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Article

Plant Growth-Promoting Rhizobacteria (PGPR) with Microbial Growth Broth Improve Biomass and Secondary Metabolite Accumulation of *Cannabis sativa* L.

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ABSTRACT: Plant growth-promoting rhizobacteria (PGPR) are a sustainable crop production input; some show positive effects under laboratory conditions but poorly colonize host field-grown plants. Inoculating with PGPR in microbial growth medium (*e.g.*, King's B) could overcome this. We evaluated cannabis plant (cv. CBD Kush) growth promotion by inoculating three PGPR (*Bacillus* sp., *Mucilaginibacter* sp., and *Pseudomonas* sp.) in King's B at vegetative and flower stages. At the vegetative stage, *Mucilaginibacter* sp. inoculation increased flower dry weight (24%), total CBD (11.1%), and THC (11.6%); *Pseudomonas* sp. increased stem (28%) dry matter, total CBD (7.2%), and THC (5.9%); and *Bacillus* sp. increased total THC by 4.8%. Inoculation with *Mucilaginibacter* sp. and *Pseudomonas* sp. at the flowering stage led to 23 and 18% increases in total terpene accumulation, respectively. Overall, vegetative inoculation with PGPR enhanced cannabis yield attributes and chemical profiles. Further research into PGPR inoculation onto cannabis and the subsequent level of colonization could provide key insights regarding PGPR-host interactions.

KEYWORDS: *Cannabis sativa L., microbial growth medium, plant growth-promoting rhizobacteria, flower yield, cannabinoid, terpene*

1. INTRODUCTION

Chemical application (*e.g.*, fertilizers and pesticides) has been widely used to improve crop production for feeding the increasing human and livestock populations; it has been a key element of the Green Revolution.[1,2] However, long-term application and over-supply of chemical inputs cause greenhouse gas emissions[3] and contribute to climate change.[4] To prevent severe climate change, it is necessary to exploit sustainable technologies to increase crop production with minimum inputs.[5]

As the lengthy co-evolution of microbes and plants is better understood, using plant growth-promoting rhizobacteria (PGPR) has become an important alternative to additional chemical fertilizers to obtain high biomass and crop yield in agriculture.[6] The acronym PGPR was first utilized in 1976 by Kloepper to define a group of bacteria that colonize the surface of the root or intercellular spaces within the root of the host plant to establish a mutualistic relationship, thus improving plant growth.[7] Studies of PGPR's potential benefits are increasing rapidly, as is the momentum to exploit them commercially as low cost and environmentally compatible crop management inoculants.[8] The significance and potential of PGPR for plant growth promotion and yield enhancement have been reported for various types of crops through many mechanisms, including (1) phytohormone production,[9] (2) nitrogen fixation and phosphorus solubilization,[10] (3) inter-organismal signal production,[11] (4) antibiotic release as biocontrol agents,[12,13] and (5) induced systematic resistance as a way to manage plant pathogen attack.[14] The effect of PGPR on plant growth differs among plant species. For instance, for Glycine max L. Merrill, inoculation with *Pseudomonas* sp. strain AK-1 and *Bacillus* sp. strain SJ-5 improved plant biomass production and led to higher proline contents.[15] It has also been illustrated that PGPR inoculation influences the accumulation of secondary metabolites, as inoculation with *Ralstonia pickettii* and *Brevibacillus invocatus* leads to increases in flavonoids and phenolics within sunflower (cv. Parsun 3) grown under polluted conditions.[16] Beneficial bacteria can be isolated from various plant species and screened for beneficial effects across a range of plant species. Fan *et al.* (2018)[17] reported that the bacteria isolated from non-crop species showed potential to enhance crop plant growth through a range of properties from IAA production to nitrogen fixation to P solubilization. Evidence also indicates that *Serratia marcescens* MOSEL-w2, *Enterobacter cloacae* MOSEL-w7, and *Paenibacillus* MOSEL-w13 isolated from *Cannabis sativa* L. play roles as biocontrol agents against the diseases caused by *Phytophthora* in other crops.[18] Some bacterial genera isolated from cannabis, including *Pseudomonas*, *Pantoea*, and *Bacillus*, also have the potential to promote plant growth.[19]

Cannabis, including industrial hemp and marijuana, has economic, social, and cultural importance around the world.[20]

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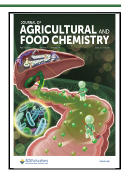

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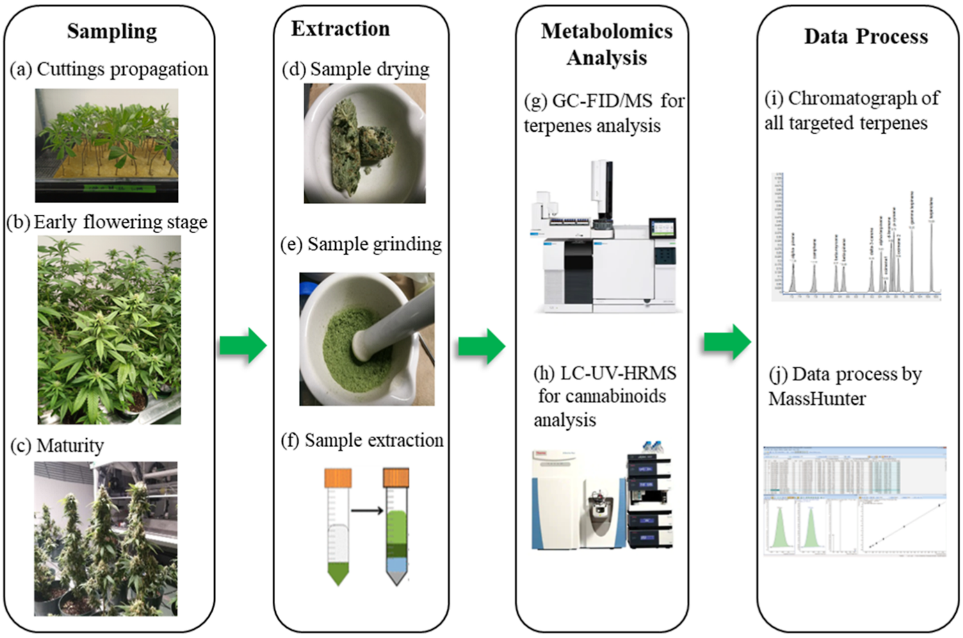

Figure 1. Workflow from plant material collection to secondary metabolite analysis. In the sampling stage: (a) cuttings growing in rockwool cubes; (b) plants at the early flowering stage for PGPR inoculation; (c) mature plants for biomass determination and secondary metabolite analysis. For the extraction step: (d) flowers were dried and (e) manually ground into fine powder; (f) then, samples were extracted. In the metabolomic analysis: (g) GC-FID/MS used for terpene identification and quantification; (h) LC-UV-HRMS used for cannabinoid analysis. In the data processing stage: (i) chromatographs of standard terpenes and (j) data process using MassHunter.

Cannabis sativa L. originated in northern China and was first domesticated and diversified in early Neolithic times in East Asia. It has successfully adapted to a wide range of climatic and edaphic conditions and proliferated around the world.[20] Cannabis is widely cultivated because it is an important source of natural fiber, seed oil, and medicinal components.[20−22] The medicinal value of cannabis depends on the levels of various cannabinoids, an important set of secondary metabolite compounds with various psychoactive and non-psychoactive effects on humans, including Δ^9-tetrahydrocannabinol (THC), and cannabidiol (CBD).[23,24] In addition, the entourage therapeutic effects have been shown to be associated with the abundance of other secondary metabolites, including the terpenes, in cannabis,[25,26] although the biological and pharmacological activities of single terpenes have already been reported.[27] It has been determined that these important components are concentrated in the trichomes of female cannabis plants.[28−30] Due to the economic and medicinal significance of this plant, it has recently been legalized in several countries for production and breeding of cannabis to allow for agro-industrial (largely fiber), medicinal, and recreational use.[31,32] Rapid maturity and high flower yield are required for both industrial and medicinal use.[33,34] Many aspects of the beneficial bacteria associated with cannabis are still unknown, and so far, only few related studies[35−37] have suggested that PGPR inoculation significantly increases the growth and secondary metabolite production (mainly THC and CBD) of *Cannabis sativa* L. How these effects occur is largely unknown, even though microbe application plays an important role in the biosynthesis of secondary metabolites in other plant species.[38−40]

As commercialization of PGPR remains somewhat hampered by the lack of reproducibility and consistency under natural conditions[41] and the understanding that added PGPR do not necessarily colonize over long-term periods when added to a new host,[42] there is a need for research exploring strategies to improve the efficiency of PGPR. To have the same level of survival of bacteria, three PGPR grown in King's B medium—two fast-growing PGPR, *Bacillus* sp. and *Pseudomonas* sp., and a slow-growing bacterium, *Mucilaginibacter* sp.—were evaluated for their effects on cannabis biomass and key secondary metabolite (cannabinoids and terpenes) profiles at different inoculation times.

2. MATERIALS AND METHODS

2.1. Plant Material and Bacterial Strains. A set of experiments was carried out at the Department of Plant Science, McGill University, Quebec, Canada (latitude 45°24′22″ N, longitude 73°56′44″ W), in a licensed growth facility (License no. LIC-5AZZW7S4GM-2019). CBD Kush (Dutch Passion, Amsterdam, Netherlands) was used as the mother plant, providing the needed cuttings. The selection of CBD Kush was based on the chemical properties, as it is a strain specifically bred to yield a balance between THC and CBD that is effective for medicinal use. Similar-sized (10 cm) clones were cut from mother plants, and the top three fully unfurled leaves were kept; all extra foliage was removed. The stem was dipped into Stim-Root rooting powder (Plant Products, Laval, QC, Canada) before being placed into a rockwool cube (3.8 × 3.8 cm) (Plant Products, Laval, QC, Canada) sitting in a plastic tray (53 × 28 × 6.4 cm) filled with diluted Remo nutrients (1.3 mL L^{-1} Velokelp, Remo Brands Inc.). Then, trays filled with cuttings were covered with a vented dome (18 cm high) to retain humidity and placed on a growth shelf under LED lights (approximately 150 μmol m^{-2} s^{-1}, 24 h photoperiod) for two weeks prior to transplanting.

PGPR strains of *Bacillus*, *Mucilaginibacter*, and *Pseudomonas* were prepared by scraping bacterial colonies into a tube containing 25 mL of sterile King's B medium (20.0 g L^{-1} protease peptone, 1.5 g L^{-1} K_2HPO_4, 10.0 g L^{-1} glycerol, 0.25 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 15 g L^{-1} agar) and then placing the tube in an orbital shaker (150 rev min^{-1}) at 28 ± 2 °C to reach the stationary phase. The inoculum was obtained by diluting the original fresh culture using King's B medium to a

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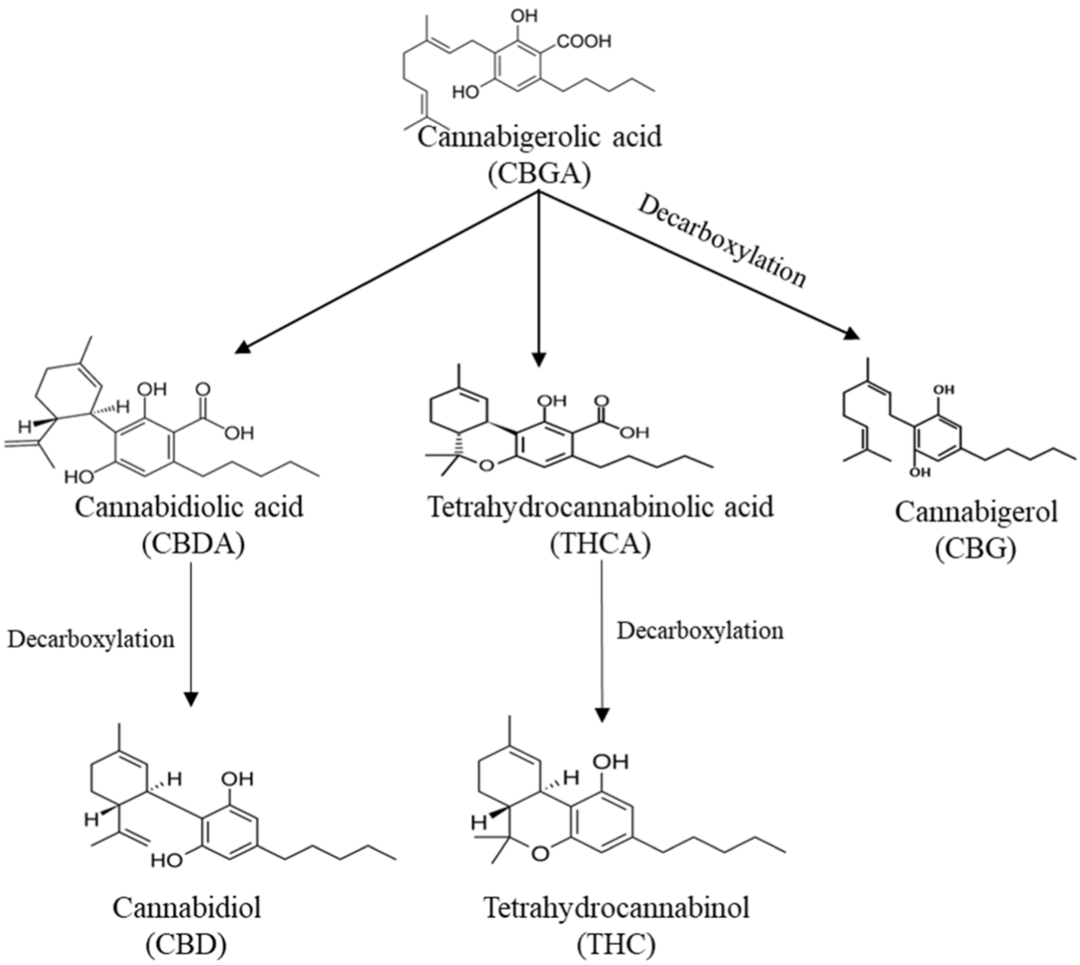

Figure 2. Biosynthesis pathway of cannabinoids found in cannabis inflorescences.[30,43,44]

concentration of 0.1 OD (optical density, about 1×10^8 colony forming units, CFU mL^{-1}).

2.2. Experimental Design. Uniformly rooted cuttings were transplanted into 15 cm pots containing 360 g of Agro Mix G2 soil well mixed with 350 mL of water. The pots with plants were placed in a growth room at 24/22 °C with an 18/6 h photoperiod/darkness regime for 21 days, keeping the plants in the vegetative stage; then, the lighting regime was changed to short day (12/12 h) from day 22 to day 70 after transplanting. LED light (VYPR Series, Fluence) was used and adjusted for light intensity during all stages of the growth of cannabis plants following Supporting Information S1. The amount of nutrients and water were supplied according to plant demand at each growth stage (Supporting Information S1). For instance, from days 1 to 7 (vegetative stage), plants were manually fertilized for three continuous days (30 mL per day), followed by one day of water (30 mL per day) alone. The nutrient preparation followed the recommendations provided by Remo Brands Inc. (BC, Canada). During the vegetative stage, 1.84 mL L^{-1} each of Velokelp, Micro, Grow, and MagNifiCal were mixed and used. During the flowering stage (days 22 to 56), 2.2 mL L^{-1} each of Velokelp, Micro, MagNifiCal, Bloom, and AstroFlower were mixed and used. The temperature and humidity in the growth room were controlled and constantly monitored. All environmental conditions are provided in Supporting Information S1 following our previous study.[37]

Inoculation with each of the PGPR strains was carried out at the following times: day 1 (vegetative stage) or day 36 (early flowering stage). 10 mL of fresh bacterial culture was poured onto the surface of the soil in each pot at the base of the plant. The experiment was arranged following a completely randomized design with five replications. For each experiment, there were 50 plants, and the treatments applied were (1) sterile water as blank, (2) fresh King's B medium as positive control, (3) *Bacillus* sp. in King's B medium, (4) *Mucilaginibater* sp. in King's B medium, or (5) *Pseudomonas* sp. in King's B medium. The whole experiment was repeated twice; thus, the values of 10 measurements for each variable were used (n = 10).

2.3. Data Collection. *2.3.1. Yield-Related Characteristics.* Nondestructive measurements of plant height and the number of axillary buds were recorded at days 21, 42, and 70 post-transplant. 10 weeks after transplanting, plants were manually sampled, separated into stems, leaves, and flowers, and weighed as fresh weight. Stem and leaves were air dried (22−25 °C) to a constant weight under room temperature to determine the dry weight of biomasses, while flowers were dried for three days using a lyophilizer (SNL216V freezing-dryer, Thermo Savant Co. Ltd., USA) at −60 °C to avoid the decarboxylation of secondary metabolites. The workflow is presented in Figure 1.

2.3.2. Chemical Responses. 2.3.2.1. Cannabinoids. Based on the cannabinoid biosynthesis pathway (Figure 2), neutral CBD and THC are decarboxylated from cannabidiol acid (CBDA) and tetrahydrocannabinol acid (THCA), which are both formed from the cannabinoid precursor cannabigerolic acid (CBGA).[30,43,44] As key cannabinoids, these compounds also showed therapeutic effects.[25,45] In this study, six key cannabinoids (CBGA, CBDA, THCA, cannabigerol (CBG), CBD, and THC) were identified and quantified.

Sample extraction and isolation: The cannabinoid analysis procedure followed Achenbach *et al.* (2018).[46] Dried cannabis flowers were manually ground into a fine powder. 100 mg of powder material was extracted twice using 5 mL of methanol and sonicating for 1 min at room temperature, followed by centrifuging for 10 min. The supernatant was combined from two extracts and filtered through a Kimwipe plug into a pre-weighed 20 mL scintillation vial. Two aliquots of 1.5 mL were removed into pre-weighed LC vials. All three

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Table 1. Effects of PGPR Treatments on Cannabis Height Collected at 21, 42, and 70 Days After Transplanting[b]

		plant height (cm)		
inoculation time	treatment	day 21	day 42	day 70
vegetative	control	18.22 ± 1.76	36.44 ± 2.25	41.64 ± 2.30
	King's B	19.60 ± 1.09	39.95 ± 2.14	44.72 ± 2.36
	Bacillus sp.	21.20 ± 1.79	41.10 ± 2.14	45.00 ± 2.49
	Mucilaginibacter sp.	20.50 ± 1.88	40.40 ± 2.60	44.25 ± 2.49
	Pseudomonas sp.	20.55 ± 1.87	42.90 ± 2.16[a]	48.55 ± 2.27[a]
flower	control	18.00 ± 1.28	35.50 ± 2.69	38.21 ± 1.88
	King's B	16.79 ± 1.13	36.64 ± 1.26	38.71 ± 1.92
	Bacillus sp.	20.43 ± 1.72	38.64 ± 2.87	42.00 ± 2.79
	Mucilaginibacter sp.	20.58 ± 1.72	38.67 ± 2.48	41.67 ± 2.75
	Pseudomonas sp.	19.64 ± 1.21	38.07 ± 2.35	41.36 ± 2.77
		p values		
inoculation time		NS	0.079	0.008
treatment		NS	NS	NS

[a]The value is significantly different from control at the same inoculation time at $p \leq 0.05$. [b]NS: no significance. Each column represents the average of 10 plants followed by the standard error (SE).

Table 2. Effect of Inoculation with Individual Bacteria on Cannabis Flower Numbers[b]

		number of flower buds		
inoculation time	treatment	day 21	day 42	day 70
vegetative	control	NA	31.30 ± 1.81	51.67 ± 2.10
	King's B	NA	33.00 ± 1.36	54.10 ± 1.95
	Bacillus sp.	NA	35.50 ± 2.49	59.90 ± 2.27[a]
	Mucilaginibacter sp.	NA	37.00 ± 2.49[a]	60.70 ± 2.43[a]
	Pseudomonas sp.	NA	31.20 ± 2.27	58.30 ± 1.90[a]
flower	control	NA	31.43 ± 1.89	51.29 ± 2.13
	King's B	NA	30.70 ± 1.92	51.40 ± 1.39
	Bacillus sp.	NA	31.00 ± 2.79	53.70 ± 2.97
	Mucilaginibacter sp.	NA	30.00 ± 1.87	51.20 ± 2.56
	Pseudomonas sp.	NA	30.60 ± 1.88	49.00 ± 1.99
		p values		
inoculation time			NS	0.015
treatment			NS	NS

[a]The value is significantly different from the control at the same inoculation time at $p \leq 0.05$. [b]NA: there were no emerged flower buds at the vegetative stage. Each column represents the average of 10 plants and is followed by the standard error (SE).

vials (LC and the remaining solution in the scintillation vial) were then dried under nitrogen (LC vials) or vacuum (scintillation vial). One LC vial was used for the cannabinoid analysis, and the rest of the LC vials were kept in a freezer for future analysis.

HPLC-MS analysis: Extracts were diluted in methanol to 10 times. The resulting solution was then separated by a liquid chromatographic system, detected by UV (280 nm), followed by high-resolution mass spectrometry (LC-UV-HRMS). The system consisted of an UltiMate 3000 UHPLC pump and an UltiMate 3000 Diode Array Detector, coupled to a Hybrid Quadrupole Orbitrap Mass Spectrometer with an electrospray ionization source (Q Exactive, Thermo Fisher Scientific). Separation was performed on a UPLC HSS-T3 column measuring 2.1 × 100 mm (Waters, 1.8 μm particle size). Mobile phase A was de-ionized water (0.1% formic acid), and mobile phase B was methanol (0.1% formic acid) at a flow rate of 400 μL min^{-1}. The elution gradient was from 80% B to 84% B from 0 to 2 min, then to 86% B from 2 to 6 min, and then to 100% B from 6 to 7 min, held at 100% B for 2 min before returning to the initial gradient. MS acquisitions were conducted with a mass range of m/z 190−1000 in the positive mode.

Quantitative analysis: Concentrations of CBDA and THCA in extracts were determined using the UV signal, with calibration curves from 0.1 to 10 μg mL^{-1}, while the HRMS signal was used for the quantitation of CBD, THC, CBGA, and CBG, with calibration curves from 0.01 to 1 μg mL^{-1}. All cannabinoid analyses were repeated twice for each sample. Data was processed using TraceFinder Clinical software (Thermo Scientific).

Total THC and CBD concentrations were determined by assuming complete carboxylation of the acid forms of the respective cannabinoids using the following equations

$$T_{\text{cannabinoid}} = (\text{acid}_{\text{cannabinoid}} \times 0.877) + \text{neutral}_{\text{cannabinoid}} \quad (1)$$

where $T_{\text{cannabinoid}}$: total cannabinoid accumulation in plant, $\text{acid}_{\text{cannabinoid}}$: the amount of the acidic form of cannabinoid, $\text{neutral}_{\text{cannabinoid}}$: the amount of neutral cannabinoid.

2.3.2.2. Terpene Profile. The method for terpene analysis was developed by the National Research Council Canada (Halifax, Nova Scotia, Canada). All the samples were (freeze) dried and stored (∼20 °C) before sample extraction and final analysis by GC-MS. 21 terpenes were targeted for identification and quantification; the compound names of all target terpenes and retention time can be found in Supporting Information S2. The terpene analysis was run once to compare the qualitative terpene among treatments rather than quantitative terpene, as some of the volatile terpenes could have evaporated partially during sample processing and storage.

Extraction: Each sample (100 mg) was solubilized in 5 mL of EtOAc spiked with 50 μL of the internal standard (tridecane) for a final concentration of 100 ng mL^{-1}. The tube containing the sample extract was sonicated for 5 min at room temperature and centrifuged

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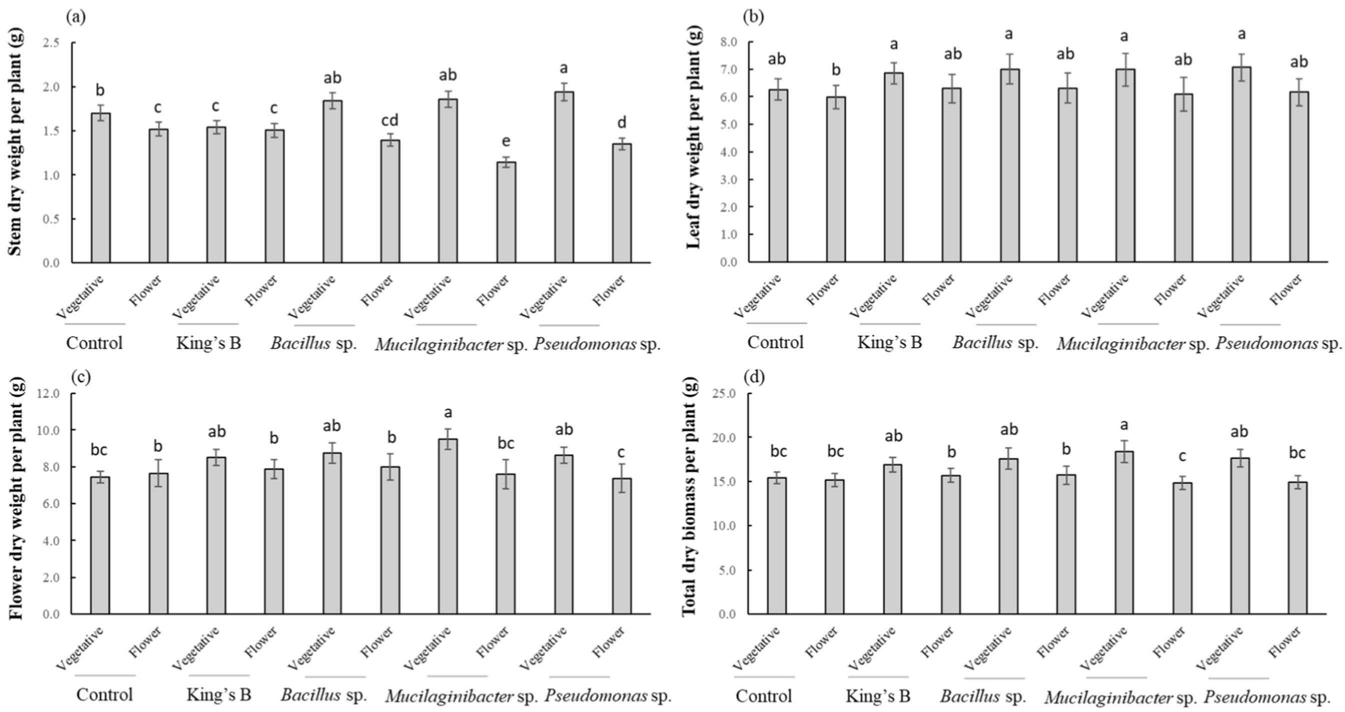

Figure 3. Final dry cannabis stem (a), leaf (b), flower (c), and total biomass (d) when PGPR inoculation treatments were applied at the vegetative and flowering stage. Note: the bar value followed by the same letter are not significantly different at $p \leq 0.05$.

at 1000 rpm for 3 min. An aliquot of the supernatant (1.5 mL) was then transferred into an HPLC vial for GC-FID/MS analysis.

GC-FID/MS: Terpene analysis was carried out using an Agilent 6890N GC system equipped with a 5975 Inert XL Series mass selective detector, a flame ionization detector (FID), and a fused silica column (30 m × 0.25 mm × 0.25 μm, Restek Rxi 624-Sil-MS). The injection volume of samples was 1 μL. The initial oven temperature was 55 °C, then 130 °C with a rate of 25 °C min^{-1} holding for 6 min, and then increased to 280 °C with a rate of 30 °C min^{-1} holding for 4 min with a total running time of 18 min. Helium was used as the carrier gas with a flow rate of 1.6 mL min^{-1}.

Quantitative analysis: Quantitation of the terpenes was determined based on the FID signal, while the MS detector confirmed the identity of the targeted analytes based on the NIST database (v. 14.1). The calibration range of terpenes was from 5 to 250 μg mL^{-1}. Data processing was conducted using MassHunter (Agilent Technologies CA, USA).

2.3.3. Statistical Analysis. The mean differences between inoculation time (vegetative and flower stages) and inoculation (PGPR treatment) on plant height and flower number were assessed using two-way ANOVA with fixed factor inoculation time and treatment in SPSS (IBM SPSS Statistics for Citrix, Version 24.0. Armonk, NY: IBM Corp.). Contrasts were used to compare control versus PGPR-treated plants at each inoculation time. The statistical significance of plant biomass and total cannabinoids/terpenes were analyzed using a post-hoc Dunnett test. Statistical significance was set at $p \leq 0.05$, and the differences between $0.05 < p \leq 0.1$ are also listed. The correlation matrix of agronomic traits and biomass were determined using bivariate Pearson correlation in SPSS.

3. RESULTS

3.1. Plant Growth Parameters and Yield. *3.1.1. Agronomic Traits.* Among the three data collection points, the average heights of plants inoculated with King's B and PGPR at the vegetative stage were all greater than plants treated at the flowering stage, especially at day 70 (p = 0.008). For three PGPR treatments, only inoculation with *Pseudomonas* sp. at the vegetative stage significantly increased plant height at days 42 and 70, which was 17.7 and 14.1% higher than control, respectively (Table 1).

The axillary bud numbers of plants inoculated with PGPR and King's B at the vegetative stage were all greater than plants treated with same inoculants at the flowering stage (Table 2). At day 42 (third weeks of the reproductive stage), the number of flowers emerged for *Mucilaginibacter* sp.-treated plants at the vegetative stage was significantly higher than the control (1.18 times higher). At day 70, all three PGPR (*Bacillus* sp., *Mucilaginibacter* sp., and *Pseudomonas* sp.) inoculations at the vegetative stage led to significantly higher final bud numbers, with increases of 15.9, 17.5, and 12.8%, respectively, more than the control.

All treatments are shown separately for the final dry biomass of the plant stem, leaf, flower, and total aboveground production in Figure 3. Specifically, stem weights were significantly increased when plants were treated with *Pseudomonas* sp. at the vegetative stage, resulting in enhancements of 13.8% compared to the control. However, inoculation with PGPR at both stages did not affect the leaf biomass. Moreover, all microbe (*Bacillus* sp., *Mucilaginibacter* sp., and *Pseudomonas* sp.) and growth medium (King's B) treatments at the vegetative stage significantly increased the final dry flower yield by 17.6, 27.9, 16.1, and 14.5%, respectively. In sum, the total aboveground dry weight of the cannabis plants was 9.8, 14.2, 19.2, and 14.6% higher than the control when plants were treated with King's B, *Bacillus* sp., *Mucilaginibacter* sp., and *Pseudomonas* sp., respectively, at the vegetative stage.

Figure 4 illustrates effects of two inoculation timings on cannabis plant growth including stem, leaf, flower, and total biomass. It suggests that the magnitude of the beneficial effect was altered by inoculation timing.

3.1.2. Correlation Matrix of Agronomy Traits. All measurable agronomic traits were positively correlated with each other (Figure 5). While total biomass was strongly

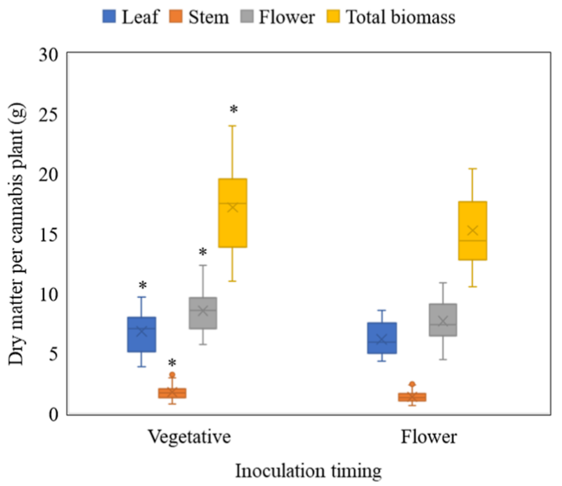

Figure 4. Boxplots showing how cannabis leaf (blue), stem (orange), flower (gray), and total (yellow) biomass differed between PGPR inoculation at vegetative and flowering stages. * indicates the value is statistically significantly different within the same variable at $p \leq 0.05$.

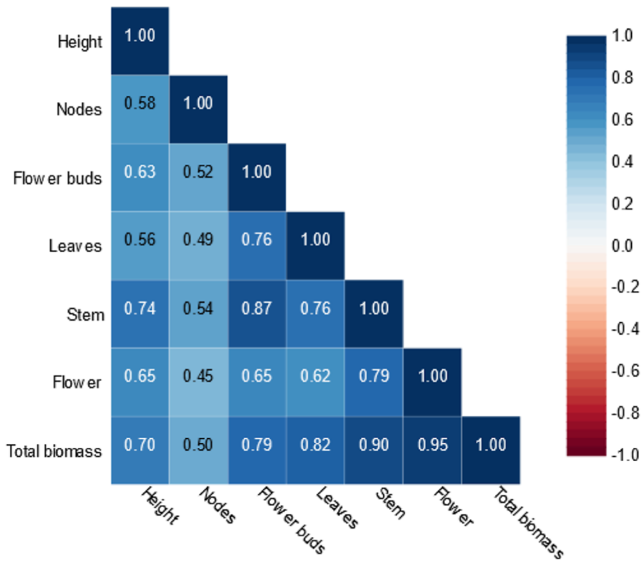

Figure 5. Correlation matrix of all agronomy traits, consisting of plant height, the number of nodes and flower buds, biomass of leaves, stems, and flowers, and total biomass of aboveground.

correlated with the weights of the flower (r = 0.95), stem (r = 0.90), and leaf (r = 0.82). The flower biomass was correlated to that of the stem and the number of flower buds.

3.2. Chemical Response. *3.2.1. Cannabinoid Profile.* CBGA is the precursor cannabinoid in the dried inflorescences; however, it was negatively related to the efficacy of any measured PGPR treatments compared with no PGPR inoculation at both inoculation time points, although no significant lead was demonstrated by both treatment and inoculation time (Table 3). A similar trend also occurred for CBG, which can be decarboxylated from CBGA. The levels of THCA and CBDA were all significantly positively correlated with PGPR inoculation at the vegetative stage, with p values less than 0.05. The content of CBDA and THCA was significantly increased from 130.67 to 144.85 and 84.75 to 94.34 mg·g^{-1}, respectively, as *Mucilaginibacter* sp. was applied at the vegetative stage. Inoculation at the vegetative stage also positively affected THC and CBD accumulations, with p values between 0.05 and 0.1. Among PGPR treatments, *Bacillus* and *Mucilaginibacter* sp. at the vegetative stage also significantly contributed to the accumulation of neutral THC and CBD. Unsurprisingly, *Mucilaginibacter* sp. performed best with regard to increases in THCA, CBDA, THC, and CBD and led to the lowest level of CBGA and the highest level of CBG, as CBGA is metabolically downstream of other cannabinoids.

Figure 6 shows total THC and CBD concentrations (mg g^{-1}) of plants, which were calculated following eq 1 in Section 2. For the vegetative stage inoculation, *Mucilaginibacter* sp. inoculation resulted in 128 mg g^{-1} of total THC and 84 mg g^{-1} of total CBD in flowers, which was 11.6 and 11.1% higher, respectively, than the control. *Bacillus* sp. and *Pseudomonas* sp. also significantly increased total THC by 4.8 and 5.9%, respectively. Moreover, there were no significant effects caused by PGPR on total THC and CBD levels when inoculation happened at the flower stage.

3.2.2. Terpenes. There were 11 terpenes (alpha-pinene, camphene, beta-myrcene, beta-pinene, D-limonene, beta-ocimene, beta-carophyllene, alpha-humulene, nerolidol, guaiol, and alpha-bisabolol) detected out of the 21 targeted in the current study. Among all treatments, *Mucilaginibacter* sp. and *Pseudomonas* sp. inoculation at the flowering stage led to statistically significant increases of terpenes of about 23 and 18% over the control, respectively. Among all terpene profiles, inoculation with *Mucilaginibacter* sp. led to the highest beta-caryophyllene (a sesquiterpene) accumulation in CBD Kush flower tissue, which was twice as high as the non-PGPR inoculation.

4. DISCUSSION

4.1. Correlation between Cannabis Agronomic Traits and PGPR Inoculation. The efficiency of PGPR application on plant growth depends on a range of factors, including the host species, rhizobacterial species, the properties of the specific PGPR, inoculum density and method, and environmental conditions.[47,48] The final cannabis biomass was highly positively correlated to plant height, axillary buds, number of nodes and branches, and also stem and flower biomass (Figure 5). Plus, under the conditions of additional PGPR application, the final biomass was affected by the timing of inoculation (Figures 3d and 4) through increased plant height, number of flower buds, and flower mass. Early inoculation with PGPR improved plant growth and yield, which can be explained in that the development of cannabis plants at the vegetative stage provides a base for reproductive growth. Thus, inoculation of PGPR at early stages of plant growth benefits all growth stages in subsequent plant growth progression. This result is supported by Naim-Feil *et al.* (2021),[49] who reported that the vegetative growth stage is a critical step in optimizing cannabis production during indoor growth. Poupin *et al.*[50] suggested that PGPR have beneficial effects across the entire life cycle of the plants by accelerating the growth rate and shortening the vegetative period. Improvement of cannabis yield by PGPR inoculation has been reported,[35,45,56] and the mechanism of PGPR cannabis growth enhancement depended on nutrient availability as PGPR can facilitate access to nutrients. Some research has reported that nutrient supplementation influences cannabis growth and chemical profiles, but these effects can be positive or negative depending on the amount of applied nutrients.[51−53]

4.2. Key Secondary Metabolite Response to PGPR. Secondary metabolites (cannabinoids and terpenes) in

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Table 3. Cannabinoid Concentration (mg g^{-1} Dry Flower Weight) of Cannabis Flower Treated with PGPR at Different Stages[b]

inoculation timing	treatment	CBGA (mg g^{-1})	CBDA (mg g^{-1})	THCA (mg g^{-1})	CBG (mg g^{-1})	CBD (mg g^{-1})	THC (mg g^{-1})
vegetative	control	6.88 ± 0.73	130.67 ± 3.30	84.75 ± 2.08	0.73 ± 0.18	0.81 ± 0.08	1.18 ± 0.12
	King's B	5.73 ± 0.45	135.38 ± 6.14	86.89 ± 2.39	0.72 ± 0.19	0.90 ± 0.08	1.29 ± 0.07
	Bacillus sp.	6.28 ± 0.55	137.53 ± 5.15	88.51 ± 4.00	0.84 ± 0.20	1.05 ± 0.14[a]	1.49 ± 0.14[a]
	Mucilaginibacter sp.	5.42 ± 0.67	144.85 ± 5.03[a]	94.34 ± 4.54[a]	0.99 ± 0.23	1.09 ± 0.11[a]	1.51 ± 0.10[a]
	Pseudomonas sp.	5.49 ± 0.56	140.10 ± 4.06[a]	89.60 ± 2.63[a]	0.74 ± 0.25	0.95 ± 0.10	1.32 ± 0.10
flower	control	6.30 ± 0.38	129.42 ± 4.05	82.39 ± 2.69	0.83 ± 0.17	0.99 ± 0.06	1.27 ± 0.08
	King's B	5.46 ± 0.48	127.78 ± 6.79	81.40 ± 2.02	0.78 ± 0.17	0.97 ± 0.09	1.32 ± 0.09
	Bacillus sp.	5.32 ± 0.67	125.16 ± 4.38	79.68 ± 3.62	0.69 ± 0.19	0.86 ± 0.09	1.18 ± 0.10
	Mucilaginibacter sp.	5.64 ± 0.70	136.53 ± 4.80	89.01 ± 3.85	0.86 ± 0.21	0.95 ± 0.08	1.38 ± 0.10
	Pseudomonas sp.	5.33 ± 0.66	126.56 ± 4.49	80.47 ± 3.18	0.81 ± 0.17	0.81 ± 0.11	1.17 ± 0.09
			p value				
inoculation time		NS	0.012	0.008	NS	0.061	0.085
treatment		NS	NS	NS	NS	NS	NS

[a]The value is significantly different from the control at the same inoculation time at $p \leq 0.05$. [b]Each column represents the average of 10 plants and the standard error (SE).

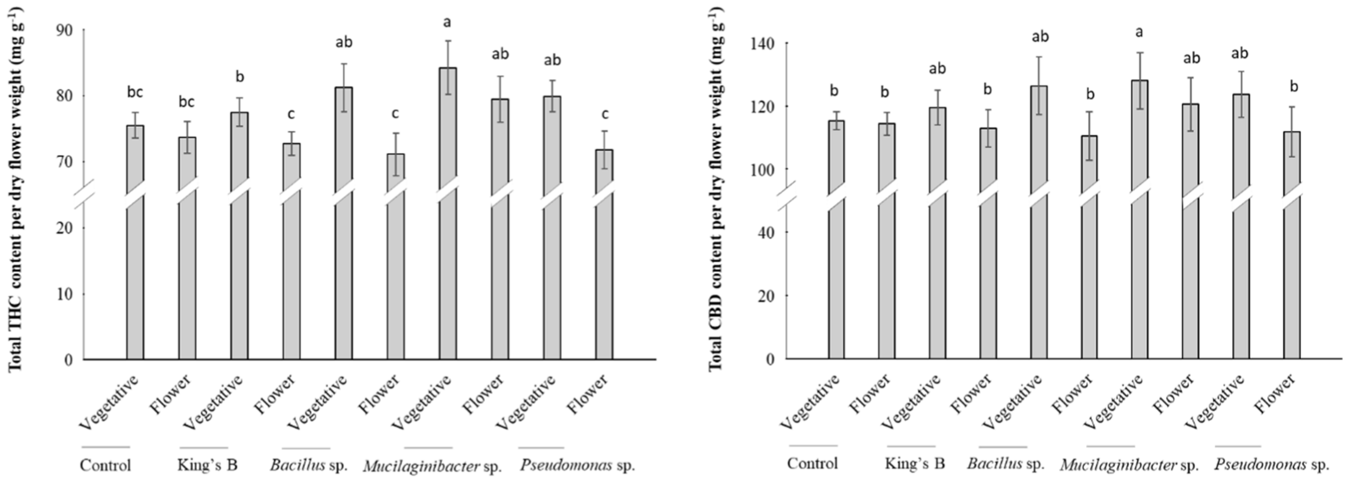

Figure 6. Contents of total THC and CBD expressed as mg g^{-1} of dry flower weight. Note: bar values followed by the same letter are not significantly different at $p \leq 0.05$.

cannabis plants are a critical component and have various pharmaceutical characteristics.[26,55] Therefore, the final content and profile of those compounds contribute to the medicinal value of cannabis. In the present study, the response of PGPR inoculation to secondary metabolite accumulation in cannabis flowers varied among the species of beneficial microbe and application timing. The effects of the PGPR on secondary metabolites in this study are consistent with Pagnani *et al.* (2018),[35] who found PGPR had positive effects on both CBD and THC contents. The author also pointed out that these effects were similar to those of nitrogen fertilizer application. It is possible that this explains why the application of King's B medium resulted in increased CBD and THC concentrations, especially at the vegetative stage inoculation, as micro- (magnesium) and macronutrients (nitrogen) are present in the medium. Research studies also reported that an increased mineral content of micro- (*e.g.,* calcium, iron, and magnesium) and macronutrients (*e.g.,* nitrogen) leads to an increase of THC content, but high nitrogen availability leads to a consistent decrease in THCA and CBDA concentrations.[53,54] Therefore, a suitable amount of King's B medium can be a source of nutrients for cannabis plant growth and development.

4.3. Impact of King's B Medium on PGPR and Plants.

PGPR have shown potential effects under laboratory conditions, while the ability to successfully colonize plant roots often determines the effectiveness of PGPR under field conditions.[56] In the field, the relative ineffectiveness of PGPR could be influenced by a range of factors including the requirement for specific signals from host plants and an inhospitable rhizosphere environment. Some genera, for example, *Bacteroidota*, *Verrucomicrobiota*, and *Acidobacteriota,* colonize host plant roots rapidly, while species within the genus *Mucilaginibacter* take a longer time to dominate in roots,[57] suggesting that growth speed could be one of the main reasons for differences in the successful colonization of PGPR.[58] King's B medium is a general type of nutrient-rich microbiological medium, containing proteose peptone, which provides carbonaceous and nitrogenous compounds for the growth of bacteria.[59] Liquid inoculant formulations using King's B medium maintained good populations of bacteria up to 90 days, and after that, there was a decrease in CFU at 120 DAS.[60] In the current study, *Bacillus* and *Pseudomonas* sp. are fast-growing beneficial microbes, while *Mucilaginibater* sp. is a slow-growing bacterium. Some studies verified that fast-growing microbes can colonize plant hosts quickly, while

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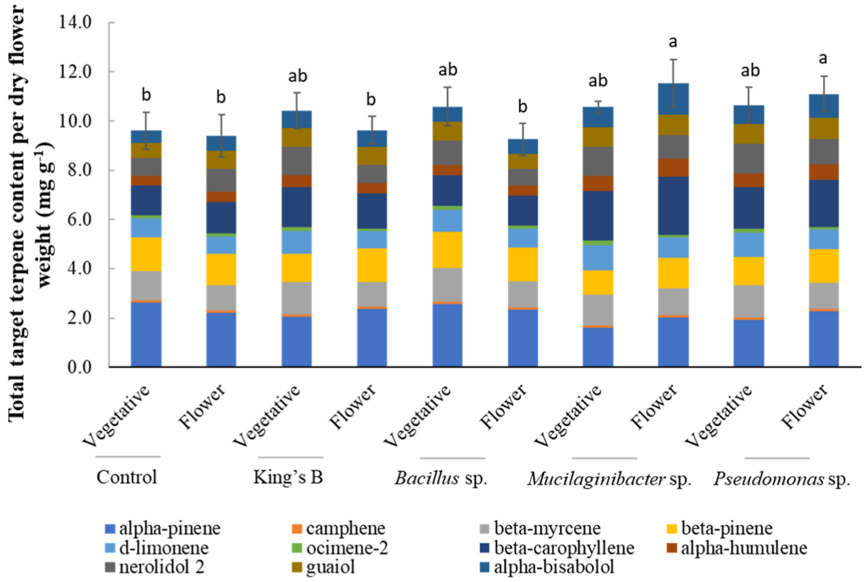

Figure 7. Concentration of all detected terpenes in cannabis flowers (mg g^{-1}). Note: the bar values followed by the same letter are not significantly different at $p \leq 0.05$.

slow-growing PGPR take longer to colonize the host but can be present for a longer time.[57] Our hypothesis proposed that the presence of microbial growth medium could improve the efficiency of PGPR colonization of cannabis roots. In the previous study, we evaluated the effects of the bacterial cells suspended in magnesium chloride (a nutrient-free matrix) on cannabis growth and reported that the slow-growing PGPR *Mucilaginiabcter* sp. had the strongest positive effect on secondary metabolite biosynthesis among the three PGPR, while the *Pseudomonas* sp. had the strongest effect on flower yield, and both PGPR positively influenced cannabis growth and secondary metabolite accumulations.[37] In contrast, *Bacillus* sp. only increased the yield but not the levels of cannabinoids or terpenes. However, in the current study, the results revealed that *Bacillus* sp. (vegetative inoculation) led to improvements in both cannabis yield and quality (Figures 3, 6, 7 and Table 3). In addition, we observed that the microbial growth medium also caused positive effects on cannabis growth and cannabinoid accumulation (Figures 3 and 4), although not at a statistically significant level.

Under the conditions used in the current study, we cannot eliminate the possibility that some PGPR may produce levels of unknown microbe-to-plant signal compounds in their growth medium that may trigger plant growth.[61] Additional research can be conducted to examine the supernatant produced by those beneficial microbes onto the growth of cannabis to explore the potential signaling exchange between plant microbes.

Overall, the current study clearly indicates that flower yield, cannabinoid, and terpene accumulations are strongly correlated with PGPR inoculation and application timings, but the efficacy of PGPR varies among microbial species. Compared with the pure cell pellets, selected PGPR in the presence of King's B medium contributed to higher biomass, yield, and quality of cannabis. From the perspective of industrial production, applying beneficial PGPR with microbe nutrient medium could be an economically and environmentally friendly approach to ultimately improving yield and quality for indoor cannabis with less chemical fertilizer use. We propose that an additional metabolomics study would be instructive by providing a clear verification of the improved efficiency and colonization into the host by some PGPR inoculation in the presence of the growth medium.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c06961.

Growth condition for cannabis from the vegetative to flower stage, including amount of nutrients, light intensity, temperature, humidity, and light cycle; the name of target terpene compounds; and the retention time for each compound (PDF)

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■ ABBREVIATIONS

CBGA, cannabigerolic acid; CBDA, cannabidiol acid; THCA, tetrahydrocannabinol acid;; CBG, cannabigerol; CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol

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