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Variation in bioactive compounds of *Glechoma longituba* and its influential factors: Implication for advanced cultivation strategies

Lin Jin[a,1], Li Liu[a,1], Qiaosheng Guo[a,*], Li Wang[b], Jun Zou[a]

[a] Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing, 210095, China
[b] Key Laboratory for Silviculture and Conservation of the Ministry of Education, College of Forestry, Beijing Forestry University, Beijing, 100083, China

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ABSTRACT

Glechoma longituba is a widespread crop in China. With high concentrations of many bioactive compounds, it has long been treated as a health beneficial ingredient for human diet and even some medicine. In order to gain comprehensive understandings of current resources of *Glechoma longituba*, samples from seventeen geographical origins in fifteen Chinese provinces were collected to reveal the connections between different influence factors and certain bioactive compounds. Firstly, high variations in the contents of ethanol-soluble extracts, total flavonoid, chlorogenic acid, caffeic acid, rosmarinic acid, oleanolic acid and ursolic acid were proved among different *Glechoma longituba* populations. Totally four parameters of soil physical properties, twelve parameters of soil chemical properties, thirty years' climate records and genetic diversity among different *Glechoma longituba* populations were thoroughly investigated. As a result, soil chemical property and climate condition with significant correlation between each other were both indicated to have the strongest positive correlations with bioactive compounds of *Glechoma longituba*, among which soil phosphorus, soil iron, temperature and moisture were showed to act as key factors. Neither the genetic variation nor the soil physical property was significantly related to the concentrations of bioactive compounds. These results laid the first theoretical foundation for further study of how to improve the quality of *Glechoma longituba* resources, as well as provided guidance for breeders to focus more on choosing planting areas with ideal climate conditions and modulating the optimum soil chemical properties for better *Glechoma longituba* cultivation.

1. Introduction

Glechoma longituba (Nakai) Kupr. (*G. longituba*) is an aromatic, perennial, evergreen creeper of the mint family Lamiaceae. Because of its high nutritional value, *G. longituba* is used as a salad green, beneficial tea, as well as food addictive for flavoring. Besides, its aerial part is commonly applied as a herbal medicine with treatments in urinary calculus, hepatic calculus, cough, flu, diarrhea, dysmenorrhea, and hysteritis (Kim et al., 2011). *G. longituba* is widely spread in most provinces of China, except for Qinghai, Gansu, Xinjiang, Tibet and Inner Mongolia (Flora of China, 1977). Though *G. longituba* has attracted considerable interest for its various applications in edible and medicinal fields, poor cultivation strategies and management caused a failure to satisfy the increasing demand. Thus, it is necessary to have a comprehensive investigation of current living situation of different *G. longituba* populations, and carry out the cultivation industry for *G. longituba* to

Abbreviations: SRAP, sequence-related amplified polymorphism; CEC, cation exchange capacity; CA, chlorogenic acid; CaA, caffeic acid; RA, rosmarinic acid; OA, oleanolic acid; UA, ursolic acid; na, observed number of alleles; ne, effective number of alleles; h, Nei's (1973) gene diversity; I, Shannon's information index; UPGMA, unweighted pair group method with arithmetic average; HCA, hierarchical cluster analysis; AAH, average annual humidity; AAP, average annual precipitation; AMP, average of maximum daily precipitation in a year; APD, average number of days with daily precipitation greater than 1.0 mm in a year; AAWD, annual average wind speed; AAAP, annual average air pressure; AAT, annual average temperature; AAMaT, annual average maximum temperature; AAMiT, annual average minimum temperature; AAEMaT, annual average extreme maximum temperature; AAEMiT, annual average extreme minimum temperature; AMaTD, average number of days with daily maximum temperature greater than 30 °C in a year; AMiTD, average number of days with daily minimum temperature less than 0 °C in a year. The following abbreviations represented the provenances where samples were collected; HZ, Hangzhou; CHZ, Chuzhou; HRB, Harbin; LY, Liaoyuan; BD, Baoding; CZ, Changzhi; NY, Nanyang; XC, Xuancheng; HA, Huai'an; NJ, Nanjing; WH, Wuhan; JA, Ji'an; ZQ, Zhaoqing; XY, Xinyang; TJ, Tianjin; BEJ, Beijing; WS, Wenshan

* Corresponding author at: Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing, 210095, China.
E-mail address: gqs@njau.edu.cn (Q. Guo).
[1] These authors contributed equally to this work.

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satisfy the market need.

Bioactive compounds in plants, which are primarily produced as secondary metabolites, are believed to help plants increase their adaptability to the surroundings, and also to operate beneficial effects on humans, directly or indirectly. For *G. longituba*, different bioactive compounds, mainly including phenolic acids, flavonoids, and triterpenoids, were identified from *G. longituba* (Ni et al., 2010). These chemical substances function in several activities, such as antioxidant (Tsai and Yin, 2008), antimicrobial (Xu and Lee, 2001), anti-inflammatory (Gamaro et al., 2011; Jennings et al., 2014), hypoglycemic (Fang et al., 2008), antiplatelet aggregative (Park, 2009), antitumor (Ohigashi et al., 1986; Wiemann et al., 2016), and liver protection (Jesus et al., 2017) etc, which are all quite valuable for human health. Therefore, the concentrations of these bioactive compounds in *G. longituba* are the most important indexes to evaluate the quality for its applicational use.

The formation and accumulation of bioactive compounds in plants would be affected by biotic and abiotic factors, including environmental factors, genetic factors, and their interactions, whose influencing mechanism is complicated. In previous study, variations in the contents of some active constituents among *G. longituba* samples was observed (Liu et al., 2012), but limited information was available to specify which influencing factors caused such chemical variations at a large scale. The objectives of this study were as follows: 1) to take a national-wide investigation of *G. longituba* resources to illustrate their current conditions of bioactive compounds, 2) to analyze the influence of genetic factors, climatic factors, and soil parameters to variations of bioactive compounds comprehensively, 3) to provide scientific guidance for future cultivation of *G. longituba*.

2. Materials and methods

2.1. Sample collection

We investigated 17 habitats of *G. longituba* covered the majority of its natural distribution in China from September to October 2015 (Fig. 1 and Table S1). For each region, at least three individual communities of *G. longituba* were collected. For determination of bioactive components, the aerial parts of the plants were washed and dried in a constant temperature drying oven at 55 °C for 12 h, then crushed and sifted through a 100-meshes sieve. For DNA extraction, young leaves were sampled and quickly frozen in liquid nitrogen before being stored at -80 °C until use.

Surface soil samples (depth of 0–20 cm) were also collected three times in each provenance from the same areas where *G. longituba* were taken for determinations of soil physical and chemical properties. Undisturbed soil was sampled to determine soil bulk density and soil texture. Besides, additional collected soil was natural air dried after removing the impurities, and finely pulverized for measuring cation exchange capacity (CEC), pH, organic matter, total nitrogen (N), available phosphorus (P), available potassium (K), calcium (Ca), magnesium (Mg), total iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn).

2.2. Quantitative analysis of bioactive components in G. longituba

Contents of ethanol-soluble extracts were determined by hot-dip method (general principle of 2201 in Part Four of Chinese Pharmacopoeia, National Pharmacopoeia Committee, 2015), and samples from each origin were measured three times.

The concentrations of three phenolic acid compounds, two triterpene acid compounds, and total flavonoid were also measured. Standard substances including lutein (92.6%), chlorogenic acid (CA, 96.2%), caffeic acid (CaA, 99.5%), rosmarinic acid (RA, 98.6%), oleanolic acid (OA, 94.9%), and ursolic acid (UA, 93.8%) were purchased from National Institutes for Food and Drug Control, Beijing, China. Before assays, the six reference solutions were configured with a concentration of 388.00 $\mu g \cdot mL^{-1}$ for lutein, 10.08 $\mu g \cdot mL^{-1}$ for CA, 20.24 $\mu g \cdot mL^{-1}$ for CaA, 117.70 $\mu g \cdot mL^{-1}$ for RA, 22.56 $\mu g \cdot mL^{-1}$ for OA, and 33.60 $\mu g \cdot mL^{-1}$ for UA.

The plant samples were ultrasonic extracted for an hour to obtain total flavonoid, phenolic acids (CA, CaA, RA), and triterpene acids (OA, UA) using 65%, 40% and 88% ethanol, respectively, and the liquid/solid ratio was 60 $mL \cdot g^{-1}$. After filtering through 0.22 μm membranes, the three extracts proceeded the measurement steps below.

The contents of total flavonoid in *G. longituba* was determined using an aluminium chloride colorimetric method, which modified the procedures reported by Woisky and Salatino (1998). 1 mL total flavonoid extract was added in a 25-mL volumetric flask, and mixed with 8 mL of 1.5% aluminium chloride and 4 mL of sodium acetate trihydrate-acetic acid buffer (pH 5.5). Before incubation for 30 min at room temperature, 65% ethanol was added to adjust the total volume and mixed well. Absorbance at 420 nm was measured using a DR5000 UV–vis spectrophotometer (Hach, Loveland, CO, USA). The total flavonoid content ($mg \cdot g^{-1}$) was estimated as lutein equivalents using the calibration curve.

The chromatographic separation in measurements of CA, CaA, RA contents was performed using a Waters Alliance HPLC system (Milford, MA, USA). The HPLC mobile phase included 0.1% phosphoric acid (A) and acetonitrile (B) with a linear gradient procedure (10%–15% B for 0–15 min, 15%–17% B for 15–20 min, 17%–25% B for 20–23 min, 25%–35% B for 23–33 min, and 35%–95% B for 33–36 min). The flow rate, detection wavelength, and injection volume was 0.8 $mL \cdot min^{-1}$, 326 nm, and 10 μL, respectively. And a Dikma Diamonsil Plus C18 column (4.6 × 250 mm, 5 μm, Dikma Technologies, Lake forest, CA, USA) was used with a column temperature of 35 °C.

The contents of OA and UA were also determined by liquid chromatography. The mobile phase consisted of 0.05% ammonium acetate (A) and acetonitrile (B) using an isocratic elution of 23:77 (A:B) at a flow rate of 0.5 $mL \cdot min^{-1}$. The detection wavelength was 210 nm. The other chromatographic conditions were same with the assay of the three phenolic acids.

2.3. SRAP-PCR analysis

Genomic DNA of single *G. longituba* individual was extracted employing TaKaRa Mini BEST Plant Genomic DNA Extraction Kit (Takara Biomedical Technology Beijing Co., Ltd., Beijing, China). DNA concentration was quantified by a Thermo Scientific NamoDrop 2000 spectrophotometer (Waltham, MA, USA), and its integrity was checked by agarose gel electrophoresis. DNA samples were diluted to a final concentration of 20 ng μL^{-1} for PCR analysis.

Fourteen primer combinations (Table 1) were synthesized by Rui Biotechnology Co., Ltd. (Beijing, China). PCR amplification was carried out in a total volume of 9.0 μL containing 5.0 μL Taq premix (Takara Biomedical Technology Beijing Co., Ltd., Beijing, China), 1.0 μL primer combination, 1.0 μL genomic DNA, and 2.0 μL ddH_2O. Amplifications were performed on a PTC-200 Thermal Cycler (MJ Research Inc., USA) under following conditions: initial DNA denaturation at 94 °C for 5 min, followed by 5 cycles of 1 min denaturation at 94 °C, annealing at 35 °C for 1 min, and extension at 72 °C for 1.5 min, for the next 35 cycles, the annealing temperature was increased to 50 °C, and followed by a 10 min final extension step at 72 °C. Polyacrylamide gel electrophoresis (8%) was performed before silver-staining, and DL2000 DNA marker (Tiangen Biotech, Beijing, China) was used to calculate the size of the bands.

2.4. Data collection of climate factors

Climate data, including parameters relative to precipitation, humidity, temperature, wind speed, and air pressure, were obtained from China meteorological data service center (http://data.cma.cn/data/cdcindex.html).