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Improved Growth and Metabolite Accumulation in *Codonopsis pilosula* (Franch.) Nannf. by Inoculation of *Bacillus amyloliquefaciens* GB03

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ABSTRACT: *Codonopsis pilosula* (Franch.) Nannf. is a traditional Chinese herbal medicinal plant and a low-cost succedaneum for *Panax ginseng* and contains various bioactivity components. In this work, we first evaluated the effects of the inoculation of the plant growth-promoting rhizobacteria *Bacillus amyloliquefaciens* strain GB03 on growth and metabolite accumulation of *C. pilosula*. The results demonstrated that application of *B. amyloliquefaciens* GB03 significantly improved the growth of *C. pilosula* compared to DH5α, Luria broth medium, and water treatment, respectively. On the other hand, we observed that the content of lobetyolin, one of the most important secondary metabolites in *C. pilosula*, was obviously improved by inoculation of GB03 and almost reached twice that compared to the other three treatments. In addition, some amino acids of roots were elevated by GB03, although not significantly. In conclusion, *B. amyloliquefaciens* GB03 could induce positive effects on the growth and further stimulate accumulation of secondary metabolites in *C. pilosula*.

KEYWORDS: *Codonopsis pilosula (Franch.) Nannf., Bacillus amyloliquefaciens GB03, growth, metabolites, lobetyolin*

■ INTRODUCTION

The genus *Codonopsis* is a perennial herb in the family Campanulaceae and has 42 species mainly distributed in Central, East, and South Asia, with approximately 40 species found in China.[1] The dry roots of *Codonopsis pilosula* (Franch.) Nannf. has been widely used as tonic medicine for centuries in China, and it could be a low-cost succedaneum for *Panax ginseng*.[2,3] It is well-known that *C. pilosula* is also used for enhancing organic immunity, promoting gastrointestinal function, nourishing spleen and lung, helping depressurization, improving microcirculation, etc. According to phytochemical analysis, *C. pilosula* contains lobetyolin (polyacetylenes), phenylpropanoids, polysaccharides, sterol, triterpenoids, saponin, alkaloid, amino acids, and other chemical compounds.[4−13] These chemicals are closely correlated to bioactivities of *C. pilosula*. Therefore, it is critical to elevate the contents of these chemical components in roots. Lobetyolin, as one important component of polyacetylenes, is used for healing gastric ulcer as one of the most important pharmacological functions of *C. pilosula*.[13]

Plant growth-promotion rhizobacteria (PGPR) are beneficial to promote the growth of host plants.[14] Obviously, plant inoculation with PGPR has been frequently used for crop health and production for decades.[15−22] On the basis of previous studies, PGPR can transport nutrients to plants, produce plant hormones such as cytokinin, gibberellins, indole acetic acid, and abscisic acid, or emit specific microbial inhibitory compounds that enhance plant biotic and abiotic stress resistance, including plant fungal pathogen resistance, salt and drought tolerance, and heavy metal endurance.[23−28] Moreover, PGPR can regulate the structure of the soil bacterial community.[29,30] Therefore, PGPR can decrease the usage rate of chemical fertilizer and insecticide in an agricultural system, induce a systematic resistance, and improve the growth and quality of plants. *Bacillus subtilis* GB03, recently renamed as *Bacillus amyloliquefaciens* (GB03), the first commercialized biocontrol strain, can improve growth of *Arabidopsis* by emitting a complex blend of volatile organic components.[31,32] A bouquet of over 25 bacterial volatile odors has been identified that triggers differential expression of approximately 600 *Arabidopsis* transcripts related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation, and other expressed proteins.[33] In addition, GB03 and other *Bacillus* sp. strains can also promote growth of some other plant species, such as sweet basil, white clover, *Puccinellia tenuiflora*, and oriental melon.[26,27,34,35] Especially, Banchio et al. found that volatiles emitted from GB03 significantly increased essential oil production in sweet basil.[34]

To date, most studies focus on the analytical methods, structural elucidation, and function of chemical compounds from *C. pilosula*. However, it has not been previously reported that PGPR induces growth enhancement and metabolite accumulation in *C. pilosula*. Therefore, the aim of this study was to verify whether *B. amyloliquefaciens* (GB03) could improve growth and metabolite accumulation in the Chinese

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medicinal plant *C. pilosula* (Franch.) Nannf. The study provides a potential application value of *B. amyloliquefaciens* (GB03) in growth promotion of important Chinese herbal plants.

■ MATERIALS AND METHODS

Bacterial Strain Culture and Plant Samples. *B. amyloliquefaciens* GB03 and *Escherichia coli* DH5α were grown in liquid Luria broth (LB) medium shielded from light for 48 h at 28 °C with 180 rpm rotation (*B. amyloliquefaciens* strain GB03 was obtained from Professor Paul W. Paré at Texas Tech University, Lubbock, TX, U.S.A., and *E. coli* strain DH5α was purchased from Takara Biotechnology (Dalian) Co., Ltd., China). The inoculum density was adjusted to 10^9 colony forming units (CFU) mL^{-1} as determined by optical density and serial dilutions.

C. pilosula (Franch.) Nannf. seeds were surface-sterilized and germinated at 25 °C in the dark. After germination, plantlets were transferred into plastic pots (diameter, 20 cm; depth, 30 cm) containing turfy soil and farm soil mix (1:1) that were previously heated at 160 °C in a constant temperature oven for 12 h. Then, the pots were irrigated with half-strength Hoagland's nutrient solution containing 2 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 0.1 mM $Ca(NO_3)_2\cdot 4H_2O$, 0.25 mM $MgSO_4\cdot 7H_2O$, 0.5 mM Fe citrate, 92 μM H_3BO_3, 18 μM $MnCl_2\cdot 4H_2O$, 1.6 μM $ZnSO_4\cdot 7H_2O$, 0.6 μM $CuSO_4\cdot 5H_2O$, and 0.7 μM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ every 3 days to keep the soil water content at 60−70%. After growth for 3 weeks, uniform seedlings were selected for the following inoculation: 1 mL of GB03, DH5α suspension culture, liquid LB medium, or double-distilled water (DDW) per plant. Plants were grown in a glass house under metal halide, and high-pressure sodium lamps were set to 14/10 h for the light/dark cycle, with a total light intensity of 800 μmol m^{-2} s^{-1}, an average temperature of 28 ± 2/23 ± 2 °C (day/night), and a relative humidity of 70 ± 10%. Plants (10 months old) were sampled for the following measurements with eight replications (eight pots) for each index (n = 8).

Plant Growth Measurement. Plants were selected and removed from pots, and roots were cleansed by water away from soil. After separating shoot from root, shoot and root fresh weights, shoot height, root volume, main root diameter, and branch number were measured immediately. The leaf area were measured by a leaf area meter (Epson Perfection 4870 Photo, Canada). Then, samples were oven-dried at 80 °C for 2 days for dry weight readings.

The content of leaf chlorophyll was measured using SPAD (Chlorophyll Meter SPAD-502, Japan). The net photosynthetic rate and intercellular CO_2 concentration of mature leaves were measured by GFS 3000 photosynthesis equipment (Germany) under natural light between 9:00 am and 10:30 am.

Amino Acid Measurement and Analysis. The samples of dried roots (0.5 g) were crushed and extracted with deionized water and 8% sulfosalicylic acid (1:1, v/v) at room temperature. The mixture was fully oscillated and centrifuged (10 000 rpm for 20 min), and then the supernatant were monitored and analyzed using an automatic amino acid analyzer (Hitachi 835, Japan).

Lobetyolin Quantitative Analysis. *Standard and Reagents.* Standard lobetyolin was obtained from Shanghai R&D Center for Standardization of Traditional Chinese Medicines. High-performance liquid chromatography (HPLC)-grade acetonitrile, ultrapure water, analytical-grade methanol, and phosphoric acid were purchased from Sangon Biotech, Ltd. (Shanghai, China).

Extraction. The dried root of each treatment specimen (three replications) was pulverized and sieved through a 300 μm mesh. A total of 2.0 g of powder of each sample was precisely weighed and extracted with 50 mL of methanol in an ultrasonic bath for 30 min. The accurate volume of 25 mL of supernatant was concentrated using a vacuum evaporator, and then the residue was dissolved with methanol with the total volume up to 10 mL in volumetric flask. Finally, the above solution was passed through a 0.45 μm Millipore filter unit, and 20 μL of sample solution was injected into HPLC for determination.

Analysis by HPLC. The solution of samples were analyzed by HPLC (Agilent 1100, Santa Clara, CA, U.S.A.) using TC-C_{18} (4.6 × 250 mm, 5.0 μm, Agilent, Santa Clara, CA, U.S.A.) at 30 °C, and the content of lobetyolin was determined as described by He et al., with the following modifications:[5] The solvent consisted of acetonitrile (A) and 0.1% phosphoric acid (B), and the linear gradient elution procedure was described as follows: 0−10 min, 95−90% B; 10−30 min, 90−80% B; 30−50 min, 80−70% B; 50−65 min, 70−50% B; and 65−75 min, 50−10% B. The flow rate was 1.0 mL/min, and the detection wavelength was 267 nm.

Statistical Analysis. All data were analyzed by variance [one-way analysis of variance (ANOVA)] using SPSS statistical software (version 13.0, SPSS, Inc., Chicago, IL, U.S.A.). Duncan's multiple range test was used to detect differences between means at a significance level of $p < 0.05$ (n = 8, except for lobetyolin quantitative analysis).

■ RESULTS

Effect of *B. amyloliquefaciens* GB03 on the Growth of *C. pilosula*. Apparent growth differences were observed between GB03 treatment and the other three treatments from 30 days in the level of the whole plant (Figure 1). The shoot height was significantly greater for GB03-inoculated

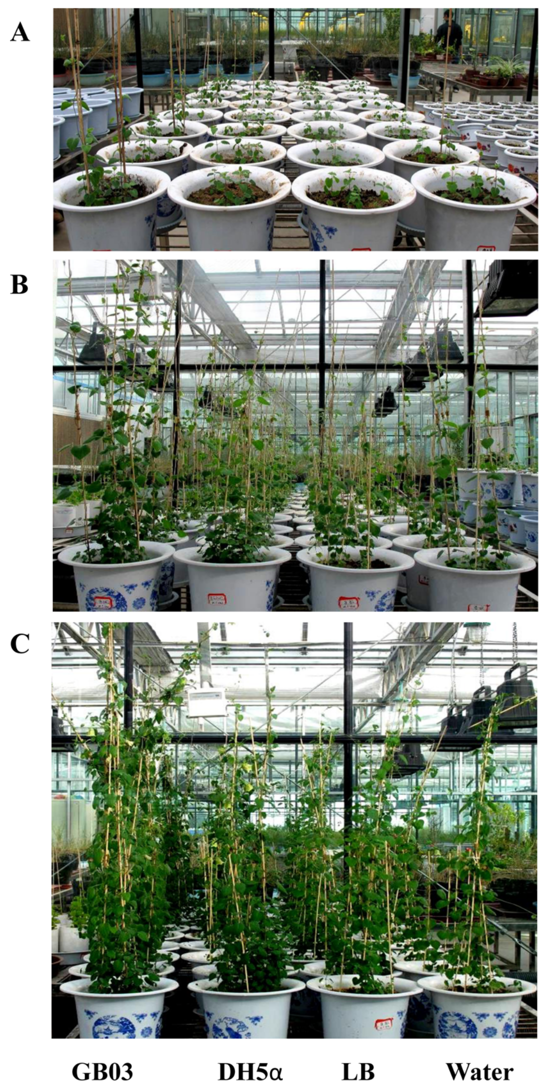

Figure 1. Effects of GB03 inoculation on the growth of *C. pilosula* (Franch.) Nannf. in pots at (A) 30 days, (B) 60 days, and (C) 90 days.

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plants ($p < 0.05$) by 18, 30, and 26% compared to DH5α, LB medium, and water treatments, respectively (Figure 2A). The

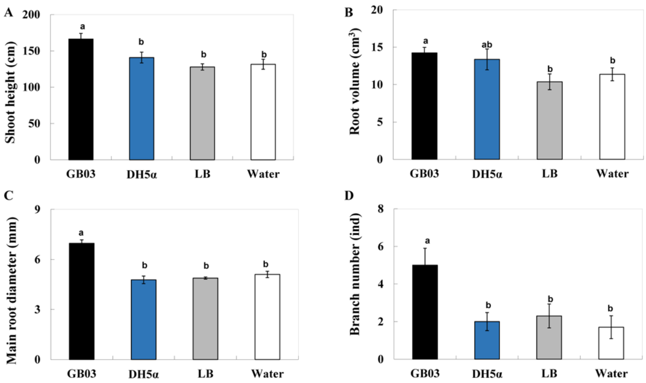

Figure 2. Effects of GB03 inoculation on (A) shoot height, (B) root volume, (C) main root diameter, and (D) branch number of *C. pilosula* (Franch.) Nannf. Values are means, and bars indicate standard deviations (SDs) (n = 8). Different letters indicate a statistically significant difference among treatments ($p < 0.05$; Duncan's test).

highest root volume and main root diameter were also observed in GB03-inoculated plants. The root volume was increased by 7 and 37% ($p < 0.05$) and 25% ($p < 0.05$) (Figure 2B), and the main root diameter was increased ($p < 0.05$) by 46, 43, and 37% (Figure 2C) compared to DH5α, LB medium, and water, respectively. The shoot branch number was increased over 2 times ($p < 0.05$) with GB03 treatment compared to DH5α, LB medium, and water treatments, respectively (Figure 2D).

Plants inoculated with GB03 have a higher biomass of shoot and root than plants inoculated with DH5α, LB medium, and water (Figure 3). The shoot fresh weight was raised with the

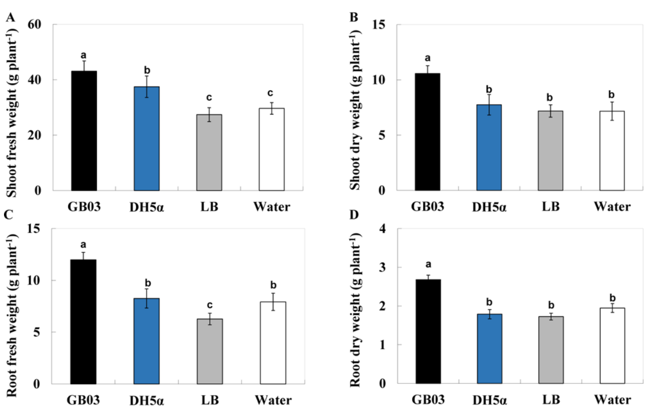

Figure 3. Comparison of plant biomass, (A) shoot fresh weight, (B) shoot dry weight, (C) root fresh weight, and (D) root dry weight, of *C. pilosula* (Franch.) Nannf. among four treatments. Values are means, and bars indicate SDs (n = 8). Different letters indicate a statistically significant difference among treatments ($p < 0.05$; Duncan's test).

GB03 group by 15, 57, and 45% and the shoot dry weight was raised with the GB03 group by 37, 47, and 48% compared to DH5α, LB medium, and water groups, respectively ($p < 0.05$) (panels A and B of Figure 3). Likewise, the root fresh weight of GB03-inoculated plants was about 45, 91, and 51% higher and the root dry weight of GB03-inoculated plants was about 50, 55, and 38% higher than those of DH5α-, LB-medium-, and water-inoculated plants ($p < 0.05$) (panels C and D of Figure 3).

Influence of *B. amyloliquefaciens* GB03 on the Photosynthetic Characteristics of *C. pilosula*. The leaf area of GB03-inoculated plants was increased by 43, 43, and 41% ($p < 0.05$) compared to DH5α, LB medium, and water, respectively (Figure 4A). The chlorophyll content of GB03-

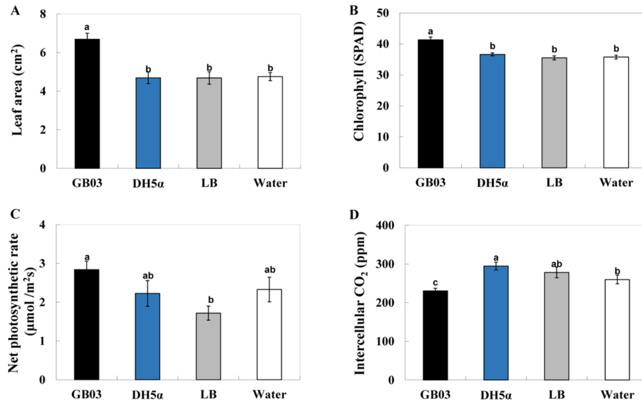

Figure 4. Comparison of photosynthetic indexes, (A) leaf area, (B) chlorophyll content, (C) net photosynthetic rate, and (D) concentration of intercellular CO_2, of *C. pilosula* (Franch.) Nannf. among four treatments. Values are means, and bars indicate SDs (n = 8). Different letters indicate a statistically significant difference among treatments ($p < 0.05$; Duncan's test).

inoculated plants was 13, 17, and 16% ($p < 0.05$) higher than that of DH5α, LB medium, and water, respectively (Figure 4B). On the other hand, the net photosynthetic rate was improved with the GB03 group by 28, 65, and 22% compared to DH5α, LB medium, and water groups, respectively, although not significant compared to DH5α and water groups (Figure 4C). The leaf intercellular CO_2 concentration of GB03-inoculated plants was reduced significantly by 22, 17, and 11% ($p < 0.05$) compared to DH5α-, LB-medium-, and water-inoculated plants, respectively (Figure 4D).

Impact of *B. amyloliquefaciens* GB03 in the Metabolite Accumulation of *C. pilosula*. *Amino Acids.* The root contents of aspartic acid, threonine, serine, glutamic acid, glycine, valine, isoleucine, leucine, lysine, and proline in GB03-inoculated plants were increased in comparison to those in DH5α-, LB-medium-, and water-inoculated plants (Table 1). In this way, the contents of root total amino acids in GB03-inoculated roots was 11, 6, and 6% higher and the contents of root essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine) was 12, 7, and 8% higher than those in DH5α-, LB-medium-, and water-inoculated roots, respectively (panels A and B of Figure 5).

Lobetyolin. The content of lobetyolin extracted from *C. pilosula* roots inoculated with *B. amyloliquefaciens* GB03 was significantly improved by 75, 72, and 52% ($p < 0.05$) compared to DH5α, LB medium, and water groups, respectively (Figure 6).

■ DISCUSSION

The beneficial effects of PGPR arouse interests and have been studied in various plants over the past decade worldwide.[36−38] *C. pilosula* (Franch.) Nannf., as a kind of natural botanical, is used in Chinese herbal medicines (CHMs). Therefore, people are highly focusing on its quantity and quality. However, available studies concerned with improving its growth and quality were rarely reported. Here, we verified the substantial

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Table 1. Effects of *B. amyloliquefaciens* GB03 on 18 Amino Acid Accumulation in Roots of *C. pilosula* (Franch.) Nannf., with Values Presented as Means ± SDs (n = 8)[a]

	contents of amino acids in four treatments (mg/g of dry weight)			
amino acid species	GB03	DH5α	LB	water
aspartic acid	6.4 ± 0.4 a	5.6 ± 0.0 a	5.9 ± 0.0 a	5.7 ± 0.0 a
threonine	2.9 ± 0.3 a	2.4 ± 0.0 a	2.6 ± 0.0 a	2.6 ± 0.0 a
serine	3.3 ± 0.3 a	2.8 ± 0.0 a	2.9 ± 0.0 a	3.0 ± 0.0 a
glutamic acid	11.2 ± 0.8 a	9.5 ± 0.0 a	10.1 ± 0.0 a	9.8 ± 0.0 a
glycine	2.9 ± 0.2 a	2.6 ± 0.0 a	2.7 ± 0.0 a	2.7 ± 0.0 a
alanine	3.1 ± 0.2 a	3.4 ± 0.1 a	3.0 ± 0.0 a	3.1 ± 0.0 a
valine	4.3 ± 0.1 a	3.7 ± 0.0 b	3.9 ± 0.0 a	3.7 ± 0.0 b
methionine	2.3 ± 0.3 a	1.5 ± 0.0 a	2.2 ± 0.0 a	2.2 ± 0.0 a
isoleucine	3.2 ± 0.2 a	2.8 ± 0.0 a	3.0 ± 0.0 a	2.9 ± 0.0 a
leucine	4.6 ± 0.3 a	4.0 ± 0.0 b	4.2 ± 0.0 ab	4.1 ± 0.0 ab
tyrosine	1.3 ± 0.3 a	1.8 ± 0.0 a	1.6 ± 0.0 a	1.9 ± 0.0 a
phenylalanine	4.3 ± 0.2 a	5.0 ± 0.1 a	4.2 ± 0.0 a	4.5 ± 0.0 a
lysine	3.6 ± 0.2 a	3.1 ± 0.0 a	3.3 ± 0.0 a	3.3 ± 0.0 a
histidine	1.4 ± 0.1 a	1.3 ± 0.0 a	1.4 ± 0.0 a	1.4 ± 0.0 a
arginine	21.1 ± 0.9 a	19.3 ± 0.1 a	20.6 ± 0.1 a	21.17 ± 0.2 a
proline	2.7 ± 0.2 a	2.3 ± 0.0 a	2.4 ± 0.0 a	2.3 ± 0.0 a
cysteine	1.9 ± 0.1 a	1.8 ± 0.0 a	1.8 ± 0.0 a	1.8 ± 0.0 a
tryptophan	1.1 ± 0.1 a	0.8 ± 0.0 a	0.9 ± 0.0 a	1.0 ± 0.0 a

[a]Different letters indicate a statistically significant difference among treatments (p < 0.05; Duncan's test).

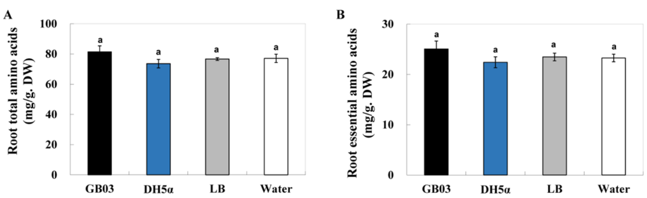

Figure 5. Comparison of the contents of (A) root total amino acids and (B) root essential amino acids in *C. pilosula* (Franch.) Nannf. among four treatments. Values are means, and bars indicate SDs (n = 8). Different letters indicate a statistically significant difference among treatments (p < 0.05; Duncan's test).

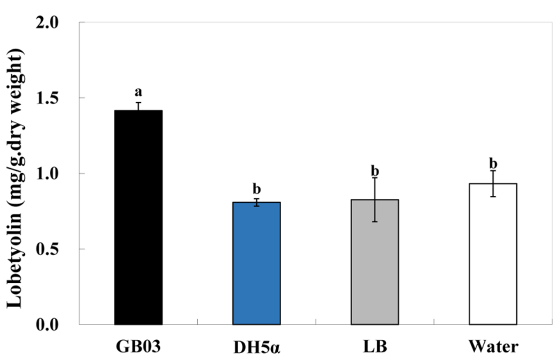

Figure 6. Comparison of the lobetyolin content in root of *C. pilosula* (Franch.) Nannf. among four treatments. Values are means, and bars indicate SDs (n = 8). Different letters indicate a statistically significant difference among treatments (p < 0.05; Duncan's test).

effects of the commercial PGPR strain *B. amyloliquefaciens* GB03 on the growth of *C. pilosula*. Inoculation of GB03 directly promoted the growth of *C. pilosula*, with a significant increase in shoot height and weight, root volume and weight, and branch number (Figures 2 and 3) and enhanced photosynthesis compared to the other three treatments (Figure 4). Recently, various studies demonstrated that PGPR can interact with plants through synthesizing a plant growth-promoting phytohormone, such as auxin, that greatly regulates the lateral and adventitious root formation and root elongation in the initial stage.[17] In our research, we found that all root parameters showed an increase in varying degrees with GB03 treatment compared to DH5α, LB medium, and water treatments. As we know, PGPR could produce various enzymes, plant hormones, and antifungal organic compounds.[39] On the basis of previous research, GB03 can produce two volatile components: 3-hydroxy-2-butanone (acetoin) and (2*R*,3*R*)-butanediol.[32] The data of *Arabidopsis* transcripts treated with over 25 bacterial volatile odors verified that GB03 volatiles induced plant growth promotion by regulating auxin homeostasis.[33] Moreover, microarray data demonstrated that the change of photosynthesis was related to GB03 downregulation of sugar signaling.[40] In addition, soil inoculation of GB03 can also promote growth of some other plant species, such as white clover and wheat.[26,43] In light of the above research results and our physical and chemical evidence, we supposed that both the volatile components from GB03 and soil inoculation of GB03 could improve plant growth and confirmed that GB03 can be qualified as a kind of PGPR for *C. pilosula*.

In recent year, secondary metabolite accumulation in different plants induced by PGPR had been studied. Wang et al. found that inoculation with endophytic microbes isolated from a Zn hyperaccumulator can result in a significant increase of plant growth and Zn accumulation in grains of rice.[37] It was found that GB03 inoculation significantly increased essential oil production in sweet basil.[34] Bharti et al. found that PGPR inoculation can improve the percentage concentration of menthol, which is the major characteristic constituent of the essential oil in *Mentha arvensis*.[41] Lobetyolin, a kind of polyacetylene, is a primary metabolite as one of the most important pharmacological functions of *C. pilosula*, and when the content of lobetyolin is higher, the quality of *C. pilosula* is better.[2] In the current research, the content of lobetyolin inoculated with GB03 was elevated almost twice as high as that inoculated with DH5α, LB medium, and water (Figure 6). In addition, the content of 14 amino acids from roots of *C. pilosula*

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was improved (Table 1), even though the difference was not statistically significant among treatments (Figure 5). *C. pilosula* roots contained a full range of amino acids, including plentiful amounts of taste-active amino acids and therapeutic amino acids.[42] Our results showed higher levels of 10 kinds of amino acids in GB03-inoculated plants of *C. pilosula* (Table 1).

Our results demonstrated that inoculation of GB03 showed both significant plant growth promotion and accumulation of lobetyolin in *C. pilosula* in the pot experiments. It will guide a new strategy for cultivating the Chinese herbal plant *C. pilosula*.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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