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.

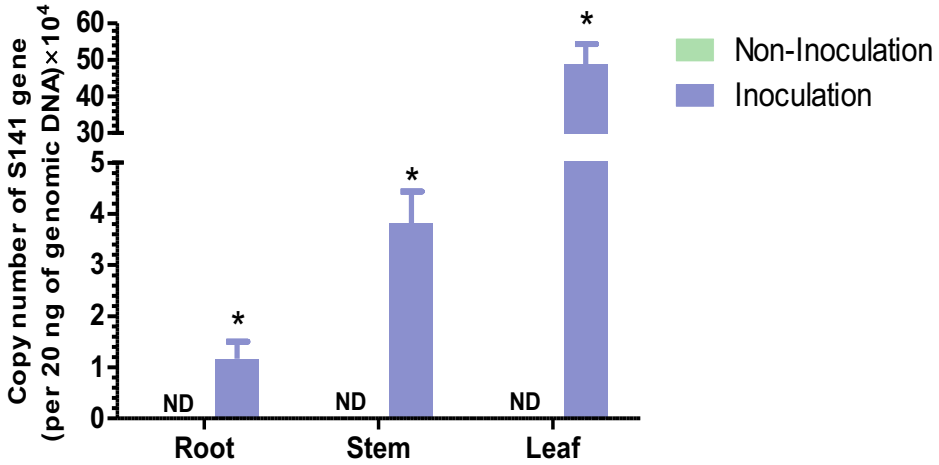

Figure 8. Localization of S141 in cannabis after inoculation with *B. velezensis* S141. Genomic DNA was collected from the leaf, stem, and root tissues at 7 days after inoculation. S141 copy levels were measured in triplicate using qRT-PCR. Data are presented as mean ± SD for n = 3 from three bio-logical 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).

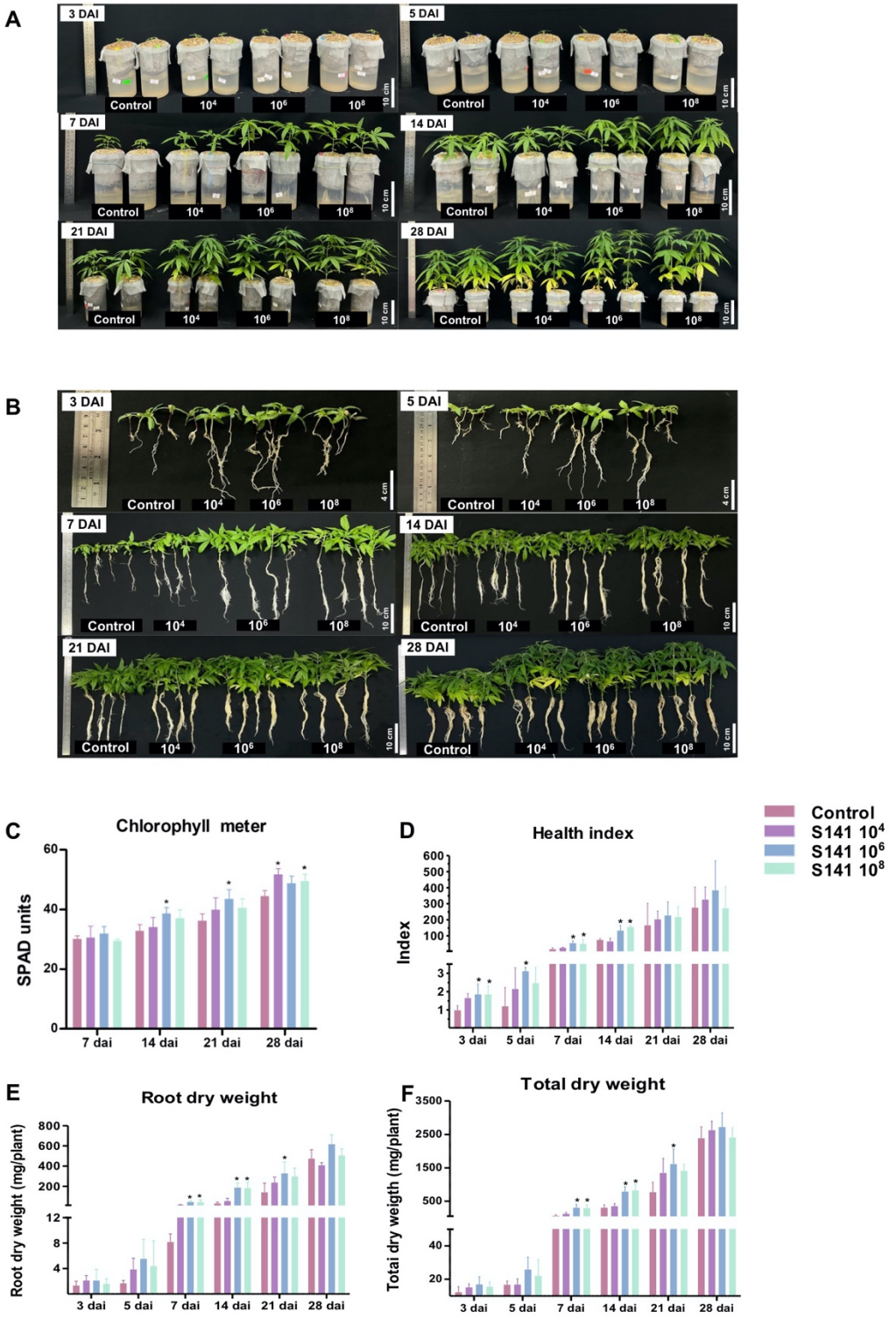

Figure 9. Evaluation of cannabis growth profiles after inoculation with *B. velezensis* S141 under laboratory conditions. Images were taken to inspect the growth Of cannabis plants cultivated in Leonard's jars after removal of the growth

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.

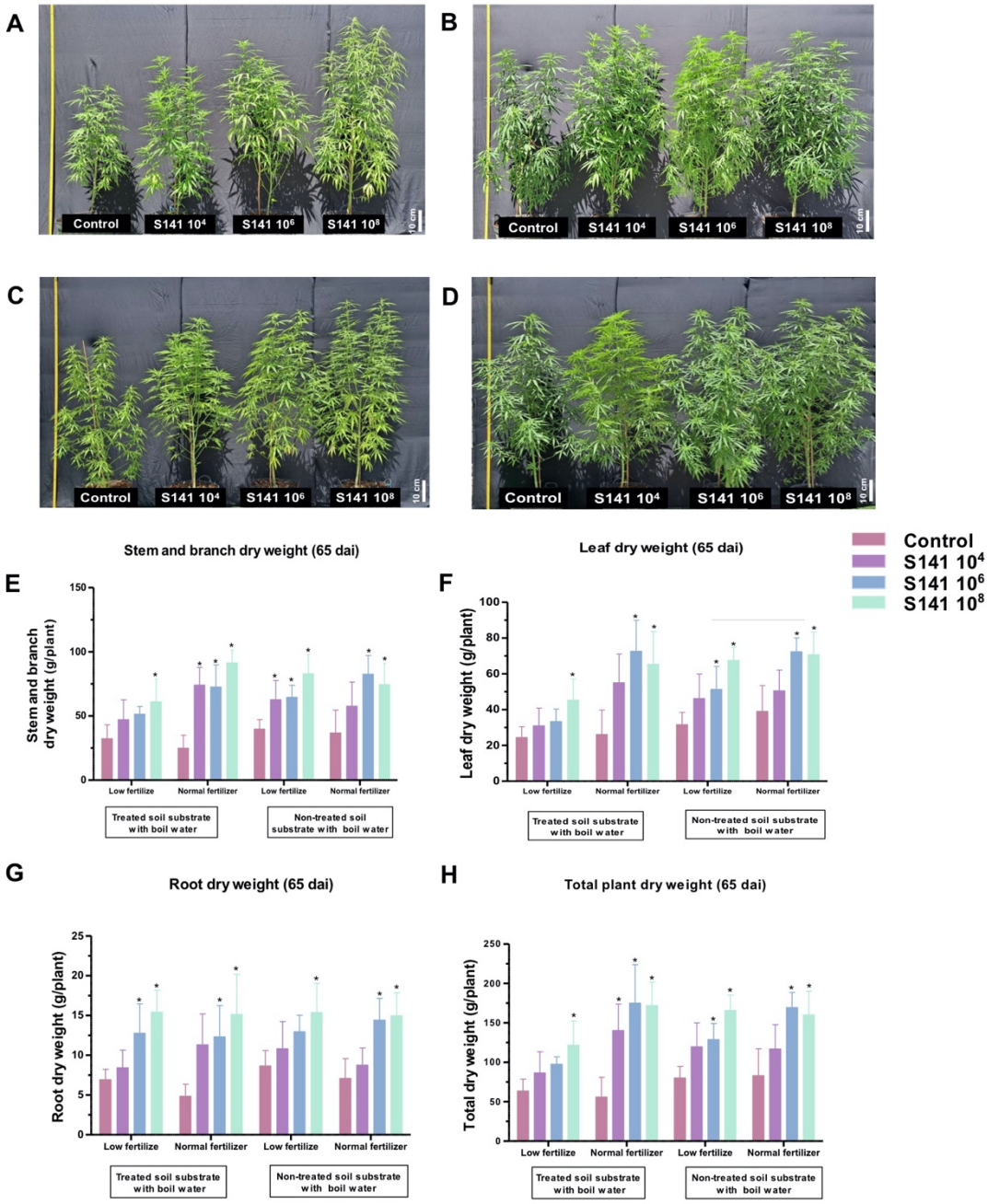

Figure 10. Assessment of cannabis growth patterns following inoculation with *B.velezensis* S141 in a greenhouse environment. The S141-inoculated and non-inoculated cannabis was cultivated in soil treated with boiled water containing either low fertilizer (A) or normal fertilizer (B) or untreated soil 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).

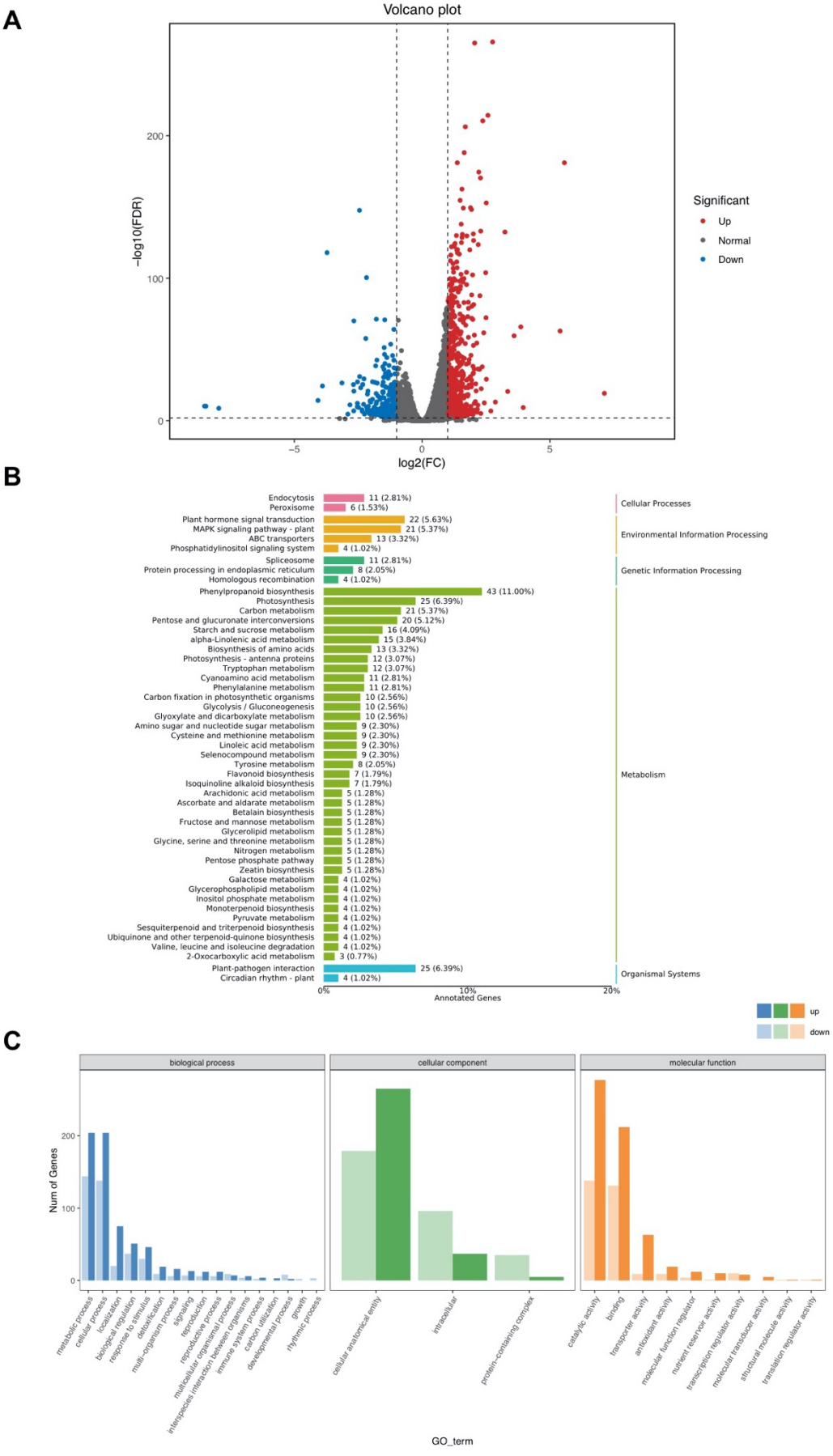
A
Volcano plot
−log10(FDR)
log2(FC)
Significant
Up
Normal
Down
B
Endocytosis 11 (2.81%)
Peroxisome 6 (1.53%)
Plant hormone signal transduction 22 (5.63%)
MAPK signaling pathway - plant 21 (5.37%)
ABC transporters 13 (3.32%)
Phosphatidylinositol signaling system 4 (1.02%)
Spliceosome 11 (2.81%)
Protein 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.

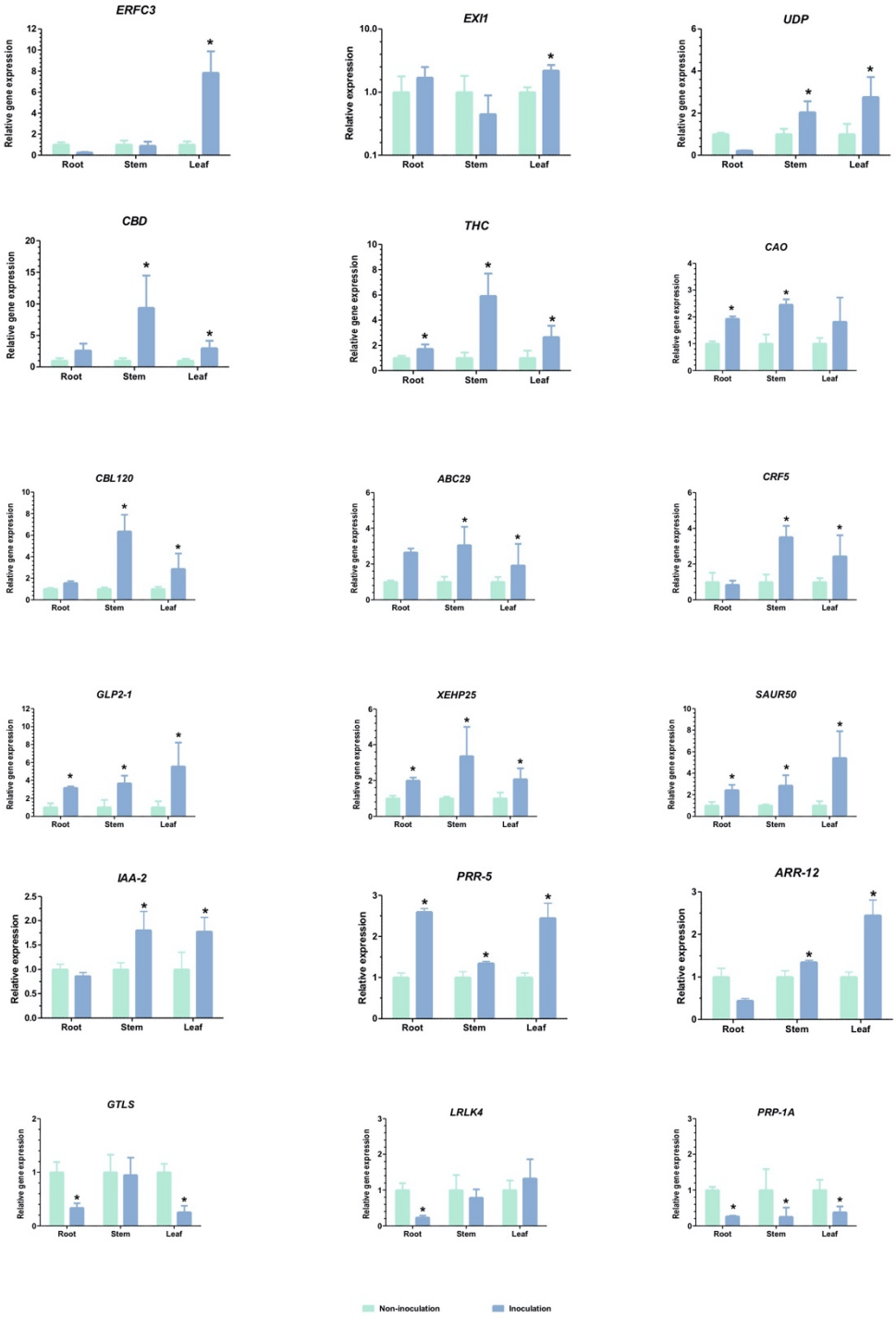

Figure 12. The qRT-PCR analysis of differentially expressed genes obtained from RNA-seq. Eighteen genes related to plant-growth-promoting impacts in cannabis were chosen, where the relative expression was calculated using the $2^{-\Delta\Delta_{Ct}}$ method and normalized against actin, an internal control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

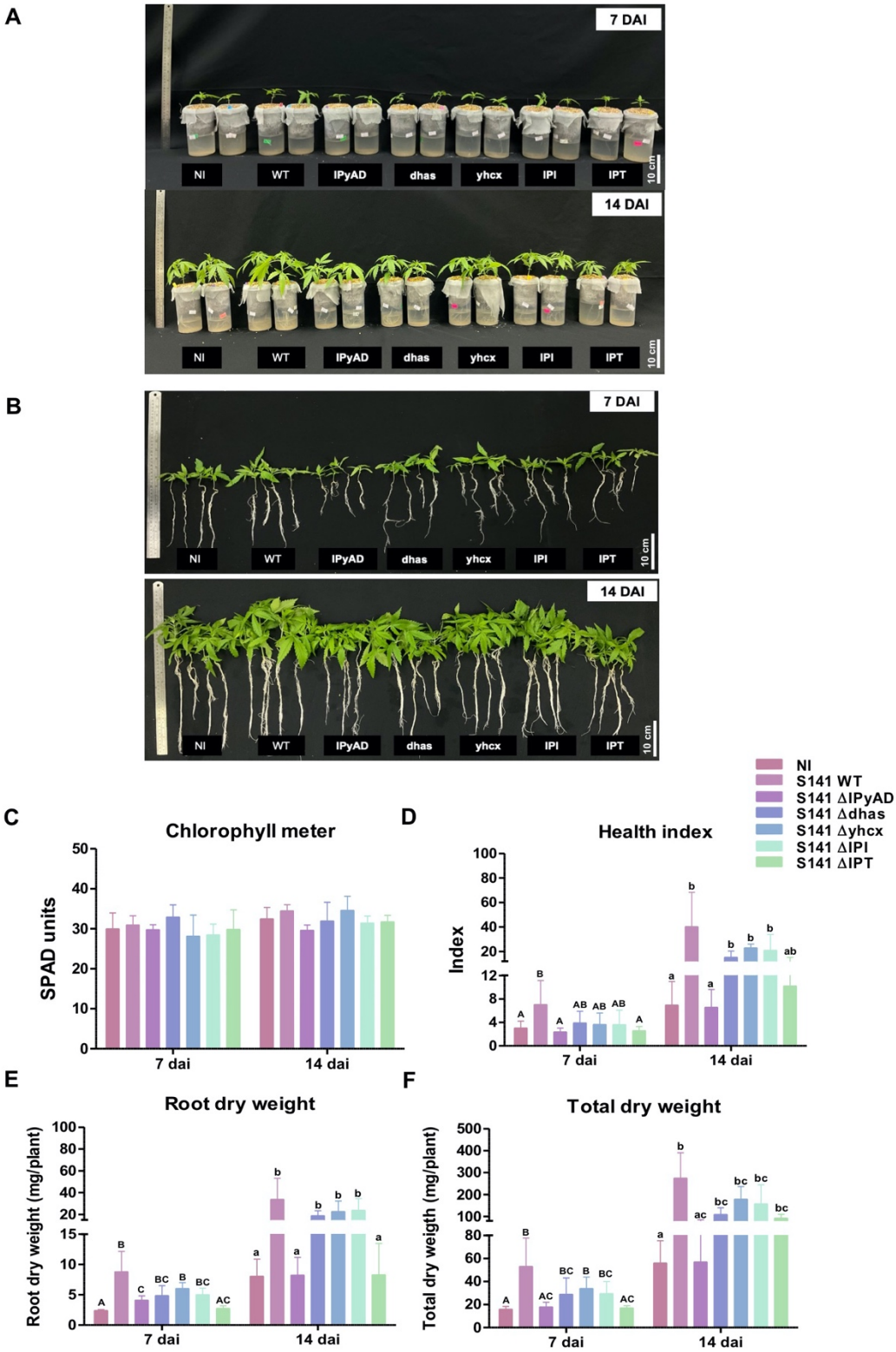

Figure 13. Examination of cannabis growth profiles following inoculation with *B. velezensis* S141 mutants. Visual inspection of the cannabis plants cultured in Leonard's jars was performed by taking images of the plants

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.