and uptake efficiency but also enhance root development and stimulate systemic resistance in plants, thereby mitigating biotic and abiotic stresses (Bashan & De-Bashan, 2010; Hayat et al., 2010). Moreover, some PGPBs can produce enzymes like 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which is able to alleviate plant stress by reducing ethylene stress levels (Glick, 2014). In the context of cannabis, the plant faces numerous challenges, such as nutrient depletion and susceptibility to pathogens. Incorporating PGPBs into cannabis farming has shown promise for enhancing plant growth, yield, and resilience. For instance, the supplementation of *Pseudomonas* and *Bacillus* during cannabis cultivation was found

to improve plant health and productivity by suppressing soil borne pathogens and optimizing nutrient availability (Backer et al., 2018)

The efficacy of *Azospirillum brasilense* B. in enhancing root development and nutrient uptake in cannabis has been noted, while *Bacillus* species have demonstrated benefits through enhanced nitrogen availability and phytohormone production (Lyu et al., 2020). Hence, integrating PGPBs into cannabis cultivation could support sustainable agriculture and enhance the crop's economic and environmental sustainability.

Among PGPBs, *Bacillus velezensis* (Ruiz-Garcia et al., 2005) has emerged as a key species with broad applications in agriculture. This bacterium is noted for its ability to produce bioactive metabolites such as indole-3-acetic acid (IAA), siderophores, and antimicrobial peptides, which collectively enhance plant growth, nutrient assimilation, and stress tolerance. Furthermore, its biocontrol properties effectively suppress phytopathogens, including fungi and bacteria, thereby reducing reliance on chemical pesticides and promoting eco-friendly practices (Cantoro et al., 2021; Chowdhury et al., 2015). The potential of *B. velezensis* in improving soil health, remediating contaminated soils, and increasing crop productivity makes it a cornerstone in sustainable agricultural initiatives (Fan et al., 2017; Wu et al., 2014).

One of the *B. velezensis* strains, specifically S141, has gained attention for its versatile applications in agriculture. It was isolated from the soybean (*Glycine max* (L.) Merr. (Fabaceae)) rhizosphere, which functions as a robust plant-growth-promoting rhizobacterium and a biocontrol agent against phytopathogens (Sibponkrung et al., 2020). Studies have reported its proficiency in enhancing crop yields and disease resistance in several crops, including rice, soybean, and maize (Kondo et al., 2023; Sibponkrung et al., 2020; Songwattana et al., 2023). However, research regarding the functional properties and utilization of *B. velezensis* S141 in cannabis, especially concerning growth-developing impacts, is scarce.

In this study, we investigated the use of S141 as a PGPB in cannabis cultivation and the potential mechanisms that activate the biological pathways in cannabis. This was achieved through a transcriptomic analysis of the bacterium's influence.

1.2 Research objectives

1.2.1 To investigate the plant growth-promoting effect of *Bacillus velezensis* S141 in *Cannabis sativa*

1.2.2 To understand the molecular mechanism of *C. sativa* after inoculation with *B. velezensis* S141 using transcriptomic technology

CHAPTER II

RITERATURE REVIEWS

2.1 Cannabis sativa

2.1.1 Taxonomy and Classification

Cannabis belongs to the Cannabaceae family, a group of flowering plants comprising approximately 102 species. Despite exhibiting significant morphological, chemical, and utilitarian diversity, the prevailing view among botanists is that cannabis constitutes a single species, *Cannabis sativa* L. However, debate persists regarding whether cannabis should be classified as multiple species, particularly *Cannabis indica* and *Cannabis ruderalis*. Attempts to further categorize cannabis into subspecies or varieties, based on morphological traits, cannabinoid content, and geographic origin, have not gained widespread acceptance and are often inconsistently applied. This is particularly evident in the use of the terms "sativa" and "indica," which are frequently employed in the cannabis industry to describe distinct cannabis characteristics but do not accurately reflect true botanical classifications.

Kingdom: Streptophyta

Subkingdom: Viridiplantae

Phylum: Streptophyta

Superdivision: Spermatophyta (seed plants)

Division: Magnoliophyta (flowering plants)

Class: Magnoliopsida

Subclass: Magnoliidae

Order: Rosales

Family: Cannabaceae

Genus: *Cannabis*

Species: *Cannabis sativa L*

2.1.2 Growth Habits

Cannabis is a versatile plant in the Cannabaceae family renowned for its wide range of growth and morphological characteristics. This variability reflects its long history of cultivation, breeding, and hybridization for various purposes, including fiber production, seed oil extraction, and medical and recreational use. Understanding the growth and morphology of cannabis is essential to improving cultivation, maximizing yield, and tailoring plant characteristics to specific applications.

Figure 1. Characteristics of *Cannabis sativa* L. (Retrieved April 29, 2024, from https://batsmg.m.wikipedia.org/wiki/Abruozdielis:Cannabis_sativa_001.JPG).

Cannabis is generally an herbaceous plant, although some varieties tend to be perennial under certain environmental conditions. Cannabis displays a wide range of growth characteristics, which are influenced by a complex interaction of

genetic, environmental, al, and cultivation factors. Plant heights can vary greatly, from dwarf varieties less than 1 m tall to giant varieties exceeding 5 m tall (Clarke & Merlin, 2016). Much of this variability is due to genetic differences between strains and environmental factors, such as light intensity, nutrient availability, and water content. There are also significant differences in branching patterns, ranging from sparse to heavily branched, which affect the overall shape and yield of the plant. Cannabis flowering is largely influenced by photoperiod. The plant begins flowering as the days shorten in autumn (Small, 2015). However, some auto-flowering varieties have been developed that flower independently of photoperiod, depending on age or stage of development.

2.1.3 Morphology

Cannabis has a strong and adaptable root system, typically characterized by a prominent taproot with extensive lateral branching. This root structure allows the plant to efficiently absorb water and nutrients from different depths of soil and allows it to tolerate a wide range of environmental conditions (Clarke & Merlin, 2016). The stem is erect, ridged, and often hollow, providing structural support for the plant and facilitating nutrient transport (Small, 2015). The leaves are palmate, typically with 5–11 serrated leaflets radiating from a central point (Clarke & Merlin, 2016). The number of leaflets can vary depending on the variety, growth stage, and environmental conditions. The arrangement of the leaves on the stem, known as phyllotaxy, can vary, with opposite or alternating patterns occurring from one variety to another.

2.1.4 Flowers and inflorescences

Cannabis is generally an unisexual plant, meaning that male and female flowers are produced on separate plants. However, separate male and female flowers may also occur. Particularly in certain strains or under specific environmental conditions (Small, 2015), male flowers are small and grouped into inflorescences, which release pollen for fertilization. Female flowers are grouped into dense, conical inflorescences, often called “buds.” These inflorescences are covered with glandular trichomes that produce resins containing cannabinoids and terpenes, which are responsible for the plant’s diverse pharmacological effects.

2.1.5 Factors affecting growth and morphology

Genetic factors play a major role in determining the growth and morphology of cannabis (Small, 2015). Different strains and cultivated varieties display different growth patterns, branching habits, leaf patterns, and cannabinoid profiles due to their specific genetic makeup. Environmental conditions, including light intensity, temperature, water content, and nutrient levels, also significantly affect the growth and morphology of cannabis (Clarke & Merlin, 2016). For example, plants grown under high-intensity light tend to be shorter and bushier, while plants grown under low-intensity light tend to be taller and longer. In addition, cultivation practices, such as pruning, training, and spacing, can significantly affect the shape, size, and yield of a plant.

2.1.6 Adaptability and Varieties

Cannabis has demonstrated remarkable adaptability to a wide range of environments. From temperate to tropical regions, this adaptability is reflected in a wide range of morphological and growth characteristics. For example, some plant species adapt to arid environments by developing thick, leathery leaves to reduce water loss, while others adapt to humid environments by developing thin, delicate leaves to increase transpiration. Plant morphology can also be influenced by abiotic factors, such as herbivory and competition. For example, some plant species develop thorns or prickles to fend off herbivores, while others develop tall, slender stems to compete for light in dense vegetation.

2.1.7 Chemical Composition

Cannabis sativa L. is a species with a long history of use by humans and possesses a complex chemical profile that includes hundreds of different compounds. This phytochemical diversity is the foundation of the plant's multifaceted effects and therapeutic potential. A comprehensive understanding of the chemical makeup of cannabis is essential for researchers, growers, and consumers to make informed decisions regarding its cultivation, use, and safety.

Cannabinoids are a group of compounds unique to cannabis and are crucial for its pharmacological activity. These molecules interact with the endocannabinoid system (ECS) in humans and other mammals, a complex signaling network involved in regulating a wide range of physiological processes, including pain

perception, appetite, mood regulation, and memory. The most widely studied cannabinoids are Δ9 - tetrahydrocannabinol (THC) and cannabidiol (CBD). THC is responsible for the psychoactive effects of cannabis, which include euphoria, cognitive changes, and impaired judgment. In contrast, CBD is non-intoxicating and has garnered significant attention for its potential therapeutic benefits, including anti-inflammatory, anti-anxiety, and anti-seizure properties (Russo, 2011). In addition to these two prominent cannabinoids, other compounds such as cannabinol (CBN), cannabigerol (CBG), and tetrahydrocannabivarin (THCV) also contribute to the overall effects of cannabis and are being actively studied for their potential therapeutic applications (Hanuš et al., 2016).

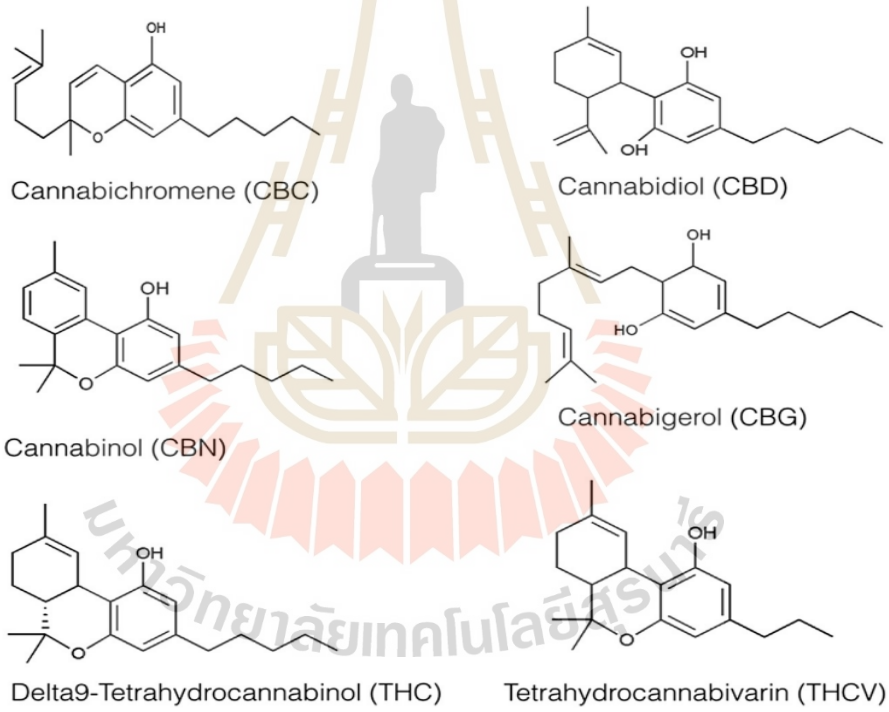

Figure 2. The structure of cannabinoids present in Cannabis (Marcu, 2016).

Terpenes are aromatic compounds commonly found throughout the plant kingdom and are responsible for the distinct aromas and flavors of different cannabis strains. However, the role of terpenes extends beyond sensory perception. They can interact with other cannabinoids and compounds in the plant, potentially modifying their pharmacological effects. This synergistic interaction, often referred to

as the “entourage effect,” suggests that the combined effects of these compounds exceed the sum of their individual effects (Russo, 2011). Common terpenes in cannabis include myrcene, limonene, pinene, and linalool, each with its unique aroma and potential therapeutic properties (Booth & Bohlmann, 2019). For example, myrcene is associated with sedative effects, while limonene is believed to enhance mood. The diverse terpene profiles of cannabis strains contribute not only to their sensory characteristics but also to their therapeutic potential.

Flavonoids, a group of polyphenol compounds found in many plant species, are another important component of the chemical makeup of cannabis. These compounds contribute to the plant’s pigmentation and have been linked to various health benefits, including antioxidant, anti-inflammatory, and anti-cancer properties (Andre et al., 2016) Cannabis contains a variety of flavonoids, including cannaflavin A, cannaflavin B, and apigenin. Cannaflavins A and B, which are unique to cannabis, have potent anti-inflammatory properties comparable to traditional non-steroidal anti-inflammatory drugs (NSAIDs) (Barrett & Bradley, 2016). These flavonoids may play a role in cannabis's effectiveness in treating inflammatory conditions such as arthritis and inflammatory bowel disease.

The chemical composition of cannabis is constantly changing and is influenced by several factors, including genetics, environment, and cultivation practices. Different strains or chemotypes exhibit unique chemical profiles due to their genetic makeup, resulting in variations in cannabinoid and terpene content. Environmental factors, such as light intensity, temperature, and nutrient availability, can affect the production of these compounds (Wanas et al., 2020). Additionally, cultivation practices, including harvest timing and drying methods, can influence the final chemical composition of the plant material. Understanding these factors is crucial for cultivating cannabis with specific chemical profiles suited to various therapeutic or recreational uses.

2.1.8 Uses and Applications

Cannabis, which includes a variety of chemovars with different concentrations of cannabinoids such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), has seen a renewed interest in its therapeutic potential. Beyond its historical use for recreational and spiritual purposes, modern research is uncovering

the diverse applications of cannabis in managing various health conditions, leading to a reevaluation of its role in contemporary medicine. One of the most promising areas for cannabis-based therapies is the treatment of neurological disorders. CBD, in particular, has gained significant attention for its anticonvulsant properties. Clinical trials have demonstrated its effectiveness in reducing the frequency of seizures in individuals with refractory epilepsy, especially in cases such as Dravet syndrome and Lennox-Gastaut syndrome. This often leads to a reduced need for multiple medications and improves the quality of life (Devinsky et al., 2017; Thiele et al., 2018) Additionally, preclinical and observational studies suggest that cannabinoids may have neuroprotective effects in conditions like multiple sclerosis and Parkinson's disease, potentially helping to slow disease progression and alleviate symptoms (Giacoppo et al., 2014).

In addition to its neurological applications, cannabis shows promise in the realm of mental health. Although the evidence is still developing and more rigorous research is needed, preliminary findings indicate that CBD may have anxiety-reducing (anxiolytic) and antipsychotic properties. Research has investigated its potential to alleviate anxiety symptoms in individuals with social anxiety disorder and post-traumatic stress disorder (PTSD) (Blessing et al., 2015). Furthermore, some studies suggest that CBD may help reduce psychotic symptoms in schizophrenia, potentially serving as an alternative or complement to traditional antipsychotic medications (McGuire et al., 2018). Cannabis also holds promise in palliative care, as its ability to relieve a variety of symptoms can significantly improve the well-being of patients with life-limiting illnesses. It is effective in managing chronic pain, reducing nausea and vomiting caused by chemotherapy, and stimulating appetite, thus enhancing the quality of life for patients with cancer and other debilitating conditions (Portenoy et al., 2012). Additionally, emerging evidence suggests that cannabinoids may have antitumor properties, inhibiting the growth and spread of cancer cells in preclinical models (Velasco et al., 2012). While further research is required to translate these findings into clinical practice, the potential benefits of cannabis in palliative care cannot be overlooked.

Despite the increasing evidence supporting the therapeutic uses of cannabis, concerns about its application persist. Significant issues include the potential

for abuse, dependence, and negative effects on cognitive function, especially among adolescents and young adults (Volkow et al., 2014). Additionally, the complex relationship between cannabinoids and the endocannabinoid system requires further investigation to fully understand the mechanisms of action, optimal dosages, and long-term effects of cannabis use. As our scientific understanding of cannabis evolves and the legal landscape changes, it is crucial to adopt a balanced approach that weighs the potential benefits against the associated risks, ensuring the safe and effective integration of cannabis into medical practice.

2.2 Residues in cannabis and other crops cultivation

Continuous sources of both leadership and observation pose significant challenges for agriculture today, particularly in the health of organisms and the quality of cannabis seeds and other crop production. Orange residues can result from the use of pesticides, heavy fertilizers, and microbial additives, which can persist and accumulate as compounds. This accumulation influences both growers and regulatory agents, especially in medical applications, where longer residue limits are often required due to potential inhalation or deficiencies (Small, 2017). Unlike traditional crops, certain products can be developed without needing to meet initial food control regulations, allowing access to a broader range of compounds. Regulatory frameworks are evolving primarily to address these issues (Eichler et al., 2023), but a crucial area for progress lies in harmonizing global standards (Upton, 2018).

Pesticide residues are the most common problem for crops, especially without synthetic lead, such as pyrethroids, organophosphates, and neonicotinoids. Control and disease systems, but most residues may persist on the processing core. Chemicals that can be monitored for quantification and filtration of the endonuclease Carcinogenic and morbidity to pain (Van Maele-Fabry et al., 2017). In the long-term control of signaling, substances can be monitored normally due to specific reasons and quality control and accumulate lipophilic substances (Citti et al., 2018). Analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and later tandem chromatography-mass spectrometry (LC-MS/MS) have been recorded for a long time for the number of residues, providing both a control system and a memory of the meat (Strashnov et al., 2024).

Fertilizer waste to fertilizer waste with important components that cause more problems. Inside most fertilizers, nitrates dissolve into water causing eutrophication and directions in drinking water sources (López-Bellido et al., 2010). In the cultivation of cannabis, Hydronic systems and the use of concentrated nutrients have clearly shown increased accumulation of manure residues. Increased nitrate and phosphate residues in cannabis products (Caplan et al., 2017) have been observed to be a good balance of toxic aromas such as nitrosamines. Scientists must strike a balance between delivering good nutrients and environmentally absorbable spices, including vegetarian,s and slow release fertilizers (Chien et al., 2009).

Residues of heavy metals such as lead, cadmium, and arsenic continue to be a concern in important plant species. Heavy metals are particularly toxic to plants, and heavy metals are particularly toxic to soils or water sources (Ahmed et al., 2022). The highest levels can be accumulated in leaves, which is why consumers may use cannabis for medical purposes (Potter, 2014). Strategies to monitor heavy metal residues to soil remediation methods such as Soil remediation with plants and the use of biochar in the re-examination of more time-consuming raw materials (Komárek et al., 2013). Developing methods to reduce heavy metal concentrations to a minimum is important for both health and agricultural purposes. Microbial residues and pathogens such as Escherichia coli and Aspergillus species pose a risk to both hardware and public health. Cannabis, which often takes time to view without processing or visible components, is the source of the investigation found to be cruise and checkpoint (McPartland & Guy, 2017). For other crops, investigations are often based on food loss at frequent or adherence to practices. Organizational microbial management strategies, the use of control agents, and post-storage inspections are most important in controlling safety standards. In standard microbial residue testing, safety aspects (Ganguly et al., 2017),quality studies using advanced techniques provide assurance and reliability (Shah et al., 2020). This highlights the long-term persistence of cannabis and other crops. It emphasizes the need to manage pesticides, fertilizers, heavy metals, and microbial residues that can be harmful to consumers and the environment. Effective management is essential in farming practices to ensure that these harmful substances are controlled. Research should

focus on developing strategies that establish and adapt safety standards compatible with both consumer health and environmental sustainability.

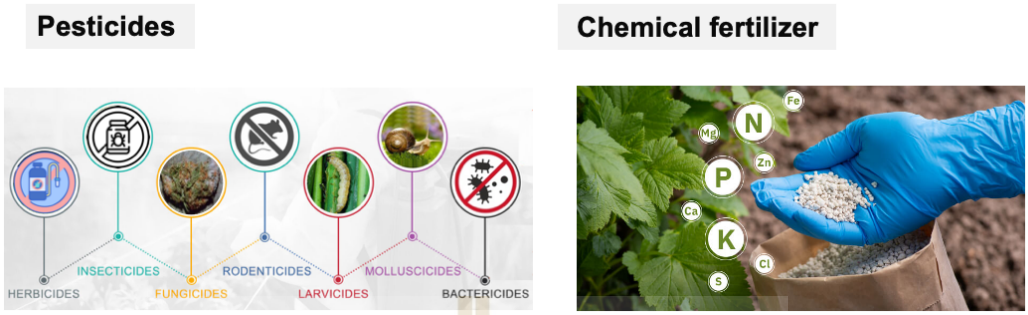

Figure 3. Residues associated with the agricultural practices of cannabis and various crops (Moscariello et al., 2021).

2.3 Plant Growth-Promoting Microorganisms (PGPM)

Plant growth-promoting microorganisms (PGPM) represent a diverse group of microbes that foster plant growth and enhance agricultural productivity by improving nutrient availability, promoting pathogen resistance, and aiding in stress tolerance. PGPM includes a wide range of organisms such as bacteria, fungi, and actinomycetes, all of which interact with plants in unique ways to improve plant health and yield. The use of PGPM in sustainable agriculture has gained significant attention due to its potential to reduce the reliance on chemical fertilizers, pesticides, and herbicides. The beneficial effects of these microorganisms on plant growth are driven by a range of mechanisms, including nitrogen fixation, phosphorus solubilization, hormone production, and disease suppression.

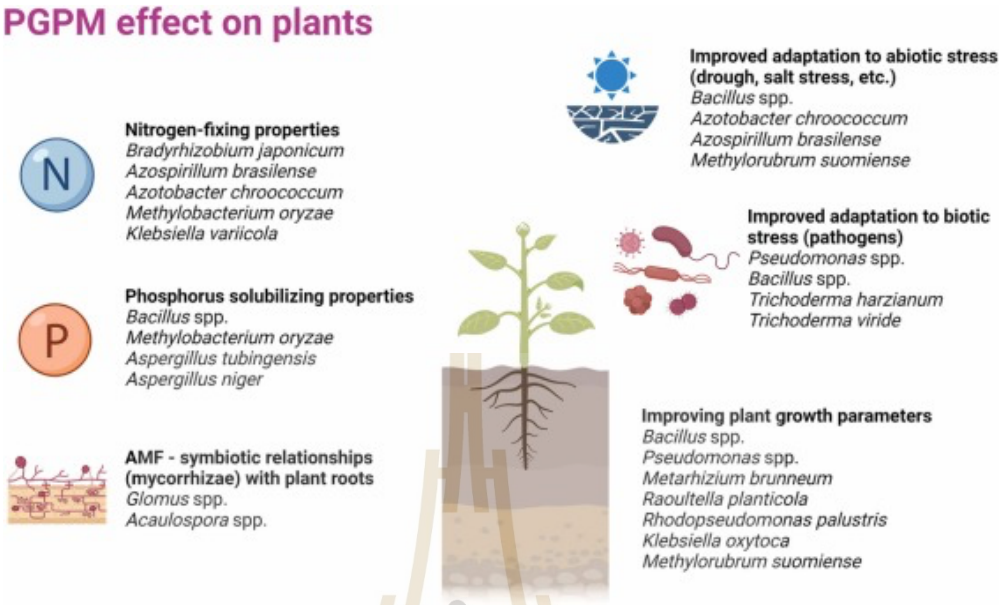

Figure 4. The impact of Plant Growth-Promoting Microorganisms (PGPM) on plant growth. Types of Plant Growth-Promoting Microorganisms (PGPM) (Szopa et al., 2022).

PGPMs can be broadly categorized based on their biological classification and mechanisms of action. The most commonly studied PGPM include bacteria, fungi, and actinomycetes.

2.3.1. Bacterial PGPM

Bacteria are the most studied and diverse group of PGPM. They inhabit various niches within the rhizosphere, endosphere, and soil, where they interact with plants. Some common genera of plant growth-promoting bacteria include Pseudomonas, *Bacillus*, *Azospirillum*, *Rhizobium,* and *Enterobacter*. These bacteria enhance plant growth through several mechanisms, such as nitrogen fixation, nutrient solubilization, production of plant growth regulators, and pathogen suppression.

Table 1. Plant growth-promoting bacterial strains are utilized as biocontrol agents to combat pathogenic microbes.

Host	Pathogen	Disease	strains	References
Apple	*Mucor piriformis*	*Mucor* rot	*Pseudomonas fluorescens*	(Wallace et al., 2018)
Banana	*Fusarium* spp.	Postharvest diseases	*Trichoderma* spp.	(Snehalatharani et al., 2021)
Wheat	*Stagonospora nodorum*	*Stagonospora nodorum*	*Bacillus subtilis* 26DCryChS	(Maksimov et al., 2020)
Soybean	*Fusarium solani, Macrophomina phaseolina*	Root rot	*Bradyrhizobium* sp.	(Parveen et al., 2019)
	Sclerotinia sclerotiorum	White mold	*Butia archeri*	(Vitorino et al., 2020)
Strawberry	*Macrophomina phaseolina*	Charcoal rot disease	*Azospirillum brasilense*	(Viejobueno et al., 2021)
	Botrytis cinerea	Gray mold	*Bacillus amyloliquefaciens* Y1	(Maung et al., 2021)
Maize	*Fusarium graminearum*	Stalk rot	*Bacillus methylotrophicus*	(Cheng et al., 2019)
Mungbean	*Cercospora canescens*	*Cercospora* Leaf Spot	*Bacillus velezensis* S141	(Songwattana et al., 2023)
Tomato	*Fusarium oxysporum* f. sp. *lycopersici*	*Fusarium* wilt	*Brevibacillus brevis*	(Chandel et al., 2010)
Tea	*Colletotrichum* sp,	Shoot necrosis	*Trichoderma camelliae*	(Chakruno et al., 2022)

2.3.2 Fungal PGPM

Fungi, especially mycorrhizal fungi, play an important role in promoting plant growth through symbiotic relationships with plant roots. These fungi enhance nutrient uptake, particularly for nutrients such as phosphorus, and also provide protection against soil-borne pathogens.

Table 2. Plant growth-promoting fungal strains are utilized as biocontrol agents to combat pathogenic microbes.

Host	Pathogen	Disease	strains	References
Rice	*Helminthosporium oryzae, Bipolaris oryzae*	*Leaf brown spot*	*Trichoderma viride, Trichoderma harzianum, Trichoderma hamatum*	(Khalili et al., 2012; Mau et al., 2022)
Tomato	*Sclerotium rolfsii* *Fusarium oxysporum f. sp. lycopersici* *Rhizophagus intraradices*	Southern blight Wilt *Verticillium* wilt	*Stenotrophomonas maltophilia PPB3* *Penicillium oxalicum* *Penicillium pinophilum*	(Sultana & Hossain, 2022) (Murugan et al., 2020) (Ibiang et al., 2021)
Sweet potato	*Ceratocystis fimbriata*	Black rot disease	*Pseudomonas chlororaphis subsp. aureofaciens SPS-41*	(Zhang et al., 2021)

2.3.3 Mechanisms of Plant Growth Promotion by PGPM

PGPM promotes plant growth through a variety of mechanisms, which can be broadly categorized into direct and indirect mechanisms.

2.3.3.1 Direct Mechanisms

- Nutrient Acquisition: PGPM enhances the availability of essential nutrients, particularly nitrogen and phosphorus, through nitrogen fixation and

phosphate solubilization. These processes increase the nutrient supply to plants, leading to enhanced growth (Malgioglio et al., 2022).

- Hormonal Regulation: Many PGPMsM produce plant hormones such as auxins, cytokinin's, and gibberellins. These hormones promote root elongation, stimulate shoot growth, and enhance flowering. By regulating these processes, PGP contributes to overall plant development (Glick, 2014)

- Improvement of Root System: The application of PGPM can stimulate root growth by producing auxins and other growth regulators. Enhanced root growth improves the plant's ability to absorb water and nutrients, leading to better overall plant health (Bashan et al., 2013).

2.3.3.2 Indirect Mechanisms

- Pathogen Suppression: PGPM can suppress plant diseases by outcompeting pathogens for space and nutrients, producing antimicrobial compounds, or inducing systemic resistance in plants. This reduces the need for chemical pesticides and supports healthy crop growth (Vinale et al., 2014)

- Environmental Stress Resistance: PGPM enhances plant resistance to abiotic stresses such as drought, salinity, and temperature extremes. These microorganisms produce Osmo protectants, modulate ion uptake, and activate plant defense mechanisms to protect plants from environmental stress (Sharma et al., 2013).

2.3.4 Applications of PGPM in Agriculture

PGPMsM are utilized in agriculture to improve soil health, enhance plant growth, and protect crops from diseases. The following are some common applications of PGPM**.**

2.3.4.1 Biofertilizers

PGPM such as nitrogen-fixing bacteria (*Rhizobium*, *Azospirillum*) and phosphate-solubilizing bacteria (*Bacillus*, *Pseudomonas*) are used as biofertilizers to supplement or replace chemical fertilizers. These microorganisms promote nutrient availability, enhance soil fertility, and reduce the environmental impact of synthetic fertilizers (Bashan et al., 2013)

2.3.4.2 Biocontrol

PGPMsPM are used as biocontrol agents to manage plant diseases caused by fungi, bacteria, and viruses. Trichoderma species, Pseudomonas species, and Bacillus species are examples of microorganisms that can suppress plant pathogens and reduce the reliance on chemical pesticides (Vinale et al., 2014)

2.3.4.3 Stress Management

PGPM can also be employed to improve crop resilience under stressful conditions such as drought, salinity, and extreme temperatures. By enhancing nutrient uptake and promoting plant growth, PG helps plants tolerate environmental stress, leading to higher yields and improved crop productivity (Malgioglio et al., 2022).

2.4 Plant growth-promoting bacteria (PGPBs)

Plant growth-promoting bacteria (PGPB) are indispensable in enhancing agricultural productivity by alleviating biotic and abiotic stressors. These microorganisms inhabit the rhizosphere, facilitating plant growth through diverse mechanisms, including nitrogen fixation, production of phytohormones, phosphate solubilization, and the synthesis of siderophores. For instance, *Azospirillum* and *Pseudomonas spp*. are renowned for promoting root architecture modification and enhancing nutrient uptake under stress conditions like salinity (Egamberdieva et al., 2019; Poria et al., 2022). Moreover, PGPB can mitigate oxidative stress in plants by enhancing antioxidant enzyme activities, contributing to improved drought and salt tolerance (Ramakrishna et al., 2020; Saikia et al., 2018).

One of the pivotal attributes of PGPB is their ability to confer resilience against saline soils, a challenge affecting approximately 20% of the world's arable land (Ondrasek et al., 2021). Salinity induces osmotic stress in plants, limiting water uptake and nutrient assimilation. Specific strains of *Bacillus* and *Halomonas* have demonstrated the ability to modulate the expression of salt-stress-responsive genes, thereby enhancing plant survival in saline environments (Qurashi & Sabri, 2012). Additionally, the production of exopolysaccharides by these bacteria helps in soil aggregation, thus improving root adhesion and water retention in the rhizosphere (Chen et al., 2016).

In heavy metal contaminated soils, PGPB enhances phytoremediation efficacy by transforming metals into less bioavailable forms or by sequestering them within plant tissues. For example, *Bacillus* and *Proteus* strains have shown significant potential in promoting plant growth while remediating lead and cadmium from polluted sites (Sorour et al., 2022).This dual role of PGPB in bioremediation and plant growth promotion underscores their utility in reclaiming degraded lands for agriculture (Kaya et al., 2024).

PGPB also demonstrates the potential to improve the productivity of marginal lands through integrated phytotechnologies. Marginal soils often suffer from nutrient deficiencies, salinity, or contamination. The use of microbial consortia tailored to specific soil conditions has proven to stabilize plant growth and optimize soil fertility. For instance, microbial formulations with strains such as Pseudomonas thivervalensis have significantly increased biomass and nutrient uptake in Brassica napus grown in copper-contaminated soils (Ren et al., 2019). Such approaches exemplify sustainable agricultural practices to rehabilitate degraded ecosystems.

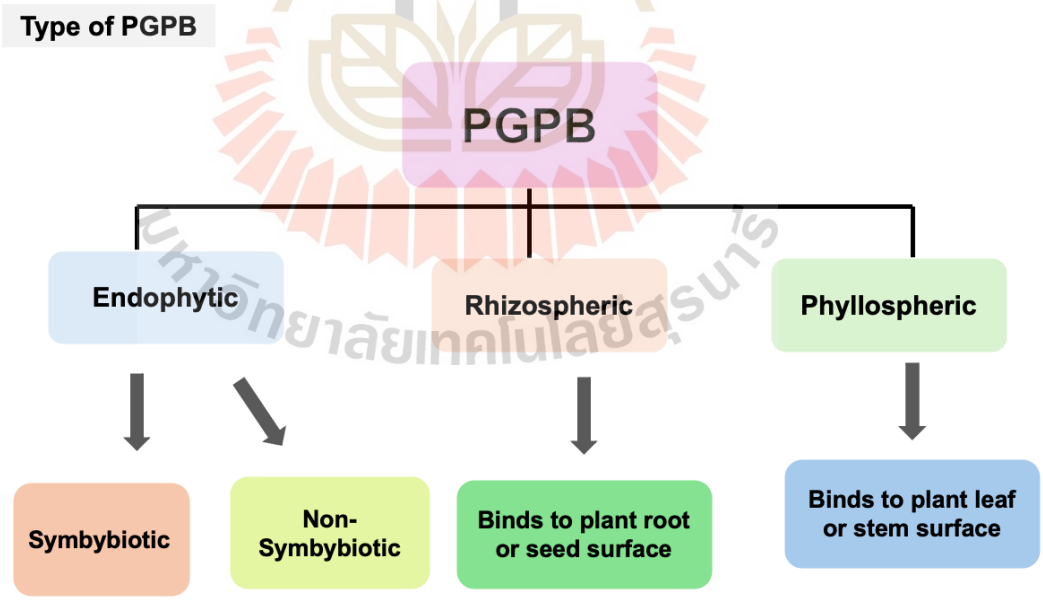

Figure 5. Plant growth-promoting bacteria (PGPB) (Stegelmeier et al., 2022).

2.4.1 Benefits of Plant Growth-Promoting Bacteria (PGPBs) in Cannabis and Other Crops

Plant growth-promoting bacteria (PGPBs) are vital for enhancing the growth, productivity, and resilience of cannabis and other economically significant crops. In cannabis, PGPBs like Bacillus and Pseudomonas species facilitate nutrient acquisition by solubilizing phosphate and fixing atmospheric nitrogen, which are critical for the plant's rapid vegetative and flowering phases (Backer et al., 2018). These microorganisms also produce phytohormones such as indole-3-acetic acid (IAA), which stimulates root elongation and branching, leading to improved nutrient uptake (Numan et al., 2018). Moreover, the inoculation of cannabis with PGPBs has been linked to higher cannabinoid yields, emphasizing their role in secondary metabolite production under controlled cultivation conditions (Lyu et al., 2020). In addition to nutrient acquisition, PGPBs protect plants from abiotic stresses like salinity and drought, challenges commonly faced in cannabis cultivation. *Bacillus subtilis* and *Azospirillum brasilense* produce exopolysaccharides that improve soil structure, enhancing water retention and alleviating salt stress in the rhizosphere (Egamberdieva et al., 2019). Furthermore, these bacteria increase the expression of stress-responsive genes, enabling crops like rice and wheat to withstand adverse conditions, a mechanism equally applicable to cannabis (Poria et al., 2022) Cannabis grown in saline soils showed enhanced growth and biomass production when treated with PGPBs capable of modulating ionic balance through sodium exclusion.

PGPBs are also instrumental in mitigating biotic stresses caused by pathogens and pests. They produce antifungal metabolites, including lipopeptides and siderophores, which inhibit the growth of phytopathogenic fungi such as Fusarium and Pythium species (Palaniyandi et al., 2014). This is particularly significant for cannabis, a crop prone to fungal infections in its humid cultivation environment. Studies on tomatoes and cucumbers have shown that Pseudomonas fluorescens reduces the incidence of fungal diseases by up to 70% (Numan et al., 2018). Similar results have been observed in cannabis cultivation systems, where PGPB application leads to reduced disease prevalence and enhanced plant vigor (Lyu et al., 2020). PGPBs also contribute to sustainable agriculture by reducing the reliance on chemical fertilizers and pesticides. In maize and wheat, PGPBs have been reported to increase nitrogen

use efficiency by up to 40%, leading to substantial reductions in synthetic fertilizer applications (Egamberdieva et al., 2019). Similarly, in cannabis cultivation, the use of microbial consortia has been shown to lower chemical inputs while maintaining high yields and quality (Poria et al., 2022). This aligns with the increasing demand for environmentally sustainable practices in commercial cannabis and other crop production. Lastly, PGPBs facilitate the phytoremediation of contaminated soils, enabling cannabis and other crops to thrive in marginal lands. By producing organic acids and chelating agents, PGPBs enhance the bioavailability of heavy metals, which plants can then accumulate or stabilize (Ledin, 2000). For instance, cannabis treated with Pseudomonas spp. has shown enhanced growth on cadmium-contaminated soils (Sorour et al., 2022). These findings underscore the potential of PGPBs not only in agricultural productivity but also in environmental remediation.

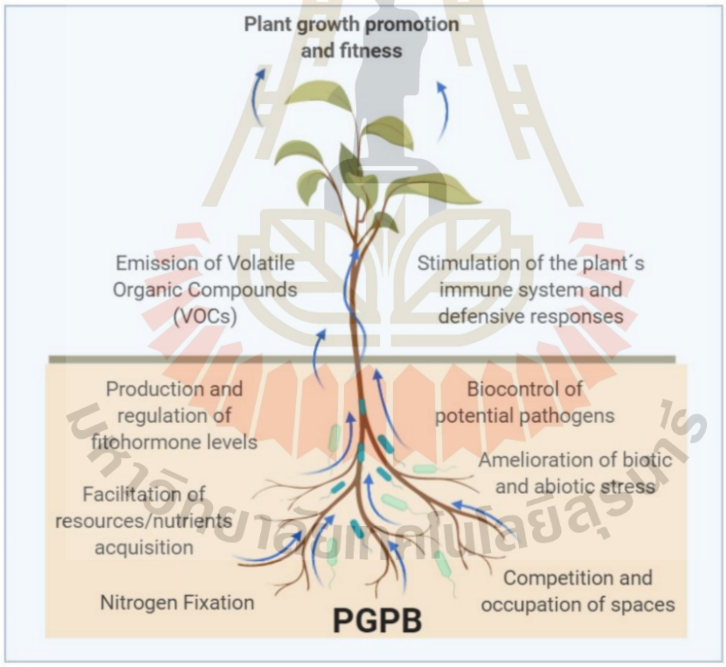

Figure 6. Beneficial activities performed by plant growth-promoting bacteria (PGPB) to promote optimal plant growth and fitness (Orozco-Mosqueda et al., 2021).

2.4.2 Mechanisms of Action of Plant Growth-Promoting Bacteria (PGPBs)

PGPBs utilize multifaceted mechanisms to enhance plant growth and resilience, acting directly or indirectly on plant physiology and soil chemistry. A primary mechanism involves nutrient acquisition facilitation, such as nitrogen fixation and

phosphate solubilization. Nitrogen-fixing bacteria, including *Rhizobium* and *Azospirillum* species, convert atmospheric nitrogen into ammonia, making it accessible to plants (Kumar et al., 2018). Phosphate-solubilizing PGPBs like *Bacillus* and *Pseudomonas* secrete organic acids that release phosphate bound in soil minerals, thereby increasing its bioavailability for uptake by plant roots (Egamberdieva et al., 2019). Additionally, potassium- and zinc-solubilizing bacteria enhance the availability of these essential nutrients, contributing to optimal plant development. Another critical mechanism is phytohormone production, which directly influences plant growth and stress response. PGPBs produce hormones like indole-3-acetic acid (IAA), gibberellins, and cytokinins that modulate root architecture, leading to improved nutrient and water uptake (Patten & Glick, 2002). IAA promotes root elongation and lateral root formation, while gibberellins stimulate shoot elongation and seed germination. Furthermore, some PGPBs regulate ethylene levels in plants under stress by synthesizing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which lowers ethylene concentrations that would otherwise inhibit plant growth under adverse conditions (Glick, 2014). PGPBs also enhance plant defense mechanisms by inducing systemic resistance and producing antimicrobial compounds. Induced systemic resistance (ISR) involves priming the plant's immune system to better respond to pathogen attacks, mediated by signaling molecules such as jasmonic acid and salicylic acid (Van Wees et al., 2008). PGPBs like *Bacillus subtilis* and *Pseudomonas* fluorescens produce lipopeptides, siderophores, and enzymes that inhibit the growth of phytopathogens, providing an effective biological control mechanism (Choudhary & Johri, 2009). These interactions reduce the dependence on chemical pesticides, promoting sustainable agricultural practices.

PGPBs influence soil structure and function through their interactions with the rhizosphere microbiome. They secrete exopolysaccharides that enhance soil aggregation, improving water retention and aeration (Qurashi & Sabri, 2012). These bacteria also alter rhizosphere microbial communities by fostering beneficial interactions and suppressing harmful microorganisms. For example, some PGPBs promote the growth of arbuscular mycorrhizal fungi, which synergistically improve nutrient uptake and plant health (Smith & Read, 2010). This microbial synergy underscores the holistic impact of PGPBs on plant-microbe-soil interactions. Finally,

PGPBs mitigate the effects of abiotic stresses such as salinity, drought, and heavy metal contamination. They achieve this by producing osmolytes, enhancing antioxidant enzyme activities, and altering ionic balances in plants under stress (Egamberdieva et al., 2019). Moreover, PGPBs facilitate phytoremediation by transforming toxic heavy metals into less bioavailable forms or by promoting their accumulation in plant tissues (Poria et al., 2022). These mechanisms not only improve plant survival under challenging conditions but also contribute to environmental sustainability by reclaiming contaminated soils.

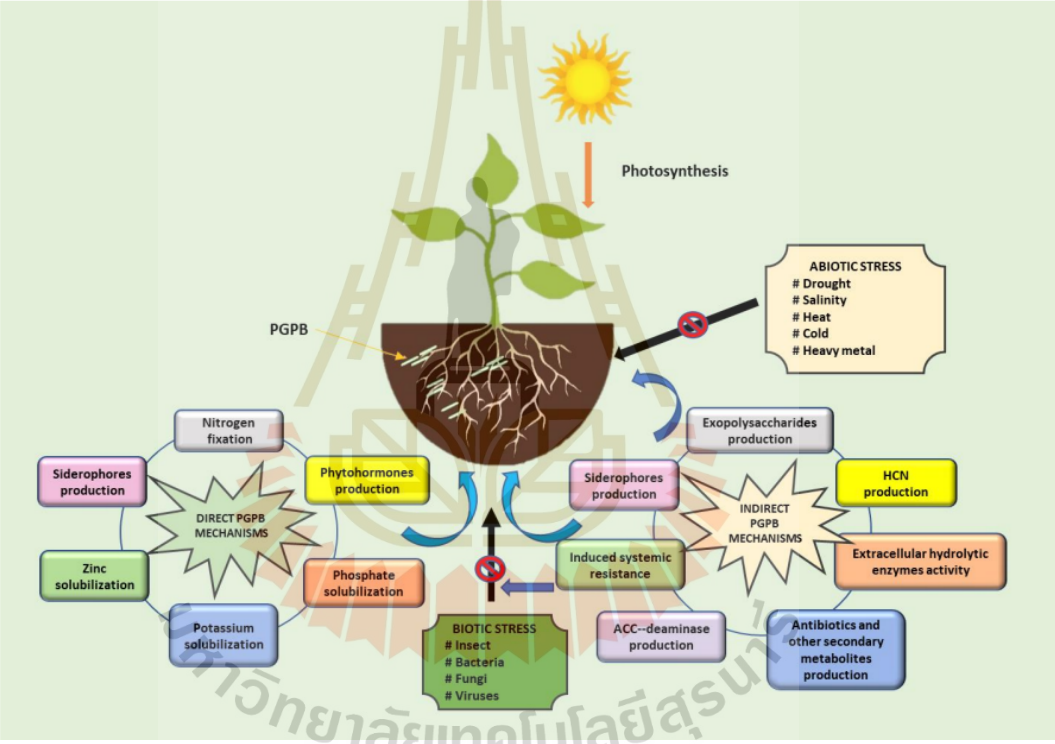

Figure 7. Mechanisms of PGPB, both direct and indirect, that contribute to plant development (Ajijah et al., 2023).

2.4.3 Application of PGPBs

The application of Plant Growth-Promoting Bacteria (PGPBs) spans diverse agricultural and environmental domains, leveraging their multifaceted benefits for sustainable development. One of their primary roles is enhancing crop productivity in nutrient poor soils. PGPBs such as *Rhizobium*, *Azospirillum*, and *Bacillus* species are used as biofertilizers to augment nitrogen fixation, phosphorus solubilization, and

micronutrient availability (Kumar & Verma, 2018). Their use has proven effective in crops like legumes, cereals, and vegetables, where they increase yields and reduce dependence on chemical fertilizers. For instance, inoculation with *Azospirillum* in maize enhances nitrogen uptake, leading to higher grain production (Egamberdieva et al., 2019).

In the context of abiotic stress management, PGPBs play a pivotal role in mitigating the adverse effects of salinity, drought, and heavy metal toxicity. For instance, *Bacillus* and *Pseudomonas* strains enhance the tolerance of crops like rice and wheat to salinity by modulating ionic balances and producing exopolysaccharides (Ramakrishna et al., 2020). Drought resilience is improved through the production of osmolytes and phytohormones, which regulate water use efficiency in crops such as tomatoes and soybeans (Glick, 2014). Additionally, in phytoremediation, PGPBs like *Pseudomonas* and *Bacillus facilitate* the reclamation of contaminated soils by aiding in the uptake or transformation of heavy metals into less bioavailable forms, enabling plants like mustard and sunflower to thrive in polluted environments (Poria et al., 2022).

The use of PGPBs in disease management highlights their potential as eco-friendly alternatives to synthetic pesticides. These bacteria produce antimicrobial compounds, including siderophores, lipopeptides, and enzymes, which inhibit the growth of plant pathogens (Palaniyandi et al., 2014). For example, *Pseudomonas* fluorescens has been shown to suppress Fusarium wilt in tomatoes, significantly reducing disease incidence (Van Wees et al., 2008). Furthermore, PGPBs induce systemic resistance in plants, priming their immune systems against biotic stressors without the need for direct microbial contact (Choudhary & Johri, 2009).

In horticulture and controlled environment agriculture, PGPBs are gaining attention for enhancing the quality and yield of high value crops like cannabis and strawberries. By stimulating root development and nutrient uptake, they improve biomass production and the concentration of secondary metabolites, such as cannabinoids in cannabis and anthocyanins in strawberries (Lyu et al., 2020). Similarly, the integration of PGPBs into hydroponic systems has demonstrated increased efficiency in nutrient cycling and disease suppression, contributing to higher productivity (Backer et al., 2018).

Beyond agriculture, PGPBs contribute to ecological restoration and land reclamation efforts. They are applied to revegetate degraded lands and enhance soil fertility in marginal environments, including saline or eroded soils (Qurashi & Sabri, 2012). The combined use of PGPBs with native plant species has been particularly effective in stabilizing soil structure and promoting biodiversity in degraded ecosystems (Smith & Read, 2010). Their broad applicability underscores their role as a cornerstone of sustainable agricultural practices and environmental stewardship.

2.4.4 Strain Selection for Plant Growth-Promoting Bacteria (PGPBs)

Strain selection is a pivotal step in harnessing Plant Growth-Promoting Bacteria (PGPBs) for agricultural and environmental applications, as the effectiveness of PGPBs depends significantly on the strain's intrinsic properties and compatibility with target crops. Ideal strains should exhibit robust plant growth-promoting traits, including nitrogen fixation, phosphate solubilization, and the production of phytohormones like indole-3-acetic acid (IAA) (Kumar & Verma, 2018). For instance, *Azospirillum brasilense* and *Pseudomonas* fluorescens have demonstrated exceptional efficacy in enhancing root development and nutrient uptake across multiple crop species (Egamberdieva et al., 2019). The ability of a strain to colonize plant roots effectively and establish a stable rhizosphere population is another critical criterion for selection (Compant et al., 2010).

Abiotic stress tolerance is a key consideration in strain selection, especially for applications in challenging environments such as saline, drought-prone, or heavy metal-contaminated soils. Strains like *Bacillus subtilis* and *Halomonas spp.* are often prioritized for their resilience under high salinity and their production of exopolysaccharides, which improve soil structure and plant water retention (Ramakrishna et al., 2020). Similarly, *Pseudomonas putida* and *Arthrobacter globiformis* are noted for their abilities to degrade organic pollutants and detoxify heavy metals, enabling plant growth in contaminated sites (Poria et al., 2022). Advanced screening techniques, including stress-specific assays and genomic analyses, are increasingly employed to identify strains with superior stress tolerance and environmental adaptability.

Compatibility with the host plant is a critical determinant of PGPB efficacy. Host-specific interactions between plants and bacterial strains often dictate the success of inoculation. For example, rhizobial strains must be compatible with the legume species they colonize to establish functional nitrogen-fixing nodules (Smith & Read, 2010). Similarly, certain strains of *Bacillus* and *Trichoderma* exhibit a preference for specific crops, enhancing growth through unique biochemical pathways (Lugtenberg & Kamilova, 2009). Researchers often perform greenhouse and field trials to evaluate the symbiotic compatibility and agronomic benefits of candidate strains.

Molecular and omics-based approaches have revolutionized strain selection by providing insights into the genetic determinants of PGP traits. Genome sequencing and comparative genomics allow for the identification of genes responsible for nitrogen fixation, ACC deaminase production, and secondary metabolite biosynthesis (Glick, 2014). Metagenomic studies of rhizosphere microbiomes further aid in discovering novel strains with untapped potential for plant growth promotion (Compant et al., 2010). These tools facilitate the development of custom-tailored PGPB consortia optimized for specific agroecological conditions.

To maximize the benefits of strain selection, regulatory considerations must also be addressed. Strains intended for commercial use must undergo rigorous safety evaluations to ensure they are non-pathogenic and environmentally benign (Authority et al., 2018). Additionally, they should exhibit genetic stability to prevent horizontal gene transfer that could compromise biosafety (Pal & Gardener, 2006). By integrating biological, molecular, and regulatory criteria, strain selection can advance the effective and sustainable use of PGPBs in agriculture and environmental management.

2.4.5 Regulations for the Use of Plant Growth-Promoting Bacteria (PGPBs)

The use of Plant Growth-Promoting Bacteria (PGPBs) in agriculture and environmental management is subject to rigorous regulatory frameworks to ensure safety, efficacy, and environmental sustainability. In the United States, the Environmental Protection Agency (EPA) regulates PGPBs under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) as microbial pesticides or biostimulants (Insecticide). Manufacturers must provide comprehensive data on the identity, mode of action, and potential environmental and human health impacts of the microbial

product. This process includes risk assessments for pathogenicity, allergenicity, and genetic stability, ensuring that only strains with minimal risk profiles are approved for commercial use (Pal & Gardener, 2006).

In the European Union, PGPBs are categorized under the Plant Protection Products Regulation (Regulation (EC) No 1107/2009) (Villaverde et al., 2014). The European Food Safety Authority (EFSA) evaluates microbial products for their safety and efficacy, requiring detailed dossiers on production processes, strain identification, and ecological risks (Insecticide). Specific attention is given to the potential for horizontal gene transfer and the impact of released PGPBs on non-target organisms, including beneficial soil microbes (Compant et al., 2010). Furthermore, the EU enforces stringent traceability and labeling requirements to ensure transparency and consumer confidence in microbial products.

Countries in Asia, such as India and China, have also established frameworks for PGPB regulation, albeit with varying levels of stringency. In India, the Fertilizer (Control) Order 1985 governs biofertilizers, including PGPBs, and mandates quality testing for microbial density, purity, and performance (Yadav et al., 1985). China, through its Ministry of Agriculture and Rural Affairs, requires that microbial inoculants undergo multi-year field trials to verify their agronomic benefits and absence of adverse environmental effects (Meng et al., 2022). Both nations emphasize the importance of aligning microbial product development with sustainable agricultural goals, reflecting global trends toward eco-friendly farming practices.

Globally, the regulatory landscape for PGPBs is evolving to accommodate advances in biotechnology and microbial genomics. The Cartagena Protocol on Biosafety provides an international framework for regulating living-modified organisms, including genetically engineered PGPBs (Diversity, 2000). It mandates risk assessments and promotes information exchange among nations, facilitating the safe cross-border movement of microbial products. This is particularly relevant for multinational corporations developing microbial solutions for diverse agro-ecological zones.

While regulations aim to safeguard human and environmental health, they also present challenges such as high costs and lengthy approval processes, potentially stifling innovation. Calls for harmonized international guidelines have emerged to streamline regulatory pathways while maintaining rigorous safety standards

(Ravensberg, 2011). Policymakers and stakeholders are increasingly advocating for risk-based, tiered approaches that balance safety with the need for rapid deployment of PGPBs to address global challenges such as food security and climate resilience.

2.5 *Bacillus velezensis*

Bacillus velezensis, a Gram-positive, spore-forming bacterium within the Bacillus subtilis species complex, has garnered attention due to its dual role as both a biocontrol agent and a plant growth-promoting rhizobacterium (PGPR). Commonly inhabiting the rhizosphere, *B. velezensis* establishes beneficial interactions with a wide variety of plant species. Its ability to adapt to different environmental conditions, coupled with the synthesis of a diverse array of bioactive compounds, makes it a highly effective and sustainable alternative to synthetic agricultural inputs, such as chemical fertilizers and pesticides. Consequently, *B. velezensis* is considered an essential tool in the development of environmentally sustainable farming practices (Fan et al., 2018; Rabbee et al., 2019).

One of the key attributes of *B. velezensis* is its production of secondary metabolites that exhibit potent antimicrobial activity. These bioactive compounds include lipopeptides such as surfactin, fencing, and iturin, as well as polyketides, including prolactin and difficidin. These metabolites play a crucial role in controlling plant pathogens by disrupting cell membranes and inhibiting essential metabolic processes. For instance, *B. velezensis* FZB42 has demonstrated substantial efficacy against soil-borne pathogens, including Fusarium spp. and Pythium spp., highlighting its utility in integrated pest management systems (Rabbee et al., 2019). Moreover, B. velezensis can form biofilms, which enhances its survival in the soil, ensuring long-term disease control and crop protection (Chowdhury et al., 2015).

In addition to pathogen suppression, *B. velezensis* significantly contributes to plant growth promotion by enhancing nutrient availability and producing phytohormones. The bacterium can solubilize phosphate, making it more accessible to plants, and synthesizing siderophores to sequester iron, alleviating micronutrient deficiencies in the rhizosphere (Singh, 2019). Furthermore, *B. velezensis* produces indole-3-acetic acid (IAA), a crucial plant hormone that stimulates root development, thereby improving the plant's ability to access water and nutrients, especially under

suboptimal conditions (Hamid et al., 2021). This multifaceted functionality of *B. velezensis* underscores its potential to improve crop productivity, particularly in challenging environmental contexts.

The genomic analysis of *B. velezensis* has revealed a wealth of biosynthetic gene clusters responsible for the production of antimicrobial peptides and growth-promoting compounds. Notably, strains such as *B. velezensis* S141 contain unique genes associated with auxin production and nitrogen fixation. These genetic traits are particularly beneficial in symbiotic relationships with legumes, enhancing nodule formation and nitrogen fixation efficiency when co-inoculated with *Bradyrhizobium diazoefficiens*. Such genetic insights facilitate the development of precision microbial formulations tailored to specific crops and environmental conditions, thereby enhancing the bacterium's efficacy in agricultural applications (Sibponkrung et al., 2020).

Field studies conducted in Thailand have demonstrated the practical effectiveness of *B. velezensis* in controlling fungal diseases and promoting plant growth. For example, *B. velezensis* S141 has been successfully used to manage fungal pathogens in mung bean crops, while also improving nodulation and nitrogen fixation in soybean plants (Prakamhang et al., 2015; Songwattana et al., 2023). Beyond its pathogen-suppressing abilities, *B. velezensis* promotes soil health by enhancing soil aggregation and increasing water retention, thus supporting sustainable farming practices (Kenfaoui et al., 2024). These ecological benefits contribute to improved crop yields and reduce environmental degradation, aligning with global efforts to reduce the ecological footprint of agriculture and promote eco-friendly farming practices. Despite the promising applications of *B. velezensis*, several challenges remain that limit its widespread adoption. Environmental factors such as soil pH, texture, and temperature can influence the bacterium's performance, and the stability of microbial formulations during storage and transportation continues to be a significant hurdle (Rabbee et al., 2019). Ongoing research is focused on developing more robust microbial consortia and advanced delivery systems to address these challenges. By overcoming these limitations, *B. velezensis* has the potential to become a cornerstone of sustainable agriculture, contributing to food security and

reducing the environmental impact of conventional farming practices (Chowdhury et al., 2015).

2.6 Sustainable agriculture

Sustainable agriculture embodies the integration of environmental stewardship, economic viability, and social equity to meet the needs of current and future generations. It focuses on preserving natural resources, enhancing soil fertility, and reducing ecological degradation while ensuring stable food production (Tilman et al., 2002). Central to this approach is the adoption of practices that maintain the balance of agroecosystems, including crop diversification, conservation tillage, and the judicious use of organic and inorganic inputs (Pretty, 2008). Such practices aim to mitigate climate change impacts and promote biodiversity, thus securing long-term agricultural productivity.

The integration of biological approaches, such as the use of Plant Growth-Promoting Bacteria (PGPBs) and natural pest control agents, exemplifies sustainable agricultural practices. PGPBs enhance nutrient acquisition, stress tolerance, and soil fertility while reducing the dependency on chemical fertilizers (Backer et al., 2018). Similarly, biological pest control using predators, parasitoids, and entomopathogenic fungi offers an environmentally friendly alternative to synthetic pesticides, mitigating the risks of pest resistance and environmental contamination (Koller et al., 2023). These approaches align with the principles of ecological intensification, which prioritize ecosystem services over external inputs.

CHAPTER III

METHODOLOGY

3.1 Ethical Compliance Statement

According to the risk levels prescribed for pathogens and animal toxins in "The Risk Group of Pathogen and Animal Toxin (2017)" by the Department of Medical Sciences, Ministry of Public Health, Pathogen and Animal Toxin Act (2015), and Biosafety Guide-lines for Modern Biotechnology, BIOTEC (2016), the biosecurity aspects of this study were reviewed and approved by Suranaree University of Technology (approval number: SUT-IBC-003/2023). Furthermore, authorization for cannabis cultivation and production was acquired (license: 13/2563), and the processing of plant materials was carried out under the supervision of the Thailand Food and Drug Administration (TFDA). The procedures followed the standard operating procedures (SOPs) for legally compliant cannabis production.

3.2 Bacterial Strains and Growth Conditions

Bacillus velezensis S141 was cultured in nutrient broth at 30 °C for 18 h, while the mutants (*lpyAD*, *dhas*, *yhcx*, *IPT*, and *IPI)* were grown in Luria Bertani medium under the same conditions, achieving cell densities between 10^4, 10^6, and 10^8 μg/ml. The media were supplemented with erythromycin (1 μg/ml), kanamycin (10 μg/ml), phleomycin (8 μg/ml), and spectinomycin (100 μg/ml) to prepare inoculum containing bacterial concentrations of 10^4, 10^6, and 10^8 CFU/ml. After incubation, cells were collected by centrifuging at 4000× g for 10 min, washed with sterile 0.85% (w/v) NaCl to remove residual media, and then resuspended in sterilized deionized water to achieve the target concentrations (10^4, 10^6, and 10^8 CFU/ml). Table 3 provides a list of the bacterial strains used in this study.

Table 3. Bacterial strains used in this study.

Bacterial Strains	Relevant Genotype or Description	References
Bacillus velezensis S141	*Wild type*	(Sibponkrung et al., 2020)
B. velezensis S141Δ*dhaS*	*dhaS deletion, Δdhas::erm*^{r}	
B. velezensis S141Δ*yhcX*	*yhcX deletion, ΔyhcX::kan*^{r}	
B. velezensis S141Δ*IPyAD*	*IPyAD deletion, ΔIPyAD::spm*^{r}	
B. velezensis S141Δ*ipt*	*IPT deletion, Δipt::phle*^{r}	
B. velezensis S141Δ*ipi*	*IPI deletion, Δipi::kan*^{r}	

3.3 *Cannabis Sativa* Strain Utilized in This Study

Foi Thong Suranaree 1 is a cannabis variety developed through the "Sub-Project Breeding and Strain Evaluation" of the Hemp-Cannabis Research, Production, and Utilization Initiative. This program operates under the Center of Excellence in Agricultural Product Innovation at the School of Agricultural Technology, Suranaree University of Technology. The strain exhibits vigorous growth, rapid maturation, and large foliage and stems. It also features an efficient canopy structure and demonstrates strong resistance to pathogens and pests. Furthermore, it is characterized by early flowering and remarkable adaptability to diverse environmental conditions. *Foi Thong Suranaree* 1 offers significant yield potential, making it an attractive option for growers focused on commercial-scale production.

3.4. Examination of Plant Growth Promotion

Cannabis was grown under laboratory and greenhouse conditions. In the laboratory setting, 200 cannabis seeds were surface sterilized using 70% (v/v) ethanol, 3% (v/v) sodium hypochlorite, and sterile water. A seedling (1 per pot) was subsequently trans-planted into sterilized vermiculite within Leonard's jars that contained AB fertilizer solution (EC: 1.8–2 mS/cm, pH 6.5–7). This cultivation took place under controlled sterile conditions, maintaining a temperature of 25 ± 2 °C, 12- h light/12h dark photoperiods, and 50% humidity. Inoculations of S141 at various concentrations, 10^4, 10^6, and 10^8 CFU/ ml, were conducted after 10 days after seedling transplantation, establishing four groups of five seedlings each: a control group that

received sterile water and treatment groups that received varying concentrations of the S141 inoculum. Plant growth was assessed by measuring chlorophyll content, health index, fresh weight, and dry weight at 3, 5, 7, 14, and 28 days after inoculation (DAI).

For the greenhouse cultivation study, four distinct soil conditions were prepared. These included soils treated with boiled water and untreated soils, each combined with either normal or low fertilizer levels, as outlined in Tables 4 and 5. Cannabis seeds were subjected to surface sterilization as described above before being planted in 1 seedling per container with these specific soil treatments and cultivated for 14 days. After this period, 1 ml of S141 inoculum (10^4, 10^6, and 10^8 CFU) was applied to the root base of each cannabis plant monthly. Sixteen experimental groups of five each were established, mirroring as the laboratory setup: a control group and a treatment group, with the latter receiving different concentrations of the S141 inoculum. The plant growth was assessed by measuring their fresh and dry weights at 65 DAI.

Table 4. Elemental analysis of planting material.

Sample	EC (ds/m) 1:5	pH 1:5	% OM	% N	% P	% K	% Ca	% Mg
Planting material	2.21	6.84	33.66	1.18	0.72	0.08	1.56	0.62

Table 5. The greenhouse experimental conditions.

Conditions	Non-treated soil normal fertilizer	Non-treated soil lower fertilizer	Boiled water-treated soil normal fertilizer	Boiled water-treated soil lower fertilizer
Pretreatment	-	-	Portions of the soil were subjected to addition by pouring through the soil volumes of boiled water (100 °C) at a ratio of 0.5 L boiled water per 1 kg of soil(Saied, 2011)	
Planting materials	- Loam soil -Coco husk chips -Rice husk-based charcoal -Manure			
plant pots	-Pot Size 30 L -Pot Diameter (Base) 13 inches - Pot Diameter 17 INCHES -Pot Height 15 INCHES			
Watering	every morning 1.5 - 2 L / plant			
Humidity	- 40 - 60% - 9 - 11 hr.			
Temperature	30 - 37 °C			
Urea Fertilizer 46-0-0-0	10 g / plant / week			
AB Fertilizer EC:1.8-2 mS/cm pH 6.5 - 7	2 L / 2 times / week	1 L / 2 times / week	2 L / 2 times / week	1 L / 2 times / week

3.5 Assessment of Chlorophyll Content and Health Index (HI) in Cannabis

The level of chlorophyll in the cannabis was monitored by a chlorophyll meter (SPAD-502Plu), and the health index (HI), a tool for evaluating factors that directly impact the survival and growth of cannabis plants, was calculated using the equation mentioned below (Fan et al., 2013). These parameters were recorded after the inoculation of wild-type S141 and S141 mutants (*IpyAD*, *dhas*, *yhcx*, *IPT*, and *IPI*). The measurements of chlorophyll content, stem diameter, stem height, and dry weight were documented at 3, 5, 7, 14, 21, and 28 DAI.

$$\text{Health index} = \frac{\text{Stem diameter}}{\text{Stem height}} \times \text{Dry weight}$$

3.6 RNA and DNA Extraction

After a 28-day cannabis cultivation period, plant tissues including roots, stems, and leaves were gathered and ground using a mortar and pestle under liquid nitrogen. Approximately 100 mg per sample was moved into a 1.5 ml microfuge tube; afterward, an RNeasy RNA Mini Kit (QIAGEN) was utilized to extract and purify total RNA from these plant tissues. The quantity and quality of the RNA were evaluated using a Nanodrop 2000 Spectrophotometer (Thermo Scientific) and agarose gel electrophoresis stained with RedSafe Nucleic Acid Staining Solution (iNtRON), respectively. For the extraction of bacterial DNA within the cannabis, plant tissues were steeped in 75% ethanol (V/V) for 1 min and subsequently rinsed with sterile distilled water. The samples were then exposed to a 3% sodium hypochlorite solution (v/v) for 2 min and cleaned again with sterile distilled water prior to DNA extraction. A QIAGEN PowerSoil DNA isolation kit was used to extract and purify the genomic DNA of the cannabis and bacteria. The quantity and purity of DNA were assessed as previously described.

3.7 Sequencing Analysis

The quality of RNA was determined using NanoDrop, Qubit 2.0, and Agilent 2100 systems before commencing the library construction. Six libraries were prepared from

2 μg each of the total RNA extracted from inoculated and non-inoculated cannabis tissues. These tissues had been exposed to S141 at a concentration of 10^6 CFU/ml. BMKGENE, China, performed the sequencing. mRNA was isolated using Oligo(dT)-attached magnetic beads, and the enriched RNAs were randomly fragmented in a fragmentation buffer. First-strand cDNAs were synthesized from the fragmented RNAs using random hexamer primers, followed by second-strand synthesis with the addition of PCR buffer, dNTPs, RNase H, and DNA polymerase I. The cDNAs were then purified using AMPure XP beads. These underwent an end-repair procedure, with adenosine added to the end while ligated to adapters. The fragments were then selected using AMPure XP beads within the size range of 300–400 bp. The cDNA library was generated through several rounds of PCR on the cDNA fragments; the qualified library was sequenced using a high-throughput platform in PE150 mode. Clean data were obtained by filtering raw data to remove the adapter sequence and read of low quantity; these filtered data were then aligned with the Cannabis_sativa.cs10 reference genome (GCA_900626175.2). The differentially expressed genes (DEGs) were selected for further analysis based on p-values < 0.05 and absolute Log2 ratios ≥ 1. The DEGs were annotated in the differential expression analysis, and Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) were then analyzed regarding the DEGs to assess the functional networks of gene products and genes within the pathways, respectively. We examined three types of GO annotation systems, which included biological process, molecular function, and cellular component. The annotation of DEGs in KEGG was built based on KEGG's database of genes within pathways, including the metabolic pathways of carbohydrates, nucleotides, amino acids, and biological degradation of organics.

3.8 Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

One microgram of total RNAs, extracted from the plant tissues of both non-inoculated and inoculated cannabis, was used as a template for cDNA synthesis using an iScript™ cDNA Synthesis Kits (Bio-Rad). Primers specific to selected candidate genes were de-signed using Primer3Plus software and are displayed in Table 6. The qRT-PCR

analysis was conducted using Luna Universal qPCR Master Mix (New England BioLabs) and the CFX96 touch real-time PCR detection system (Bio-Rad). Each sample underwent triplicate examination, with amplification conditions as follows: 98 °C for 2 min and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Relative expression was calculated based on $2^{-\Delta\Delta Ct}$, with actin used as an internal control (Livak & Schmittgen, 2001; Pfaffl, 2001).

3.9 Quantification of Endophytic S141 in Various Plant Tissues

Quantitative real-time PCR (qRT-PCR) was used to determine the copy numbers of S141 quantitatively. The reaction was carried out using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and Luna Universal qPCR Master Mix (New England Biolabs), in addition to specific primer pairs (Table 6). The copy numbers were quantified according to a standard curve established using a plasmid containing the S141 sequence (Limkul et al., 2022).

Table 6. Summary of primers for qRT-qPCR used in this study.

Primers	Accession	Sequence (5′to3′)	Annealing temperature (°C)
Actin -F	XM_030632129.2	TTGCTGGTCGTGATCTTACTG	60
Actin -R		GTCTCCATCTCCTGCTCAAAG	60
THCAS-F	XM_030649882.2	GCTCTCTTCGTTGCTGGACT	60
THCAS-R		TGTTCCCACCTCTATGCCCA	60
CBDAS-F	XM_030623918.2	CTTAGTTTGGCGGCTGGGTA	60
CBDAS-R		CTTTGGGACAGCAACCAGTCT	60
SAUR50-F	XM_030643469.2	GCTCTCTTCGTTGCTGGACT	60
SAUR50-R		TGTTCCCACCTCTATGCCCA	60
XEHP25-F	XM_030654082.2	CTTAGTTTGGCGGCTGGGTA	60
XEHP25-R		CTTTGGGACAGCAACCAGTCT	60
GLP2-1-F	XM_030625170.2	AGGCCTTGGGACTTGCTTTC	60
GLP2-1-R		GACCGTGTACAAGAGCAGCT	60
ABC29-F	XM_030635527.2	TGGATGGTGCTCCTTTTCTTCG	60
ABC29-R		AGGCTCCAGACCAAATCCCA	60
CBL120-F	XM_030646708.2	TGTGTGGACTCCTCAACTCCA	60
CBL120-R		ACTCAAACATGCGACCACGT	60
GTLS-F	XM_030629393.2	AGCTGTGACGGGTCAACTTC	60
GTLS-R		AGAGATTGGGCCGATTGGTG	60
LRLK4-F	XM_061114175.1	AGAAGCTAAGGCACCTGCAG	60
LRLK4-R		CGGTATGGACTTGGTGCAGT	60
PRP-1A-F	XM_030629932.1	TGGATGGTGCTCCTTTTCTTCG	60
PRP-1A-R		AGGCTCCAGACCAAATCCCA	60
EIX1-F	XM_030640350.1	TGTGTGGACTCCTCAACTCCA	60
EIX1-R		ACTCAAACATGCGACCACGT	60
CRF5-F	XM_030632056.1	AGCTGTGACGGGTCAACTTC	60
CRF5-R		AGAGATTGGGCCGATTGGTG	60
CAO -F	XM_030634090.1	AGAAGCTAAGGCACCTGCAG	60
CAO -R		CGGTATGGACTTGGTGCAGT	60
UDP -F	XM_030628925.1	TGGATGGTGCTCCTTTTCTTCG	60
UDP -R		AGGCTCCAGACCAAATCCCA	60
ERFC3 -F	XM_030647537.1	TGTGTGGACTCCTCAACTCCA	60
ERFC3 -R		ACTCAAACATGCGACCACGT	60
S141-F	AP018402.1	TGATTGCCGGCACAGAAAATAACAGG	60
S141-R		GGTTTCCGGTACCACGTCTGTC	60

3.10 Assessment of cannabis growth profiles following Inoculation with *Bacillus velezensis* S141 mutants

To evaluate the effects of plant hormones synthesized by S141 on cannabis growth profiles, we utilized mutants Δ*IpyAD*, Δ*dhas,* Δ*yhcx*, Δ*IPT,* and Δ*IPI*, as studied by Sibponkrung et al. (Sibponkrung et al., 2020), Sterilized vermiculite, contained within Leonard jars, was used for cultivation, and all equipment was sterilized by autoclaving at 121 ℃ for 30 min preceding seedling transplant. Surface-sterilized cannabis seedlings were subsequently placed onto the sterilized vermiculite within culture trays. Ten days following transplantation, each seedling received 1 ml of S141 mutant strain (*IpyAD*, *dhas*, *yhcx*, *IPT*, and *IPI*) at a concentration of 10^6 CFU/ml, while the control group received 1 mL of sterile water. Cultivation conditions were strictly controlled: temperature 25 ± 2 ℃, photoperiod of 12 h light/12 h dark, and humidity at 50%. On days 7 and 14 after inoculation, fresh and dry weights of plants were recorded to assess growth patterns.

3.11 Statistical Analysis

A paired sample t-test was used to identify significant differences between the means of each group ($p < 0.05$). The data are presented as the average ± standard deviation (SD), which were derived from three biological replicates.

CHAPTER IV

RESULTS

4.1 Colonization of *Bacillus velezensis* S141 in Cannabis After Inoculation

Prior to inoculating the cannabis with *B. velezensis* S141, cannabis seedlings were grown for 10 days in vitro. At 28 days after inoculation, the plant tissues, including the leaves, stems, and roots, were harvested, surface-sterilized, and subjected to genomic DNA extraction. These extracts were then used as templates for qPCR analysis to confirm the presence of S141. The highest number of S141 copies were found in the leaves (4.89×10^5 copies/20 ng DNA), followed by the stems (3.82×10^4) and roots (1.17×10^4) in the S141-inoculated cannabis. No S141 was found in the non-inoculated group (Figure 1). These findings suggest that S141 is an endophyte and PGPB in cannabis.

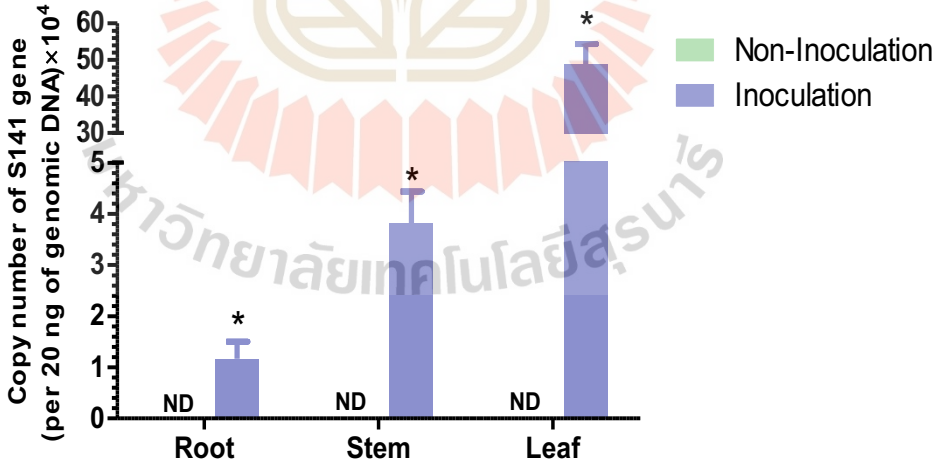

Figure 8. Localization of S141 in cannabis after inoculation with *B. velezensis* S141. Genomic DNA was collected from the leaf, stem, and root tissues at 7 days after inoculation. S141 copy levels were measured in triplicate using qRT-PCR. Data are presented as mean ± SD for n = 3 from three bio-logical replications, with significant differences ($p < 0.05$) indicated by asterisks. ND denotes not detected.

4.2 Enhanced Growth Performance of Cannabis Inoculated with *Bacillus velezensis* S141: Laboratory Conditions

To explore the influence of S141 on cannabis growth at the laboratory scale, this study varied the amounts of S141 added to the cannabis plants in Leonard's jars. We then monitored the quantity of chlorophyll and the health index (HI). To initially evaluate the growth influenced by S141 supplementation, images of the plants were taken at different points after S141 inoculation. From 3 to 14 day after inoculation (DAI), the overview of plant characteristics displayed noticeable increases in root length, plant height, root dry weight, and total dry weight when compared to the control (Figure 9A–F). Similarly, S141 supplementation led to a significant increase in chlorophyll content, as measured by SPAD units. At 28 DAI, all experimental groups inoculated with S141 showed an approximately 20% higher chlorophyll content compared with the control (Figure 9C). Moreover, the HI values of the 10^4, 10^6, and 10^8 groups at 3, 5, 7, and 14 DAI were about 2-fold higher than that of non-inoculated ones, while no significant differences were observed at 21 and 28 DAI (Figure 9D). For root dry weight and total dry weight, only groups supplemented with S141 at 10^6 and 10^8 CFU/ml displayed approximately 2-fold-higher mg/plant in both categories (Figure 9E, F).

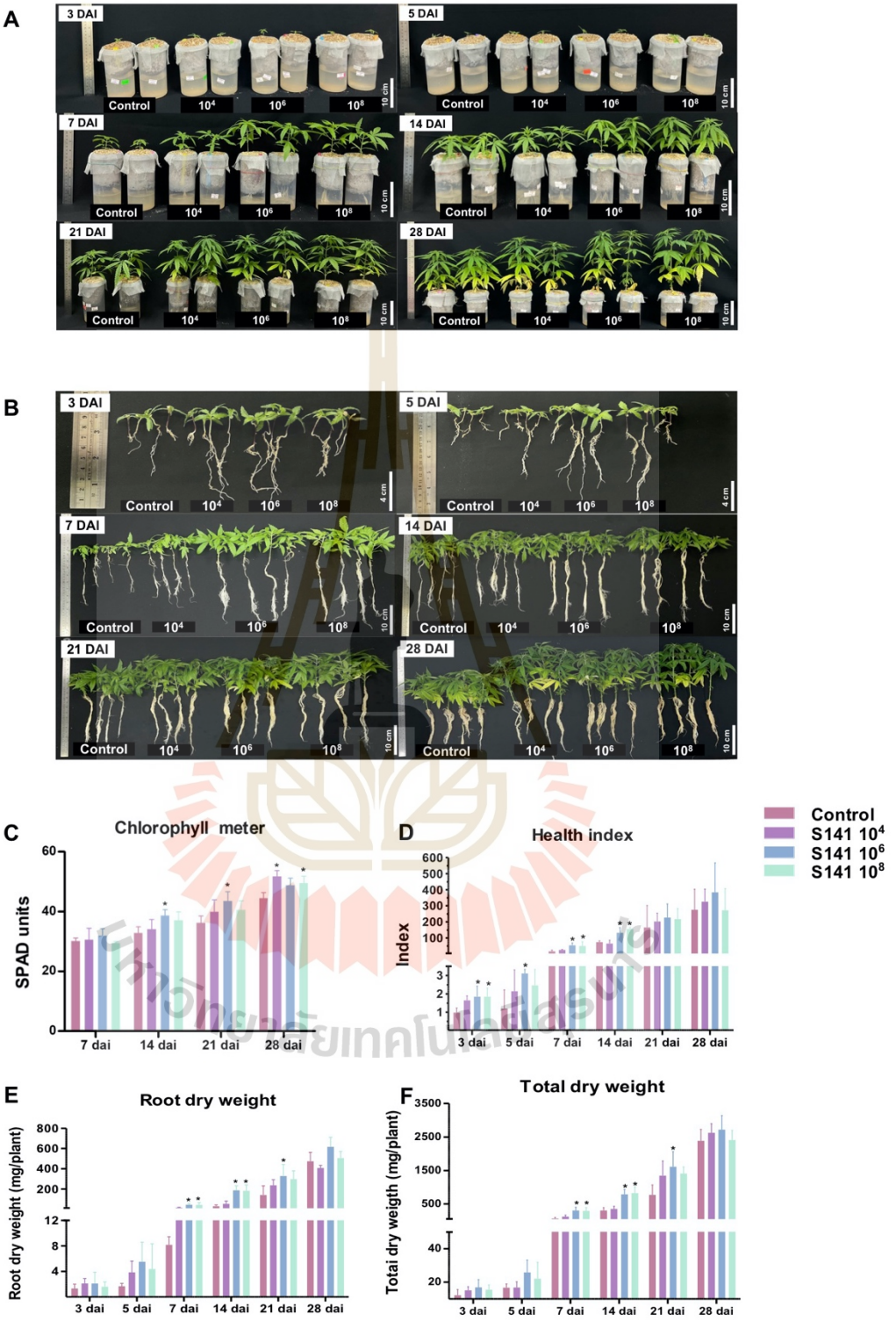

Figure 9. Evaluation of cannabis growth profiles after inoculation with *B. velezensis* S141 under laboratory conditions. Images were taken to inspect the growth Of cannabis plants cultivated in Leonard's jars after removal of the growth

medium (A,B). Parameters representing the cannabis growth profiles including chlorophyll content (C), heath index (D), root dry weight (E), and total dry weight (F) were examined. Bars display means ± SD calculated from biological triplicate (n = 4), and aster-isks denote statistically significant differences between treatment groups and control group ($p < 0.05$).

4.3 Enhanced Growth Performance of *Bacillus velezensis* S141-Inoculated Cannabis: Greenhouse Conditions

To evaluate the impact of S141 on the growth performance of cannabis in a green house, an experiment was designed using the same protocols as stated above. The plants were transferred into pots containing different types of soils and various levels of fertilizer and nurtured for 65 days after S141 inoculation. Upon examining the images collected at the end of the experiment, it was revealed that the growth of cannabis treated with S141 exhibited notable differences in the 10^4 to 10^8 CFU/ml group cultivated in both boiled soils supplemented with normal and low fertilizer (Figure 10A–H). As for the dry weight of the leaves, roots, stems, and branches, as well as the total plant, no significant different was observed between the control and S141 inoculation at 10^4 CFU/ml. For S141 inoculation at 10^6 and 10^8 CFU/ml, we found an approximately 2- to 4-fold increase in weight compared to the non-inoculated group (Figure 10E–H). This trend is similar to that observed with laboratory cultivation.

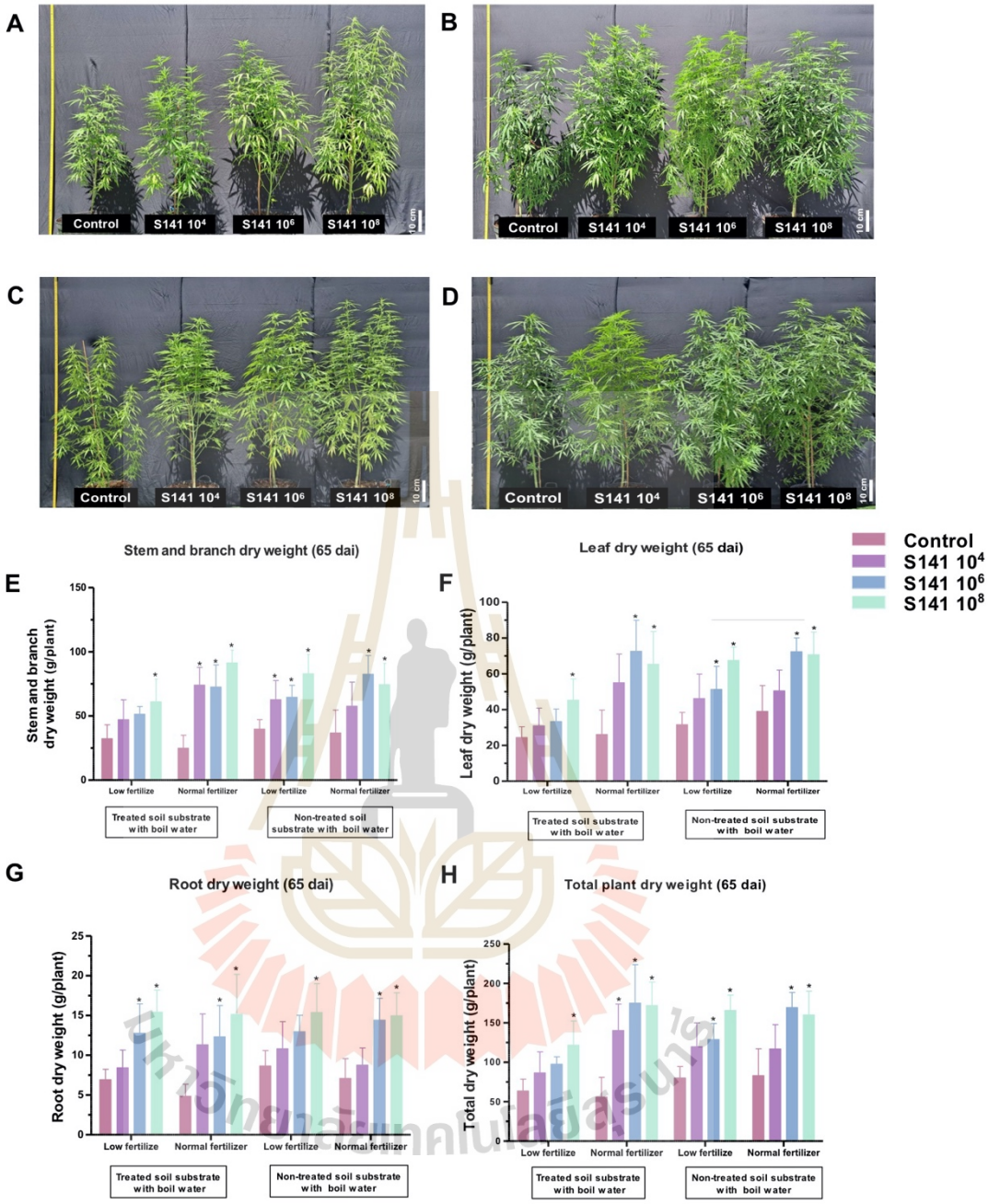

Figure 10. Assessment of cannabis growth patterns following inoculation with *B.velezensis* S141 in a greenhouse environment. The S141-inoculated and non-inoculated cannabis was cultivated in soil treated with boiled water containing either low fertilizer (A) or normal fertilizer (B) or untreated soil comprising low fertilizer (C) or normal fertilizer (D), from which features images of individually cultivated cannabis plants in pots were collected. Specific growth parameters, including leaf dry weight (E), stem and branch

dry weight (F), root dry weight (G), and total dry weight (H), were monitored. Bars indicate mean values ± SD (n = 5) from three biological replications. Significant differences between control group are indicated by asterisks ($p < 0.05$).

4.4 Transcriptomic Analysis, GO Terms, and KEGG Pathways

To uncover the molecular mechanism by which the S141 inoculum promoted cannabis growth, a transcriptomic analysis was conducted from three biological replications of each group. About 40 million total reads were generated from six libraries. Of these, clean reads were identified from Q20, constituting more than 96% (Table 7). A total of 976 DEGs were detected, the data for which were filtered at a false discovery rate (FDR) of less than 0.05 and an absolute Log2 (fold change) value of greater than one for comparison between the non-inoculated and inoculated groups. The findings indicated that the number of up-regulated genes exceeded that of the downregulated ones, 606 to 370, respectively (Figure 11A). The DEGs associated with molecular functions, cellular components, and biological processes were then examined in GO categories. Increased activities in metabolic processes, cellular processes for biological processes, cellular anatomical entities for cellular components, and catalytic activity and binding for molecular functions were found (Figure 11B). Finally, KEGG analysis led to the identification of potential growth-promoting pathways in cannabis impacted by S141, such as the biosynthesis of phenylpropanoid, plant–pathogen interaction, and plant hormone signal transduction (Figure 11C).

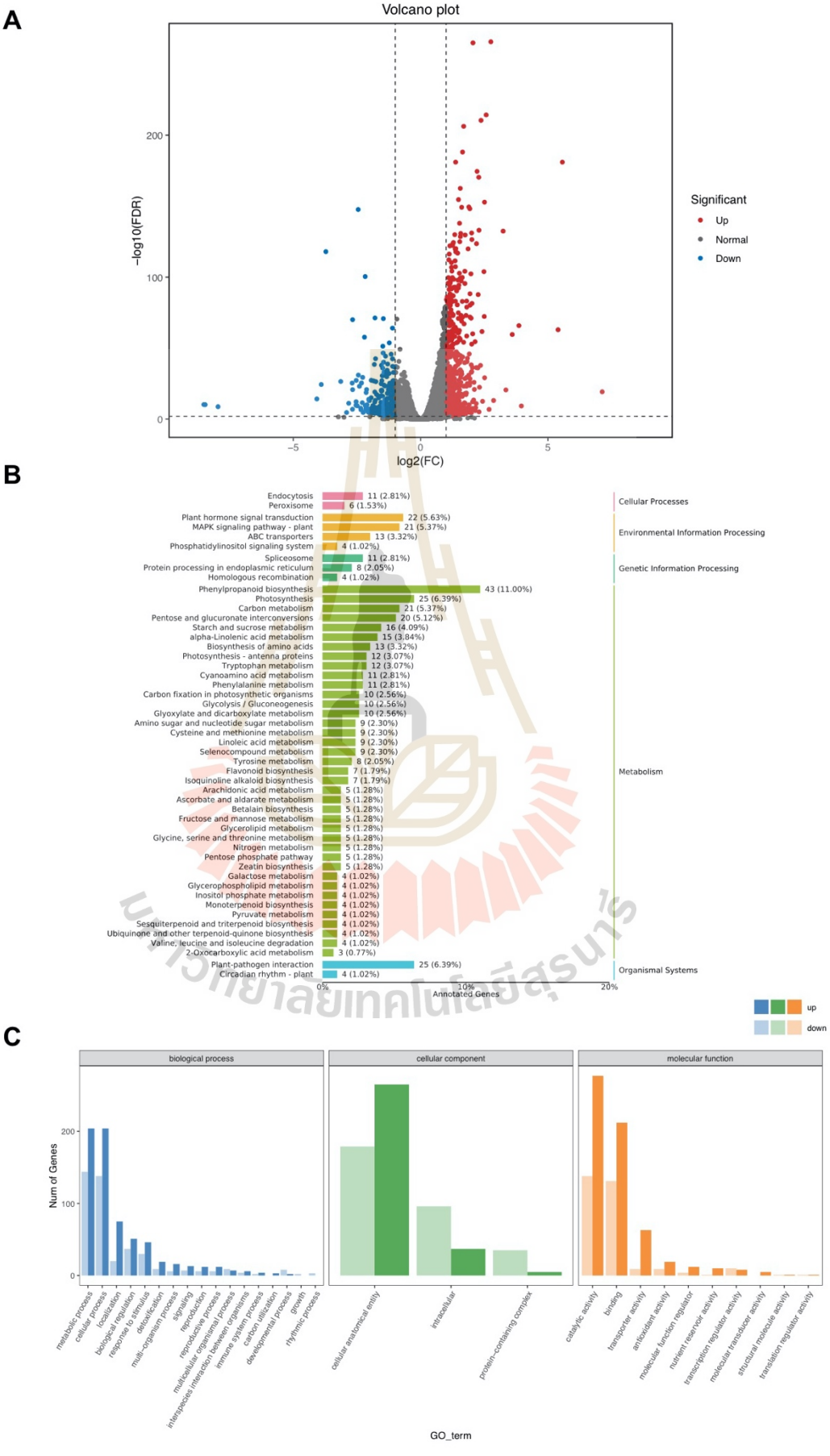
A
Volcano plot
−log10(FDR)
log2(FC)
Significant
Up
Normal
Down
B
Endocytosis 11 (2.81%)
Peroxisome 6 (1.53%)
Plant hormone signal transduction 22 (5.63%)
MAPK signaling pathway - plant 21 (5.37%)
ABC transporters 13 (3.32%)
Phosphatidylinositol signaling system 4 (1.02%)
Spliceosome 11 (2.81%)
Protein processing in endoplasmic reticulum 8 (2.05%)
Homologous recombination 4 (1.02%)
Phenylpropanoid biosynthesis 43 (11.00%)
Photosynthesis 25 (6.39%)
Carbon metabolism 21 (5.37%)
Pentose and glucuronate interconversions 20 (5.12%)
Starch and sucrose metabolism 16 (4.09%)
alpha-Linolenic acid metabolism 15 (3.84%)
Biosynthesis of amino acids 13 (3.32%)
Photosynthesis - antenna proteins 12 (3.07%)
Tryptophan metabolism 12 (3.07%)
Cyanoamino acid metabolism 11 (2.81%)
Phenylalanine metabolism 11 (2.81%)
Carbon fixation in photosynthetic organisms 10 (2.56%)
Glycolysis / Gluconeogenesis 10 (2.56%)
Glyoxylate and dicarboxylate metabolism 10 (2.56%)
Amino sugar and nucleotide sugar metabolism 9 (2.30%)
Cysteine and methionine metabolism 9 (2.30%)
Linoleic acid metabolism 9 (2.30%)
Selenocompound metabolism 9 (2.30%)
Tyrosine metabolism 8 (2.05%)
Flavonoid biosynthesis 7 (1.79%)
Isoquinoline alkaloid biosynthesis 7 (1.79%)
Arachidonic acid metabolism 5 (1.28%)
Ascorbate and aldarate metabolism 5 (1.28%)
Betalain biosynthesis 5 (1.28%)
Fructose and mannose metabolism 5 (1.28%)
Glycerolipid metabolism 5 (1.28%)
Glycine, serine and threonine metabolism 5 (1.28%)
Nitrogen metabolism 5 (1.28%)
Pentose phosphate pathway 5 (1.28%)
Zeatin biosynthesis 5 (1.28%)
Galactose metabolism 4 (1.02%)
Glycerophospholipid metabolism 4 (1.02%)
Inositol phosphate metabolism 4 (1.02%)
Monoterpenoid biosynthesis 4 (1.02%)
Pyruvate metabolism 4 (1.02%)
Sesquiterpenoid and triterpenoid biosynthesis 4 (1.02%)
Ubiquinone and other terpenoid-quinone biosynthesis 4 (1.02%)
Valine, leucine and isoleucine degradation 4 (1.02%)
2-Oxocarboxylic acid metabolism 3 (0.77%)
Plant-pathogen interaction 25 (6.39%)
Circadian rhythm - plant 4 (1.02%)
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Metabolism
Organismal Systems
Annotated Genes
up
down
C
biological process
cellular component
molecular function
Num of Genes
metabolic process
cellular process
localization
biological regulation
response to stimulus
detoxification
multi-organism process
signaling
reproduction
reproductive process
multicellular organismal process
interspecies interaction between organisms
immune system process
carbon utilization
developmental process
growth
rhythmic process
cellular anatomical entity
intracellular
protein-containing complex
catalytic activity
binding
transporter activity
antioxidant activity
molecular function regulator
nutrient reservoir activity
transcription regulator activity
molecular transducer activity
structural molecule activity
translation regulator activity
GO_term

Figure 11. Transcriptomic analysis of S141-inoculated and non-inoculated cannabis. (A) A volcano plot dis-plays differentially expressed genes, in which red- and blue-colored dots indicate up- and down-regulated genes, whereas grey-colored spots display non-differentially expressed genes, demon strating a log2(Fold change) of more or less than 1 between libraries generated from inoculated and non-inoculated cannabis with an adjusted p-value < 0.05. (B) KEGG pathways classify these genes into cellular processes (pink), environmental information processing (orange), genetic information processing (dark green), metabolism (green), and organismal systems (blue). (C) Gene Ontology organizes them into biological processes (blue), cellular components (green), and molecular functions (orange), wherein dark and light colors represent categories with upregulated and downregulated genes, respectively.

Table 7. Sequencing data analysis RNA sequencing (RNA-Seq) was analyzed and indicated of percent of GC, Q20 and Q30 of each sample.

#SampleID	Total Reads	Mapped Reads	GC (%)	Q20(%)	Q30(%)
CBRC1	41,813,660	37,584,995 (89.89%)	43.4	97.89	95.97
CBRC2	40,907,318	37,014,927 (90.48%)	43.63	97.82	95.78
CBRC3	40,852,224	36,650,102 (89.71%)	43.71	96.72	94.24
CBRI1	46,624,092	41,736,266 (89.52%)	43.59	96.88	94.54
CBRI2	42,244,200	37,838,633 (89.57%)	43.56	96.87	94.45
CBRI3	41,335,878	37,093,945 (89.74%)	43.53	96.8	94.39

4.5 Gene Expression Assessment by qRT-PCR

A total of 18 randomly chosen genes related to cannabis growth were studied using qRT-PCR. These included auxin-responsive protein SAUR50 (SAUR50), xyloglucan endotransglucosylase/hydrolase protein 25 (XEHP25), germin-like protein 2-1 (GLP2-1), ABC transporter G family member 29 (ABC29), carboxylesterase 120 (CBL120), receptor-like protein EIX1 (EIX1), ethylene-responsive transcription factor CRF5 (CRF5), in-dole-3-acetic acid (IAA2), response regulator (ARR5), response regulator (ARR12), caffeic acid 3-O-methyltransferase (CAO), UDP-glucose flavonoid 3-O-glucosyltransferase 7-like

(UDP), ethylene-response factor C3 (ERFC3), tetrahydrocannabinolic acid synthase (THCAS), cannabidiolic acid synthase (CBDAS), G-type lectin S-receptor-like serine/threonine-protein kinase At2g19130 (GTLS), leucine-rich repeat receptor-like protein kinase At5g49770 (LRLK4), and pathogenesis-related protein 1A-like (PRP-1A). The latter was used to validate the RNA-Seq results (Figure 12).

Significantly higher expressions of THC, GLP2-1, XEHP25, IAA2, and SAUR50 (about 2- to 6-fold) was found in all tissue's roots, stems, and leaves than in the uninoculated group. The UDP, CBD, CBL120, ARR12, ARR5, and CRF5 were significantly upregulated 2- to 10-fold in stems and leaves when compared to the control. An increase of about 8-fold in ERFC3 and EX11 expressions was observed only in leaf tissue. Root and stem tissues showed an about 2.5-fold upregulation of ABC29 expression. GTLS, LRLK4, and PRP-1A were significantly downregulated around 2- to 5-fold. It is worth noting that the expression pattern determined from the RNA sequencing (RNA-Seq) data was similar to the ex-pression pattern of the selected DEGs determined from the RT-qPCR results.

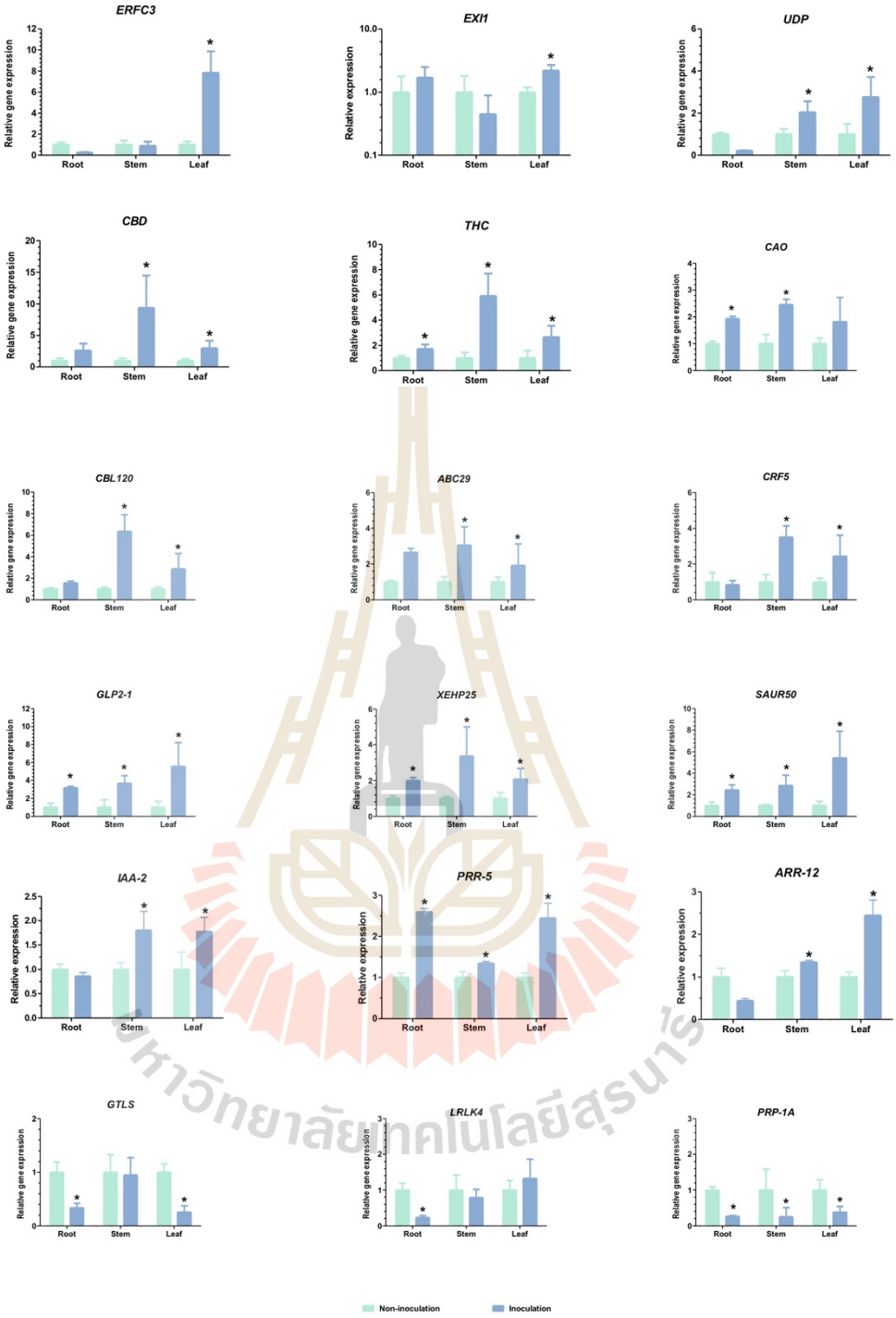

Figure 12. The qRT-PCR analysis of differentially expressed genes obtained from RNA-seq. Eighteen genes related to plant-growth-promoting impacts in cannabis were chosen, where the relative expression was calculated using the $2^{-\Delta\Delta_{Ct}}$ method and normalized against actin, an internal control (Livak & Schmittgen, 2001; Pfaffl, 2001). method and normalized against actin, an

internal control [47,48]. Bars indicate means ± SD analyzed from three biological replications (n = 4), and asterisks indicate significant differences at $p < 0.05$.

4.6 Impact of *Bacillus velezensis* S141 Mutants on the Growth of the *Foi Thong Suranaree* 1 Cannabis Strain Under Controlled Laboratory Conditions

S141 was previously characterized by its genetic repertoire, which comprises various genes associated with plant growth promotion and biocontrol activities. The beneficial effects can be traced to key genes, including those that encode enzymes for indole-3-acetic acid (IAA) synthesis, such as *yhcx*, *IPyAD*, and *dhaS*. These genes play an important role in IAA production from indole-3-pyruvic acid. In addition, the cytokinin biosynthesis pathway includes *IPT* and *IPI* genes. This pathway responds to the IPI gene encoding the isopentenyl pyrophosphate isomerase (IPI) enzyme, which converts isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP). This DMAPP then acts as a substrate for the enzyme isopentenyl transferase (IPT), which is responsible for cytokinin biosynthesis (Sibponkrung et al., 2020).

To examine the impact of the genes related to the production of plant hormones in S141, *B. velezensis* S141 mutants (*IpyAD*, *dhas*, *yhcx, IPT*, and *IPI*) and wild-type S141 were inoculated onto cannabis plants at a concentration of 10^6 CFU/ml. At 7–14 DAI, no significant differences in chlorophyll content were observed across all experimental groups inoculated with S141 and S141 mutants when contrasted with the control group (uninoculated). Investigations into the characteristics of the plants from S141 and S141 mutants (*dhas*, *yhcx*, and *IPI*) revealed noticeable increases in root length, plant height, root dry weight, total dry weight, and HI compared to those of the uninoculated group (Figure 13A–F).

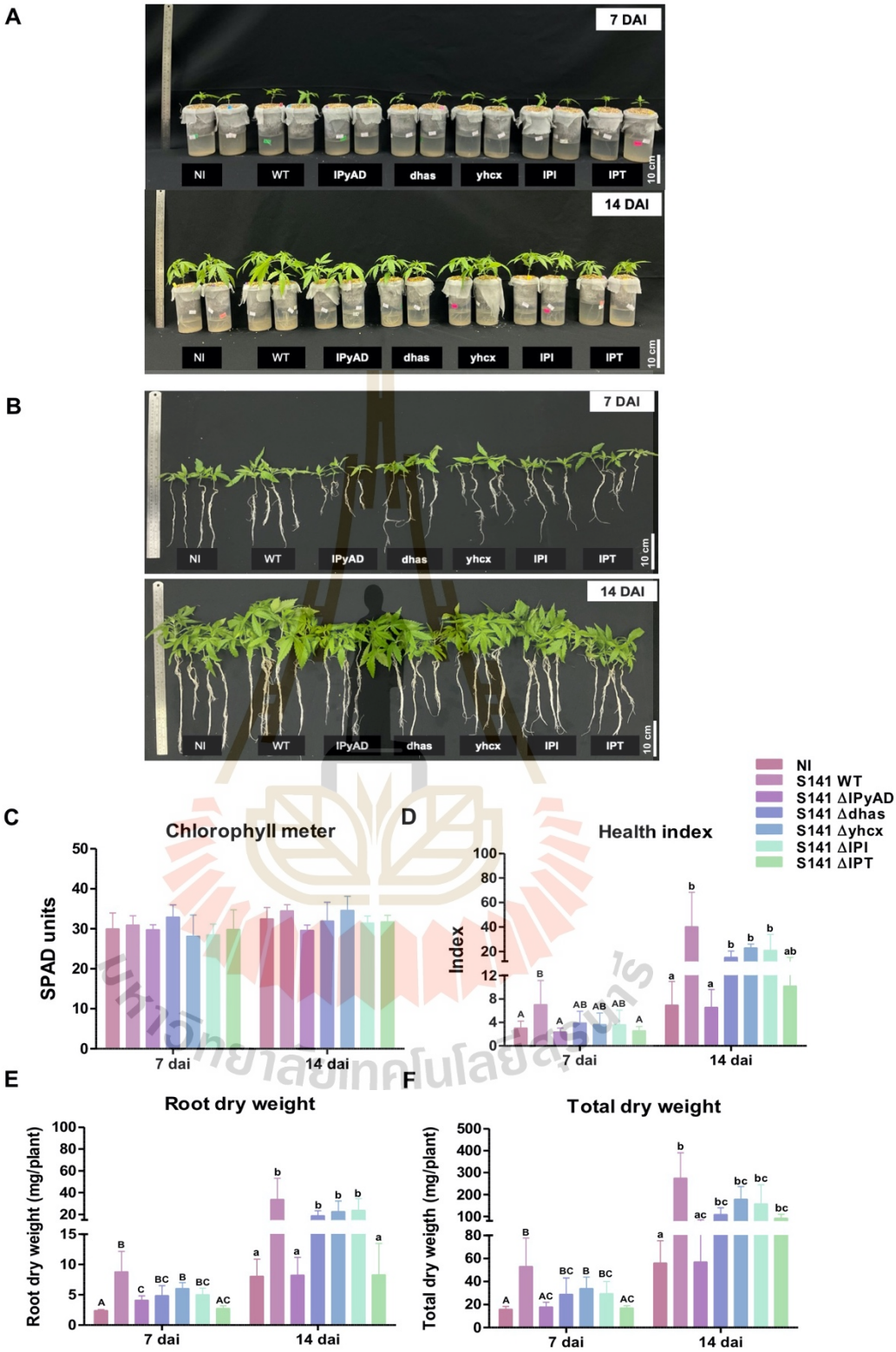

Figure 13. Examination of cannabis growth profiles following inoculation with *B. velezensis* S141 mutants. Visual inspection of the cannabis plants cultured in Leonard's jars was performed by taking images of the plants

after removing the growth medium (A,B). Parameters representing the cannabis growth profiles including chlorophyll content (C), heath index (D), root dry weight (E), and total dry weight (F) were examined. Bars display means ± SD calculated from biological triplicates (n = 4), and different letters indicate significant differences between treatment groups ($p < 0.05$).

Interestingly, in groups inoculated with S141 mutant genes (*IpyAD* and *IPT*), no significant changes were observed in the root length, plant height, root dry weight, total dry weight, or HI when compared to the uninoculated group. These findings suggest that the genes encoding for plant hormones in S141 (*IpyAD* and *IPT*) play a significant role in promoting growth in cannabis.

CHAPTER V
DISCUSSION

Bacillus velezensis is recognized for its diverse applications as a PGPB and a plant-growth-promoting rhizobacterium (PGPR) (Bagheri et al., 2022; Bai et al., 2023; Balderas-Ruíz et al., 2020). *B. velezensis* has been identified as a PGPB in cannabis, similar to in this study, which discovered the highest S141 copy number in surface-sterilized tissues and leaves, followed by the stems and roots of inoculated cannabis. No S141 was present in the non-inoculated group. This result highlights that S141 might be an endophytic bacterium in cannabis (Figure 8). Several strains of *B. velezensis* are known for their capacity to enhance plant growth through various mechanisms. *B. velezensis* FZB42, renowned for producing phytohormones such as indole-3-acetic acid (IAA), improves nutrient uptake, leading to increased biomass and stress tolerance in crops like *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) (Chowdhury et al., 2015; Liu et al., 2017). *B. velezensis* 83 promotes maize growth by stimulating root development through phosphate solubilization, auxin production, and volatile organic compound emissions, thus boosting overall plant vigor (Balderas-Ruíz et al., 2020).]. Moreover, *B. velezensis* BS1 has demonstrated biocontrol efficacy against fungal pathogens in pepper plants, contributing to enhanced plant health and growth by reducing disease stress (Shin et al., 2021). S141 is seen as a promising plant-growth-promoting rhizobacterium with multifaceted benefits for agricultural systems. It enhances plant growth, nodulation, and nitrogen fixation efficiency, particularly in collaboration with crops like soybean, highlighting its potential as a beneficial tool in sustainable agriculture (Sibponkrung et al., 2020). Additionally, S141 showcases the capacity to boost arbuscular mycorrhizal symbiosis, increasing nutrient absorption and usage in host plants. By activating key plant marker genes linked to mycorrhizal symbiosis and upregulating the genes essential for nutrient absorption, S14 contributes to improved plant performance and resilience.

Its effect on the cost benefit balance in mycorrhizal symbiosis underscores its complex interactions within plant–microbe systems (Kiddee et al., 2024). In this study, S141 inoculation substantially enhanced cannabis growth, particularly at an optimal concentration of 10^6 CFU/ml, as demonstrated by increased trunk circumference, height, chlorophyll content, and dry weight (Figure 9). Furthermore, greenhouse experiments with four separate soil conditions, including soils treated with boiled water and untreated soils, combined with either normal or low fertilizer levels, validated these findings. The results showed the practical benefits, such as improved growth parameters and reduced fertilizer needs, of treatments with this bacterium (Figure 10). These findings emphasize the potential of *B. velezensis* S141 to advance cannabis cultivation practices, promote cannabis growth, encouraging further exploration of its action mechanisms and optimization for extensive agricultural applications.

Transcriptomes have been utilized in cannabis to decipher how the plant responds and defends itself against biotic and abiotic stresses (Balthazar et al., 2020; Huang et al., 2019; Miotti et al., 2023). This study found transcriptomic analysis useful in identifying the plant genes associated with S141 inoculation (Figure 11). The GO term analysis showed that the upregulation of biological processes such as metabolic process, cellular process, localization, and biological regulation was higher than downregulation following *B. velezensis* inoculation. This suggests a connection to the network of metabolic pathways responsible for growth, development, and environmental responses (Liao et al., 2023). Consequently, differential gene expression observed in S141-treated *C. sativa* might be linked to its enhanced growth rate. Pathway analysis through the Kyoto Encyclopedia of Genes and Genomes (KEGG) highlighted several metabolism-related processes, including phenylpropanoid biosynthesis, plant-pathogen interactions, and plant hormone signal transduction. Previous studies have shown that transcription factors such as MdMYB88 and MdMYB124 regulate the accumulation of phenylpropanoid metabolites by modulating MdCM2 expression, aiding plants in combating pathogens and withstanding drought conditions (Geng et al., 2020). Effectors in plant–pathogen interactions operate in key ways: they break through physical barriers to invade the plant, create an environment that supports their survival inside the plant, and even employ tactics to evade or deceive plant defenses, while weakening the plant's immune responses (S. Zhang et

al., 2022). Our study observed such behaviors in plant–pathogen interactions after inoculation with the bacterium, which suggests that these interactions might develop and respond to the environment of the organismic system of plants. In the realm of plant hormone signal transduction pathways, membrane or transmembrane receptors display specificities and diversities. Acting as gateways, these receptors play an essential role in recognizing various hormones and transmitting signals into the cell (Song et al., 2017). Recent studies showed that the upregulation of phytohormone signal transduction is crucial in the response of Jerusalem artichoke (*Helianthus tuberosus* L., (Asteraceae)) seedlings to salt stress. The genes studied included those for abscisic acid, auxin, ethylene, and jasmonic acid (Yue et al., 2022). Plant growth regulators have been studied for their ability to enhance the growth and yield of rice under high-temperature conditions both day and night, with the most significant production found via hormone treatments that boosted photosynthesis (Fahad et al., 2016). Our results suggested that plant hormone signal transduction was represented by KEGG pathways during *B. velezensis* inoculation in *C. sativa*. This implies that biological processes related to gene metabolism, previously identified as pivotal in growth pathways, could be highly beneficial for plant growth in conjunction with plant–pathogen interactions and plant hormone processes. (Figure 14).

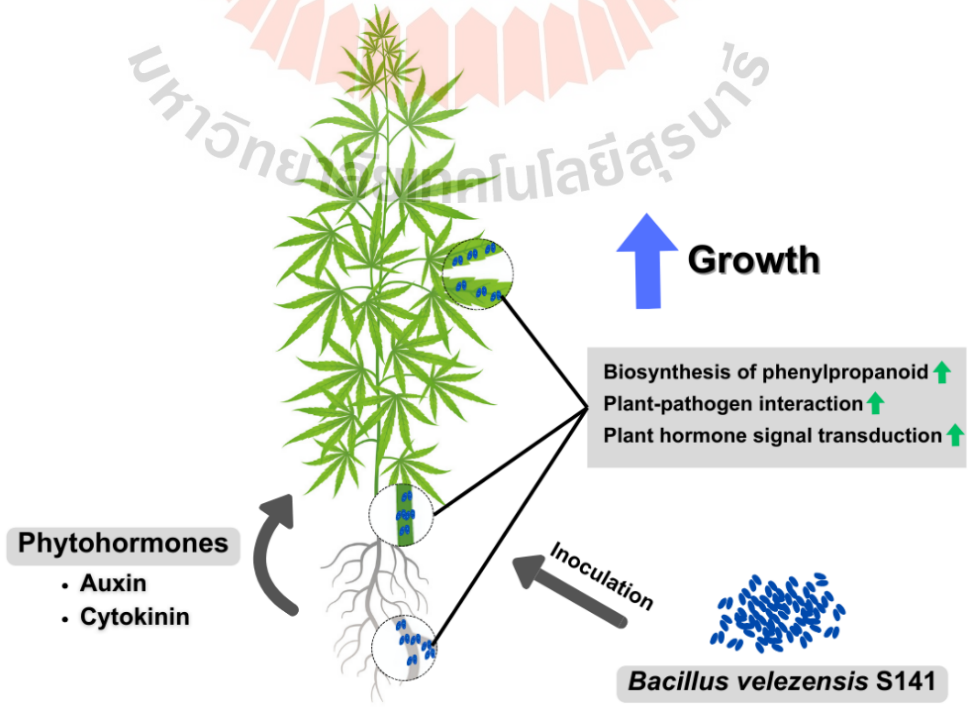

Figure 14. Schematic overview of mechanisms of cannabis growth promotion by *Bacillus velezensis* S141. S141, an endophytic cannabis bacterium, promotes cannabis growth by producing phytohormones and triggering genes involved in the biosynthesis of phenylpropanoid, plant–pathogen interaction, and plant hormone signal transduction pathways. This figure was created using https://www.canva.com/ (accessed on 21 August 2024).

In this study, we used qRT-PCR to explore the expression profiles of 18 genes related to cannabis growth (Figure 12), with a particular focus on their modulation by phytohormones involved in developmental processes. Genes such as SAUR50, IAA2, and ARR5 showed significant upregulation across all tissues (root, stem, and leaf). Previous studies have shown that auxins like IAA2 are essential in root and shoot development by regulating cell elongation and differentiation (Wu et al., 2017). SAUR proteins, such as SAUR50, have been associated with auxin-mediated growth responses, suggesting their conserved role across plant species (Ren & Gray, 2015). The observed upregulation of these genes aligns with their established roles in enhancing vegetative growth, a key factor in optimizing cannabis cultivation and biomass production. Genes such as UDP, CBD, CBL120, ARR12, ARR5, and CRF5 displayed tissue-specific expression, with predominant activity in stems and leaves, reflecting their roles in regulating growth pathways. For instance, cytokinin-responsive genes like ARR5 and ARR12 are implicated in shoot branching and delaying leaf senescence (Mason et al., 2005), potentially impacting cannabis plant architecture and yield. The upregulation of CBD-related genes in the stems aligns with the biosynthesis of cannabinoids, essential compounds with pharmaceutical relevance, thus emphasizing the economic significance of understanding their regulatory mechanisms in different plant tissues (Andre et al., 2016).]. Comparative analyses with previous studies emphasize both the evolutionarily con-served and divergent regulatory mechanisms of phytohormone-responsive genes across plant species. Studies on germinlike proteins (e.g., GLP2-1) involved in cell wall modification and growth regulation further highlight their conserved roles in hormonal signaling pathways affecting plant architecture and development (Cosgrove, 2018; Takahashi et al., 2018). The observed downregulation of genes like GTLS and LRLK4 suggests their potential roles in modulating receptor-

mediated signaling pathways and stress responses, indicative of complex hormonal crosstalk governing growth and development in cannabis and related species (Afzal et al., 2016; Gou et al., 2010). These findings underscore the intricate interplay between hormonal signals and gene regulation, emphasizing the importance of understanding these mechanisms for optimizing crop productivity and metabolic pathways in cannabis cultivation. Future research could examine in greater detail the specific molecular interactions and signaling pathways involved in phytohormone-mediated growth processes, thereby facilitating targeted breeding strategies and biotechnological advancements in cannabis agriculture.

The roles of the genes involved in the production of plant hormones, auxin and cytokinin, in bacteria are pivotal for understanding and optimizing plantmicrobe interactions. Genes such as *yhcx*, *IPyAD*, and *dhaS*, which are integral to auxin biosynthesis, enhance plant growth by promoting root elongation, nutrient uptake, and stress resistance (Lee et al., 2019; Q. Zhang et al., 2022). Likewise, cytokinin biosynthesis genes like IPT and IPI contribute to shoot growth, cell division, and delay in leaf senescence (Glanz-Idan et al., 2022; Kant et al., 2015). Mutations in these genes can drastically reduce hormone production, leading to reduced bacterial colonization, plant growth promotion, and disease resistance. In prior studies, co-inoculation of *Bradyrhizobium diazoefficiens* F (*Xanthobacteraceae*) strain USDA110 with S141Δ*yhcx* and S141Δ*IPI* reduced the number of nodules, indicating the significant impact of *yhcx* and *IPI* on promoting soybean growth (Sibponkrung et al., 2020). In this study, we elucidated the distinct roles of the genes involved in plant hormone production in cannabis growth via the investigation of S141 and its mutant strains. Significant differences in growth parameters emerged between the inoculated and control groups. Specifically, both wild-type S141 and mutants possessing *dhaS*, *yhcx*, and *IPI* genes exhibited marked increases in root length, plant height, root dry weight, total dry weight, and the health index (HI), underlining the importance of these genes in promoting robust cannabis growth (Figure 13A–F). Conversely, mutants lacking *IpyAD* and *IPT* genes displayed no significant improvements in these metrics compared to the controls, implying a crucial role of *IpyAD* and *IPT* in mediating cannabis growth promotion. These findings contribute to our understanding of how specific genetic

elements in S141 enhance plant growth, potentially through the modulation of the hormone signaling pathways essential for developmental processes in cannabis.

This study offers substantial evidence of the growth-promoting effects of S141. However, further research is necessary to thoroughly understand its mechanisms of action and to optimize its use in cannabis cultivation. Future research could concentrate on understanding the distinct metabolic pathways and signaling mechanisms engaged in plant–microbe interactions, as well as investigate potential synergies with other beneficial microorganisms or agricultural inputs. Moreover, conducting field trials over several growing seasons and under varied environmental conditions could help confirm the effectiveness and consistency of S141 inoculation across different cannabis cultivars and cultivation practices.

CHAPTER VI

CONCLUSION

This study underscores the significant impact of *B. velezensis* S141 inoculation on the growth profiles and gene expression of *C. sativa* inoculated with S141. Both laboratory and greenhouse cultivation trials evidenced that S141 could positively impact various facets of cannabis growth, comprising increased stem size, height, chlorophyll content, and the dry weights of the leaves, stems, and roots. Furthermore, RNA sequencing analysis detected considerable modifications in gene expression, notably in metabolic processes, cellular components, and catalytic activities, underlining the intricate mechanisms governing the symbiotic relationship between S141 and cannabis. Moreover, pathway enrichment analysis signaled key pathways linked to plant growth and defense, accentuating the potential of S141 as a bioinoculant for enhancing cannabis cultivation practices.

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BIOGRAPHY

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