Analysis of amino acids in Meconopsis quintuplinervia by HPLC-MS and exploration of geographical distribution pattern

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Abstract

The amino acids in Tibetan medicine herb *Meconopsis quintuplinervia* from sixteen different populations on the Qinghai-Tibet Plateau were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) with derivative reagent 10-ethyl acridine-2-sulfonyl chloride (EASC). Among all the amino acids, phenylalanine was the most abundant, followed by glycine, tryptophan, and lysine. These results suggest that the use of EASC provided a fast and sensitive method for amino acid detection and that the concentrations of essential amino acids were relatively high. Mass spectrometry results verified the accuracy of the HPLC results. To reveal the influence of the ecological environment on the contents and type of amino acids, we analyzed the HPLC determination results in combination with environmental factors. Specifically, cluster analysis was used to classify groups, and Principal Component Analysis (PCA) and Pearson correlation analysis methods were used to quantify and analyse the influence of environmental factors on the types and contents of amino acids. The results show that the sixteen populations were divided into 4 groups, with the distribution of amino acid components in *M. quintuplinervia* affected by the content of total N (A-N), available K (S-K). Pearson correlation analysis shows that soil organic matter (OR), A-N, available P (S-P) and available N (S-N) in soil positive correlation between most amino acids, and negative correlation between A-K and most amino acids. The derivatization method established in this study can be used for rapid determination of various amino acids, and the influence of environmental factors on amino acid content in *M. quintuplinervia* was revealed. The research results can provide basic data for the protection, development, and utilization of *M. quintuplinervia*.

Introduction

Meconopsis quintuplinervia Regel., a perennial herb of the Papaveraceae family, is a widely used Tibetan medicine herb throughout the Qinghai-Tibet Plateau (Northwest Institute of Plateau Biology 2019). Due to the special geographical environment of growth, many plateau authentic Tibetan medicine biological resources contain unique chemically active components. Modern pharmacological studies have found that *M. quintuplinervia* has antioxidant, anti-inflammatory, analgesic and hepatoprotective effects (Miao Yang et al. 2010; Zhiwang Wang et al. 2011). This herbal medicine also contains flavonoids, alkaloids, amino acids and other active ingredients. Generally, amino acids are indispensable substances in organisms. Recent studies have shown that amino acids not only serve as substrates for protein synthesis but also as regulatory factors for protein and energy metabolism (Yoshiharu Shimomura and Yasuyuki Kitaura 2018). Similarly, amino acids can also be used as very effective antioxidants (Alexandrina Guidea et al. 2020). Amino acids are of importance in many physiological and pathological processes and have attracted wide attention as promising therapeutic drugs (Long Binh Vong et al. 2020). However, previous studies on the components of *M. quintuplinervia* mainly focused on flavonoids, alkaloids, terpenoids and volatile oil, and more research is needed on amino acids (Yu Gong et al. 2020).

High-performance liquid chromatography (HPLC) is a useful method for the determination of amino acids (Xinrui He et al. 2016). Because most amino acids lack chromophores and can not produce fluorescence,

it is necessary to derivatize amino acids and connect them with chromophores or fluorescent groups to improve the sensitivity of amino acid detection (Zhaolai Dai et al. 2014). Dansyl chloride (DNS-Cl), o-phthaldialdehyde (OPA), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and fluorescein isothiocyanate (FITC) are widely used in the derivatization of amino acids, but they have different drawbacks, such as limited selectivity and sensitivity. Only several amino acids can be detected at the same time, unstable derivative products, many interference factors and a time-consuming derivatization procedure (Liyuan Zhang et al. 2012; Sándor Kéki et al. 2008; Karen A. Corleto et al. 2019; Rana M. Kazan et al. 2019; Mar Castellanos et al. 2016; Zhihong Shi et al. 2013). In recent years, many new derivatization reagents have also been developed. Rita Gatti reported that 2,5-dimethyl-1H-pyrrole-3,4-dicarbaldehyde (DPD) is a precolumn derivatization reagent for HPLC/UV detection of amino acids and can derivatize more than a dozen amino acids in ten minutes (Rita Gatti et al. 2010). Amir Hayat carried out simultaneous HPLC determination of amino acids in selected Pakistani rice varieties by precolumn derivatization with 2-hydroxynaphthaldehyde (Amir Hayat et al. 2014). Xian-En Zhao carried out simultaneous determination of monoamine and amino acid neurotransmitters in rat endbrain tissues with 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) (Xian-En Zhao and You-Rui Suo 2008). Amir Hayat used 4-fluoro-7-nitrobenzofurazan (NBD-F) as a derivatization reagent of amino acids, and the derivatization reaction was easy to perform(Mayu Onozato et al. 2018; Yuanqi Du et al. 2018). However, these reagents still have the problem of insufficient simultaneous detection of amino acids. Therefore, a rapid and sensitive method for the determination of amino acids in 10-ethyl acridine-2-sulfonyl chloride (EASC) (Huaixin Zhao 2009) was established. It can detect 20 kinds of amino acids at the same time. Additionally, the derivation process is simple and rapid.

Although the activity of *M. quintuplinervia* has been recognized on a large scale, the ecological reasons for the difference in the content of its active components, especially amino acids, are still unclear (Tao Wei et al. 2018; Yuting Xu and Huifang Xu 2014). To reveal the influence of the ecological environment on the contents and type of amino acids, we recorded the environmental conditions in different populations of *M. quintuplinervia* and analyzed them in combination with the determined amino acid content and types. In addition, we used cluster analysis, Principal Component Analysis (PCA) and correlation analysis to analyze the difference in amino acid content among different populations and the environmental factors that cause these differences, to provide a certain basis for the evaluation of amino acids and then contribute to the rational development and utilization of medicinal plant resources in the Qinghai-Tibet Plateau (Chen Jin et al.2014).

Materials and methods

Sample collection

Samples of *M. quintuplinervia* were collected from 16 different geographical locations distributed in the Qinghai-Tibet Plateau, China, during the florescence in 2013. The geographic information of each population was also recorded. All sampling sites were marked in Fig. 1, with samples 1 and 11 from Menyuan County, 2 from Maqin County, 3 and 10 from Xunhua County, 4 and 6 from Tongren County, 5

from Hualong County, 7 from Zema County, 8 from Datong County, 9, 14 and 15 from Qilian County, and 12 from Guide County. Weeds and dust were removed and samples were then air-dried and crushed into powder. Each powder was individually filtered using an 80 mesh sieve. All samples were identified by Professor Jing Sun as *Meconopsis quintuplinervia* Regel. These samples were numbered and stored in glass desiccators prior to use.

Chemicals and reagents

Amino acid standard sample (Sigma company), spectrally pure acetonitrile (Sigma company), the purified water was Watsons distilled water. Other reagents, such as hydrochloric acid, were analytical or HPLC grade. The derivatization reagent EASC was made by our laboratory.

Methods

Determination of soil physical and chemical values

The soil pH value (NY/T 1377–2007, China), soil total salt content (NY/T 1121.16–2006, China), soil available phosphorus content (NY/T 1121.7–2014, China), soil available potassium content (NY/T889-2004, China) and soil organic matter content were determined according to the recommended standards of the Ministry of Agriculture of China. Soil total nitrogen (LY/T 1228–2015, China), total phosphorus (LY/T 1232–2015, China), total potassium (LY/T 1234–2015, China) and sodium content (LY/T 1254–1999, China) were determined with reference to forest soil determination standards. The soil available nitrogen content was determined according to a local standard (DB13/T 843–2007, China).

Preparation of reference solution

A quantitative amino acid standard was accurately weighed and dissolved in a small amount of 6 mol/L hydrochloric acid. The pH was adjusted to neutrality with 6 mol/L sodium hydroxide solution and prepared into a 1.0×10^{-2} mol/L solution with a pH 9.0 sodium borate buffer solution (0.1 mol/L). EASE (0.078 g) was weighed, dissolved in acetonitrile and fixed to 25 mL, and its concentration was 3.0×10^{-3} g/mL.

Extraction of sample

An amount of approximately 0.0500 g (± 0.0001) *M. quintuplinervia* sample powder was accurately weighed into a 3 mL ampere bottle and hydrolyzed at 100 ℃ with 1 mL of 6 mol/L hydrochloric acid for 24 h. The extract was cooled to room temperature and then filtered. The pH of the filtrate was adjusted to 8.0 ~ 9.0 with 6 mol/L sodium hydroxide solution, and the filtrate was used as the test solution.

Derivatization

Took 20 μL mixed amino acid standard solution or 100 μL test solution. Then, 100 μL 0.01 mol/l borate buffer solution (pH = 9.5), 200 μL derivatization reagent solution and 200 μL acetonitrile were added. The

mixture was reacted in a 65°C water bath for 10 min, removed and allowed to cool after derivatization. Then, acetonitrile-water solution was added to dilute the solution to 1 mol/L, and the solution was injected into the HPLC system for analysis.

HPLC determination

HPLC analysis was performed with HPLC–MS (Agilent 1100, Agilent Company, USA). An Akasil C18 column (250 mm × 4.6 mm, i.d. 5 μm, Elite Co., Ltd., Dalian, China) was used. The mobile phase consisted of 5% acetonitrile (containing 30 mmoL/L $NH_4H_2PO_4$) (A) and 95% acetonitrile (B) with a gradient elution of 100–85% B (0–1 min), 85–75% B (1–3 min), 75–62.5% B (3–8 min), 62.5–50% B (8–14 min), 50–40% B (14–15 min), 40–0% B (15–17 min), and 100% A (17–20 min). The flow rate was 1.0 mL/min, the column temperature was 30°C and the injected volume was 10 μL. The excitation and emission wavelengths of fluorescence are 262 nm and 425 nm, respectively.

MS detection

The experiments were carried out using an Agilent 1100 ion trap mass spectrometer (Agilent, USA) equipped with an electrospray ion (ESI) source. The solution was analyzed in positive ionization mode under the following conditions: spray pressure, 35 p.s.i (1 p.s.i = 6894.76 Pa); capillary voltage, 3500 V; dry gas flow rate, 9 L/min; dry gas temperature, 350 ℃.

Data analysis

The injection concentrations were in the range of 0.21 ~ 896.78 nmoL/L, and the linear regression equation and linear correlation coefficient were obtained according to the peak area and the actual injection quantity. The detection limit of amino acid derivatives was calculated according to S/N = 3:1, and the detection limit LOD (Limit of detection) and quantitative limit LOQ (Limit of quantitation) of the method were obtained. Under the same elution conditions, amino acid derivatives were analyzed six times in parallel, and the relative deviation RSD (Relative standard deviation) of peak area was obtained.

Relevant verification parameters are listed in Table 1. Most of the linear correlation coefficients of amino acid derivatives were above 0.9990. The LOD was between 0.07 nmoL/L and 1.71 nmoL/L, and the LOQ was between 0.29 nmoL/L and 5.43 nmoL/L. The relative standard deviation RSD of the peak area was less than 1.89%.

Table 1
Linear regression equation, correlation coefficient, detection limit and reproducibility, MS data of amino acid derivatives.

Amino acid	Linear regression equation	Correlation coefficient r	LOD (nmoL/L)	LOQ (nmoL/L)	Peak area RSD(%)	MS [M + H]+
Cys	Y = 10731.84X + 50.13	0.9990	0.54	1.83	1.61	407.2
His	Y = 7940.97X + 13.66	0.9993	0.82	2.46	1.29	441.0
Orn	Y = 11042.36X + 81.75	0.9995	0.15	0.57	0.38	418.2
Arg	Y = 8132.39X − 40.22	0.9994	0.25	0.82	0.97	460.2
Lys	Y = 5681.66X -116.39	0.9993	1.05	3.64	1.06	432.2
Ser	Y = 8995.99X − 30.68	0.9995	0.40	1.16	0.51	391.1
Asp	Y = 10980.24X − 204.30	0.9987	0.15	0.50	1.89	419.2
Glu + Thr	Y = 9448.3X − 9.82	0.9991	0.26	0.87	0.34	433.2, 405.1
Gly	Y = 9469.87X − 7.33	0.9996	0.07	0.29	1.69	361.1
Trp	Y = 7597.84X + 50.13	0.9992	0.38	1.27	0.65	490.2
Ala	Y = 6852.57X + 65.41	0.9994	0.94	3.28	0.73	375.0
Tyr	Y = 9190.65X − 57.86	0.9995	0.19	0.65	0.86	467.2
GABA	Y = 8531.26X − 21.65	0.9991	1.71	5.43	0.47	389.1
Pro	Y = 7634.78X + 16.67	0.9989	0.22	0.74	0.88	401.0
Met	Y = 10838.99X + 6.52	0.9997	0.11	0.39	0.11	435.0
Val	Y = 6520.04X − 3.99	0.9993	0.16	0.56	0.35	402.0
Phe	Y = 3496.77X + 19.69	0.9992	0.85	2.83	1.72	451.1

Note: Linear equation Y: peak area, X: injection volume (nmoL/L)

Amino acid	Linear regression equation	Correlation coefficient r	LOD (nmoL/L)	LOQ (nmoL/L)	Peak area RSD(%)	MS [M + H]+
Ile	Y = 5348.06X − 98.83	0.9989	0.12	0.39	1.21	417.2
Leu	Y = 6454.56X + 6.52	0.9994	0.06	0.21	0.36	417.2
Note: Linear equation Y: peak area, X: injection volume (nmoL/L)						

Results and discussion

Soil physical and chemical values analysis

The heat map of soil physical and chemical values at each population was shown in Fig. 2. Among all sites, the soil physical and chemical values of sampling point 15 (Youhulu Ditch, Qilian county) were quite different from those of other sites, and the values of S-N, S-P, A-N, OR and A-S were the highest, but A-K value of sampling point was the lowest. Sampling point 1 had the highest S-K value, while sampling point 7 had the highest A-P value. From the sampling distribution, it can be seen that the sampling point 15 is the most northwest, so the content of soil elements may be quite different from other sites. The sampling point 7 was farthest from each sampling point, so the soil physical and chemical values also varies greatly.

HPLC determination and MS identification

Derivatization was mainly affected by the pH value of the buffer, reaction time and temperature, and the amount of EASC also affected the derivatization yield (Xian-En Zhao et al. 2015). First, the pH value was evaluated, and the results showed that the amount of derivatives increased with increasing basicity, but derivatives decreased slightly when basicity reached a certain degree. It was found that the peak areas of amino acids were the largest in borate buffer solution with pH 9.5. Then, the temperature and time of derivatization were evaluated from 20 to 80℃ and 1 to 20 min, respectively. When derivatization was carried out at 65℃ for 10 min, the maximum peak area was reached.

The sample solution was tested under the aforementioned chromatographic conditions. The chromatographic separation diagram is shown in Fig. 3. Amino acids were well separated under this chromatographic condition. Twenty amino acids can be detected simultaneously under the action of EASC. The determination results of amino acid components in M. quintuplinervia samples are presented in Fig. 4. Among the 19 kinds of amino acids, 17 kinds of amino acids were detected in the samples, and 4-amino butyric acid and leucine were not detected in each spot.

Among all detected amino acids, phenylalanine was the most abundant, followed by glycine, tryptophan and lysine. The total content of essential amino acids (EAAs) ranged from 0.16 mg/g to 0.80 mg/g (average 0.51 mg/g), and that of amino acids from 0.45 mg/g to 2.53 mg/g (average 1.72 mg/g). The

proportion of EAAs to total amino acids ranged from 23.012–35.62% (average 11.17%). EAAs play an important role in human health, such as promoting normal digestion of the intestinal tract, maintaining and regulating protein synthesis and metabolism, and regulating the nerve center (Xiaokun Yu et al. 2013). Compared with other medicinal materials, its amino acid content is low, while the content of essential amino acids in total amino acids is relatively high (Liangjun Xiao et al. 2015; Xiaoyuan Wang et al. 2019; Meihui Zhang and Ruihai Li 2019).

The coefficients of variation of the total EAA content, total amino acid content and ratio of total EAAs to total amino acids were 0.402, 0.405 and 0.112, respectively, and the coefficient of variation of each amino acid ranged from 28.23 to 384.75. This result showed that the content of the same kind of amino acids changed greatly among different spots and that the environment had a great influence on the amino acid content. Among them, the difference in Arg content among different points is the smallest.

An electrospray ionization source was used for online postcolumn mass spectrometry identification. The first-order mass spectrometry (a) and the second-order mass spectrometry (b) taking glycine derivatives as an example are shown in Fig. 5. A schematic diagram of the mass spectrometry fragmentation mode is shown in Fig. 6. It can be seen from the figures that glycine was well distinguished, and the MS results verified the accuracy of the HPLC results.

Cluster analysis

Cluster analysis was carried out on the content of amino acids in various samples of *M. quintuplinervia* by the baverage method under Seuclid measurements using OriginPro 2023 software. The results are shown in Fig. 7.

When the distance was 16800, 16 samples were grouped into 2 groups; when the distance reached 9100, samples were grouped into 3 groups; and when the distance was approximately 6000, samples were grouped into 4 groups, which is a good classification number. Among them, 6 sampling points of 1, 12, 11, 16, 14 and 13 were clustered into group 1. The two samples, 2 and 4, were grouped into Group 2. Seven sampling sites 3, 5, 8, 10, 9, 6, and 7, were clustered into Group 3. The sampling point at the 15 was separately grouped into group 4 (Baohai Liu 2018).

Judging from the types and contents of amino acids detected at each sampling location, the average content of amino acids detected in group 1 was higher than the overall average of amino acids, and group 1 had better amino acid quality. Cysteine, histidine, and alanine were not detected at the two sampling points of group 2, but they were rich in other amino acids. In group 3, the content of each detected amino acid was lower than the overall average of each amino acid. In group 4, most kinds of amino acids were detected in 15, and the content was higher than the overall average value of each amino acid. Combined with environmental factors, 15 had the highest soil organic matter content, available phosphorus content, available nitrogen content, and relatively low altitude, so the content and types of amino acids were rich. The relationship between the specific environment and amino acids needs further analysis.

PCA analysis

PCA is a mathematical tool that aims to represent the variation present in the dataset using a small number of factors (Daniel Granato et al. 2018). PCA analysis was carried out by OriginPro 2023 software.

Firstly, the content of amino acids in *M. quintuplinervia* was analyzed by PCA. Using a correlation matrix to make a PCA diagram, the distribution diagram and factor load diagram of the main components of *M. quintuplinervia* and amino acid compounds were obtained as shown in Fig. 8A. The cumulative variance contribution rates of the first two common factors were 73.6% and 10.2% respectively, and the cumulative contribution rate was close to 85.0%. Therefore, the first principal component (PC1) and the second principal component (PC2) were extracted to judge the overall characteristics of amino acids in *M. quintuplinervia* (Huan Chen et al. 2014). In the distribution map obtained by PCA, 15 was separated from other samples, in the first quadrant, 2 and 4 were also clearly separated from other samples, in the fourth quadrant, which was similar to the results of cluster analysis, the other sampling points were close. Orn, Cys, His, Ala were distributed in the first quadrant, These highly positively correlated amino acids were the characteristic amino acids of *M. quintuplinervia*.

Using covariance matrix to make PCA diagram, the distribution diagram and factor load diagram of the main components of *M. quintuplinervia* and soil physical and chemical values analysis were obtained as shown in Fig. 8B. The cumulative variance contribution rates of the first two common factors were 91.6% and 5.5% respectively, and the cumulative contribution rate was more than 85.0%. Therefore, the first principal component (PC1) and the second principal component (PC2) were extracted to judge the overall characteristics of soil physical and chemical values. The distribution of PCA samples based on soil physical and chemical values was different from that of amino acids. In the distribution map, 15 was still separated from other samples, 1 was far away from other sampling points. PC1 had a large load value at A-N(0.87), PC2 had a large load value at S-K(0.95), They can be used as characteristic physical and chemical values affecting *M. quintuplinervia*.

Correlation analysis

Pearson correlation coefficient method was used to analyze the correlation between environmental factors and amino acid content. Pearson correlation coefficient was carried out by OriginPro 2023 software.

Firstly, the correlation between latitude and longitude, altitude, soil physical and chemical values and total amino acids was analyzed (Fig. 9A). For total amino acids, S-N, A-N, or in soil had extremely significant positive correlation ($p < 0.01$), A-K had extremely significant negative correlation ($p < 0.01$) and A-S had significant positive correlation ($p < 0.05$). Among soil factors, S-N and A-N, S-N and OR, S-N and A-S, OR and S-P, A-S and A-N, A-S and OR had extremely significant positive correlations ($p < 0.001$), A-N and A-K had extremely significant negative correlations ($p < 0.001$). There was no obvious correlation between environmental factors and total amino acid content. Latitude had a positive correlation with the contents of soil A-S and S-P ($p < 0.05$), and a significant negative correlation with the contents of soil A-P ($p < 0.01$).

Then, the correlation between environmental factors and soil factors on different amino acids was analyzed(Fig. 9B). In environmental factors, longitude had a negative correlation with Lys and Orn content($p < 0.05$), latitude had a significant positive correlation with Phe content ($p < 0.01$), and altitude has a negative correlation with Phe content($p < 0.05$). Among soil factors, OR was positively correlated with most amino acids($p < 0.01$), A-N and S-N were positively correlated with most amino acids($p < 0.05$), and A-S and S-P were positively correlated with some amino acids($p < 0.05$). A-K was negatively correlated with most amino acids($p < 0.01$). Ile, Ala, Gly, His were greatly affected by soil factors, while Phe, Val, Arg, Orn, Cys were less affected by soil factors.

Conclusions

In this study, EASC was used to establish a rapid and sensitive HPLC method for the determination of amino acids. The established amino acid determination method was accurate and reliable, most of the linear correlation coefficients of amino acid derivatives were above 0.9990, and MS results verified the accuracy of the HPLC results. Seventeen kinds of amino acids were simultaneously detected in the samples, and the proportion of total EAAs to total amino acids ranged from 23.012–35.62%, which is relatively high. Then, cluster analysis was performed to classify the sample points, 16 samples were grouped into 4 different groups.The average content of amino acids detected in group 1 was higher than the overall average of amino acids; cysteine, histidine and alanine were not detected at the two sampling points of group 2; in group 3, the content of each detected amino acid was lower than the overall average of each amino acid; and most kinds and contents of amino acids were detected in 15. PCA showed the positive correlation amino acids Orn, Cys, His and Ala were determined as the characteristic amino acid of *M. quintuplinervia.* Through the correlation study between the origin of *M. quintuplinervia* and its soil physical and chemical values, A-N and S-K were considered as characteristic physical and chemical values affecting *M. quintuplinervia*. Pearson correlation analysis showed that OR, A-N, S-P and S-N in soil were positively correlated with most amino acids, while A-K was negatively correlated with most amino acids. Compared with latitude and longitude and altitude, soil physical and chemical values had greater influence on amino acid content.

Declarations

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Figures

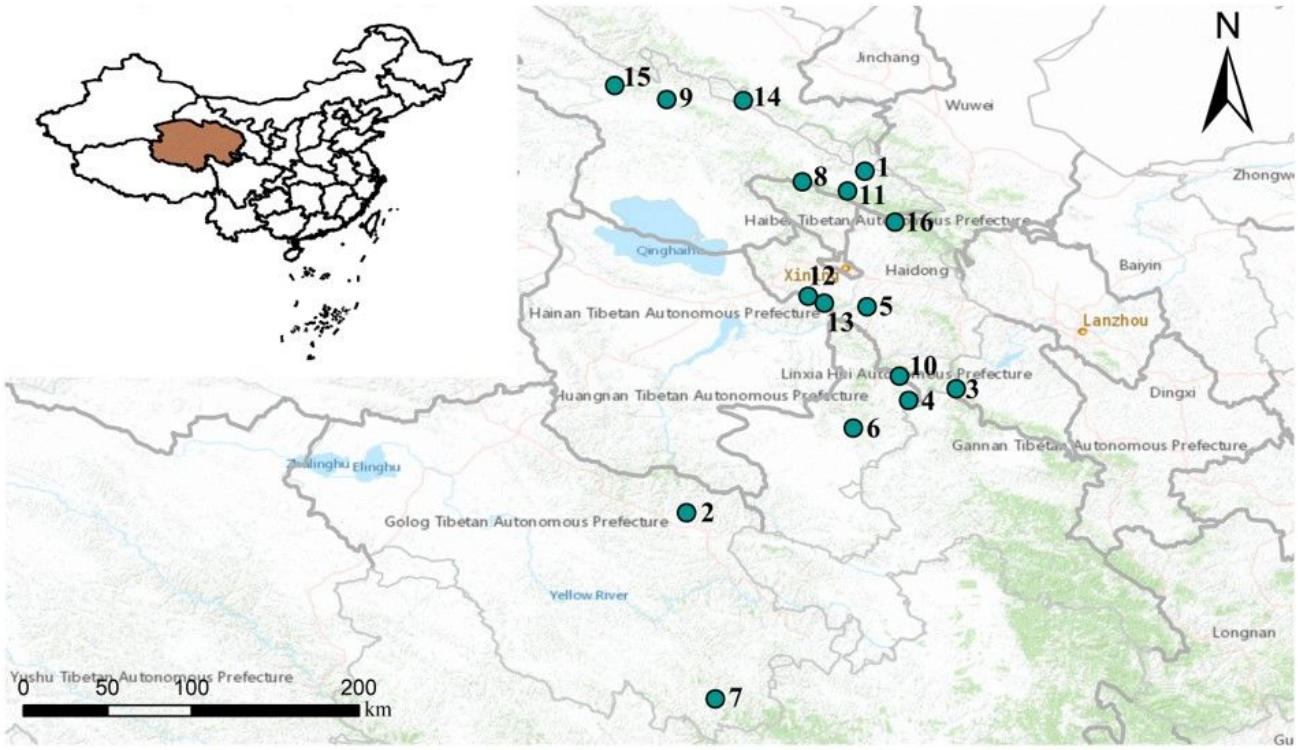

Figure 1

Location map of sampling points of *M. quintuplinervia*

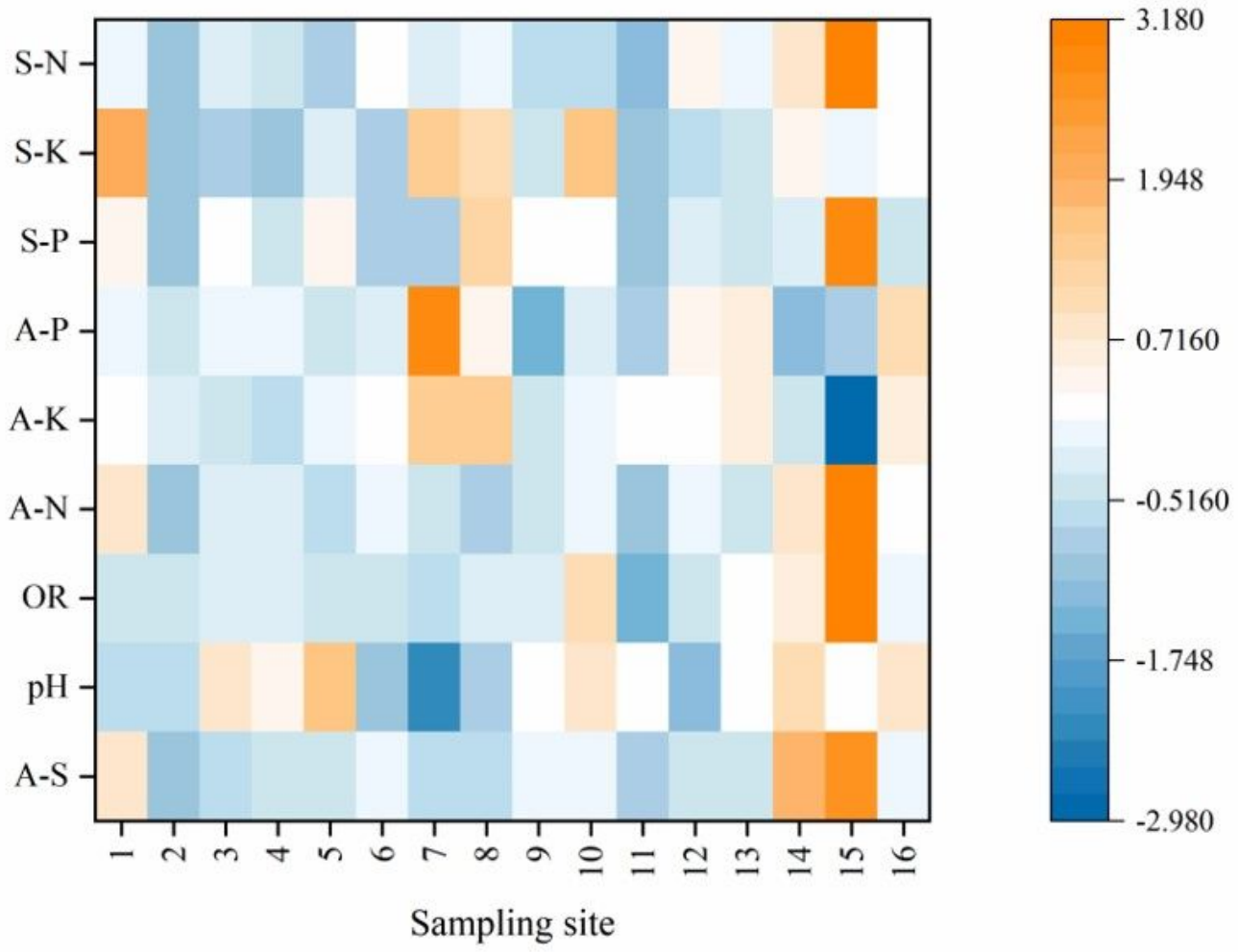

Figure 2

heat map of soil physical and chemical values

Note: A-S: total soil salt, OR: soil organic matter, A-N: total N, A–K: total K.

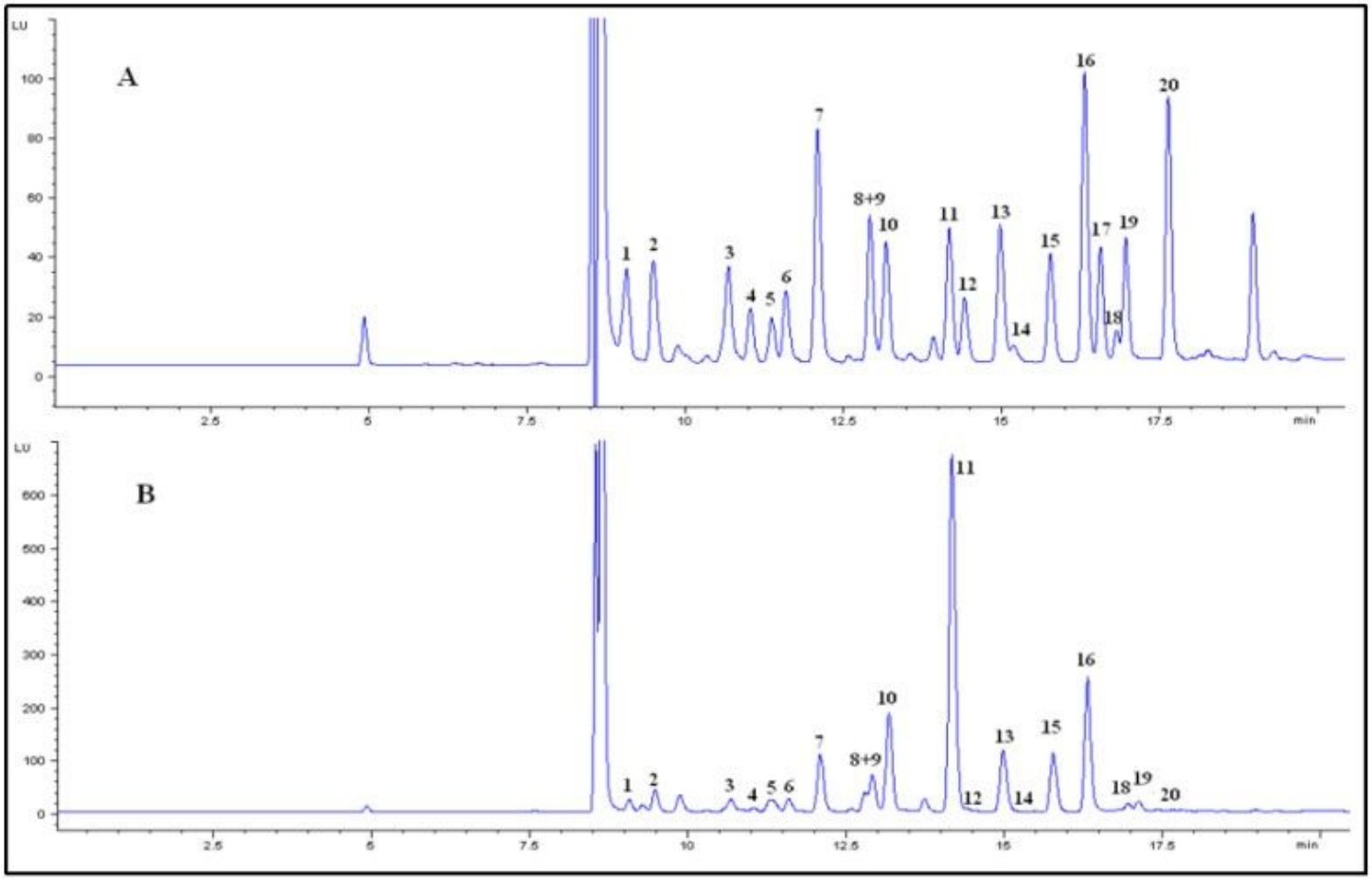

Figure 3

Chromatograms of Amino Acid Derivative Standard (A) and Sample (B)

1 Cysteine 2 Histidine 3 Ornithine 4 Arginine 5 Lysine 6 Serine 7 Asparagine 8 Glutamine 9 Threonine 10 Glycine 11 Tyrosine 12 Alanine 13 Tryptophan 14 4-Aminobutyeic acid 15 Proline 16 Methionine 17 Valine 18 Phenylalanine 19 Isoleucine 20 Leucine

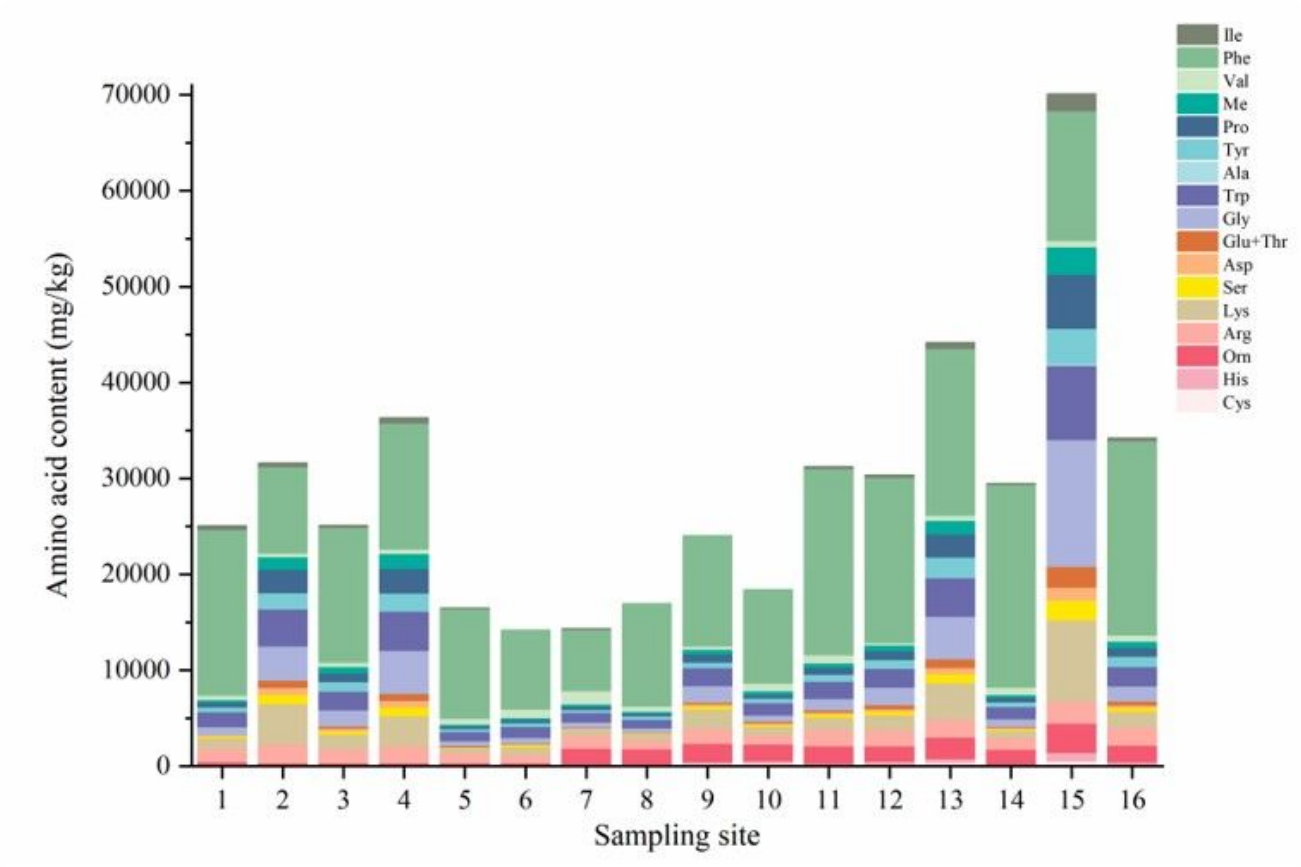

Figure 4

Amino acid content in each population of *M. quintuplinervia*

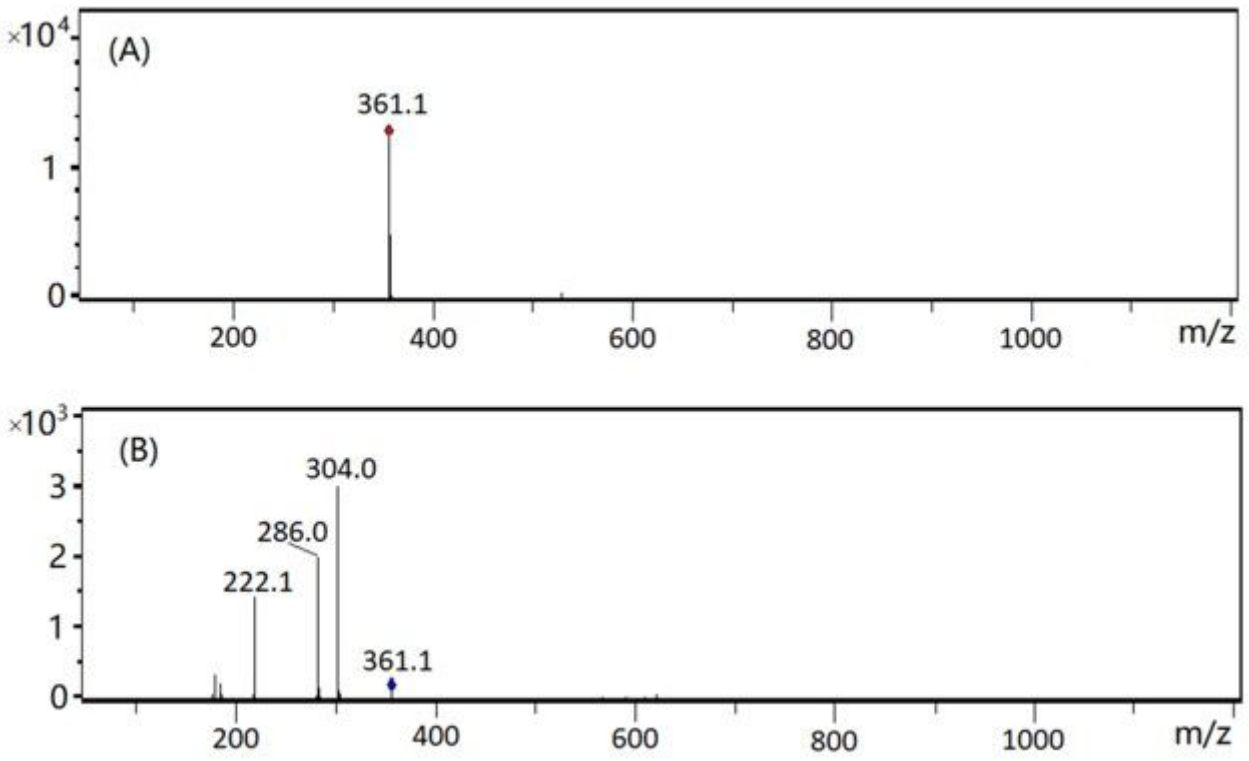

Figure 5

Glycine derivatives as examples of primary mass

spectrometry (a) and secondary mass spectrometry (b)

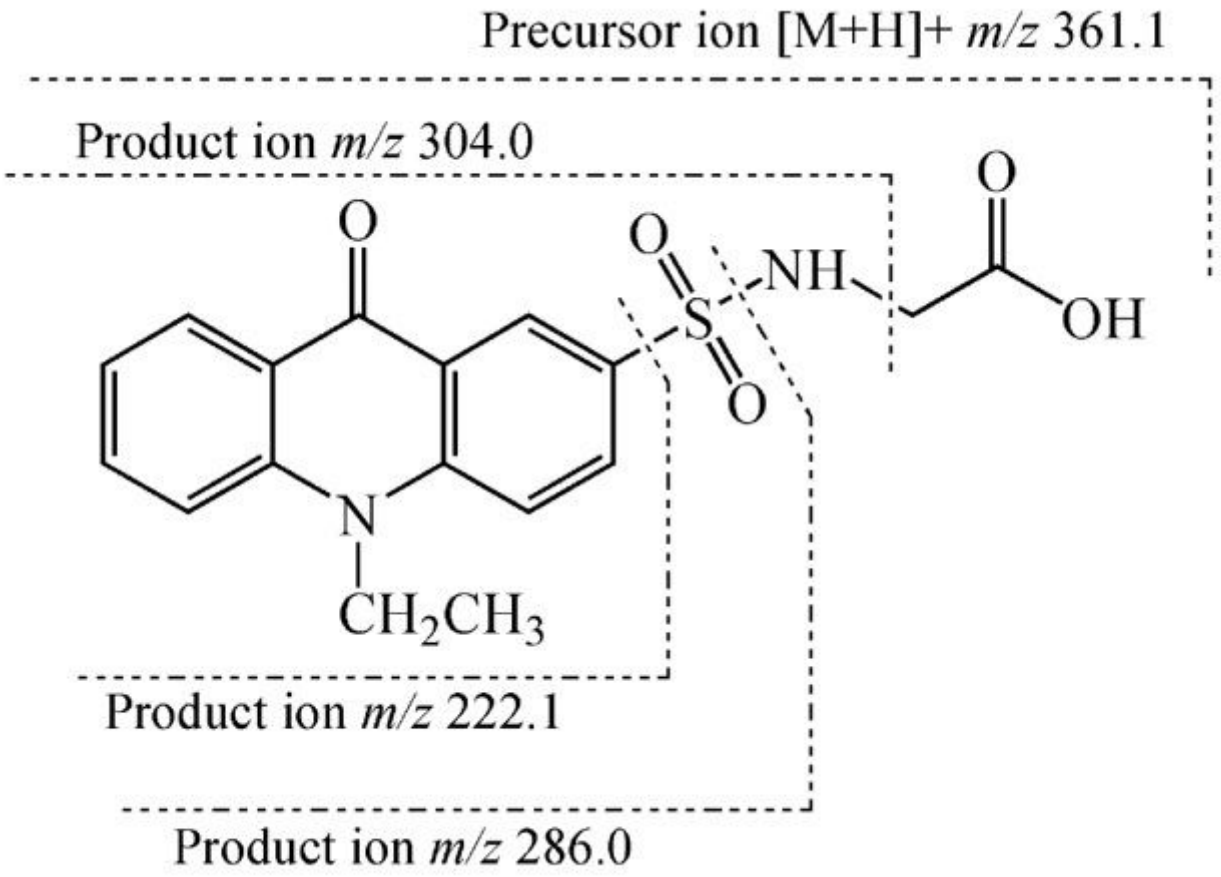

Figure 6

Schematic diagram of fragmentation mode of glycine EASC derivatives by MS

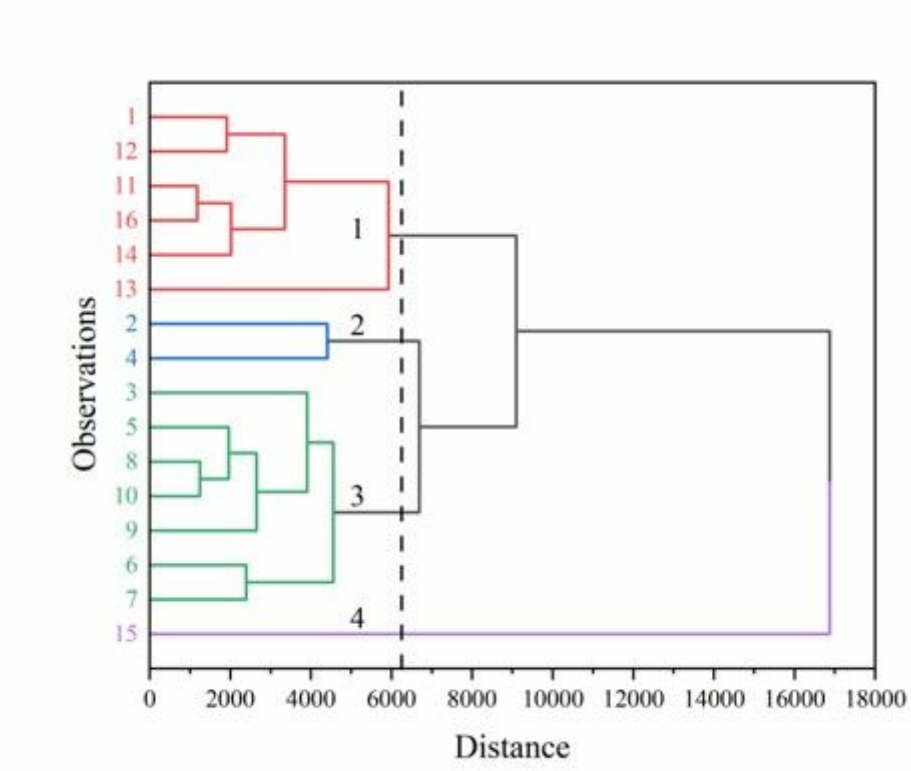

Figure 7

Cluster diagram of amino acid composition of *M. quintuplinervia*

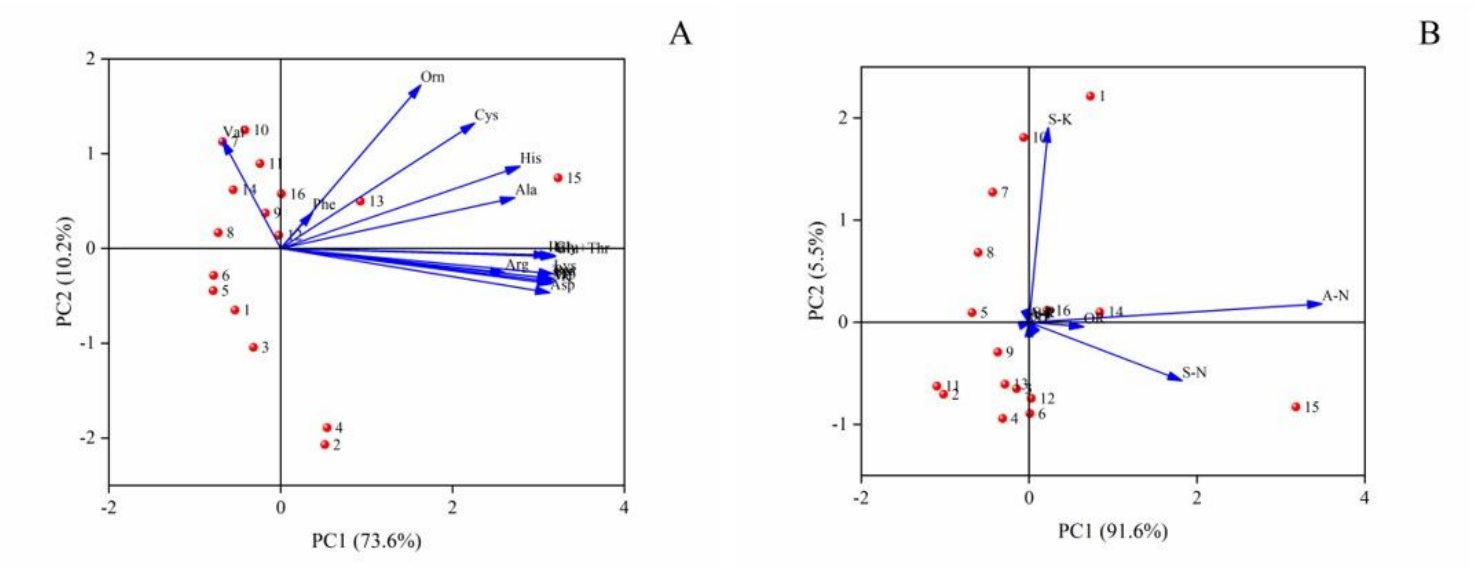

Figure 8

Distribution map and factor load map in PCA analysis

A: PCA analysis of amino acid content in *M. quintuplinervia*; B: PCA analysis of soil physical and chemical values

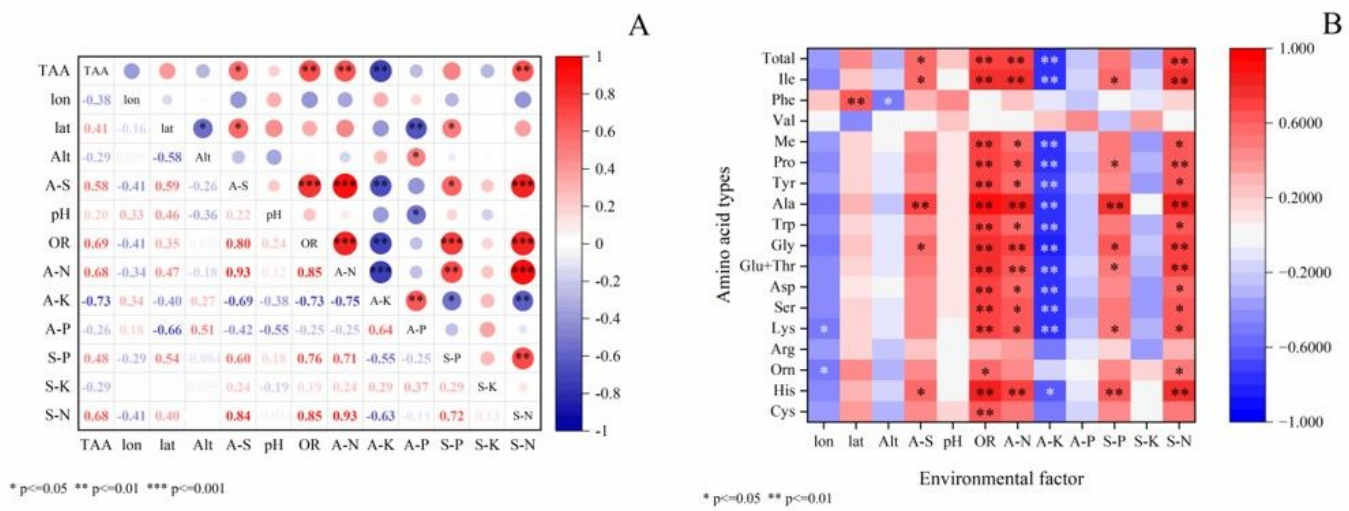

Figure 9

Pearson correlation analysis of geographical factors, soil factors and total amino acid content

A: correlation analysis between total amino acids and environment; B: correlation analysis between amino acids and environment