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 °C 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 °C, 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.