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Geographical variations in fatty acid and steroid saponin biosynthesis in *Dioscorea zingiberensis* rhizomes

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ABSTRACT

Dioscorea zingiberensis is an important source of diosgenin. However, the low environmental adaptability of plants producing bioactive components impedes their industrial development. In this study, *D. zingiberensis* rhizomes were collected from 20 populations covering a wide geographical area from northwestern to southwestern China. Variations in metabolites of steroid saponins biosynthesis pathway (steroid saponins, diosgenin and phytosterols) and fatty acids were determined. Spearman analysis showed that fatty acids were strongly correlated with steroid saponins. Furthermore, the specialized metabolites showed regional variations across a large geographical scale. Redundancy analysis illustrated that environmental variables had a profound impact on specialized metabolites. Among all environmental variables evaluated, latitude was the factor that contributed the most to the variation of metabolites in steroid saponins biosynthesis, while sunshine duration and mean annual temperature contributed the most to the variation in fatty acids. This study provides valuable information for understanding the effects of environmental factors on the production of bioactive chemicals by medicinal crops.

1. Introduction

Natural steroid compounds are crucial for the industrial production of steroidal drugs. Diosgenin is a major precursor of numerous steroidal hormones and is derived mainly from the acid hydrolysis of saponins extracted from *Dioscorea* plants (Zhang et al., 2018a). With increasing industrialization, the demand for diosgenin has sharply increased in recent years (Shen et al., 2018). However, the yields of specialized metabolites from plants tend to fluctuate due to changing environment conditions (Yang et al., 2013). More importantly, the yields of specialized metabolites from medicinal plants are significantly influenced by their natural environments (Xue et al., 2021). Thus, enhancing the environmental adaptability of medicinal plants will facilitate their industrial applications in pharmaceutical production.

Dioscorea zingiberensis, belonging to the *Dioscorea* genus of the family Dioscoreaceae, is an important source for the production of diosgenin. *D. zingiberensis* is widely distributed throughout southern and western China, whose rhizome is extensively utilized on account of its high content of saponins. Saponins, especially steroid saponins, are not only the primary bioactive components in the rhizome of *D. zingiberensis*, but also the main sources for diosgenin production (Zhang et al., 2009). Nevertheless, the fluctuating yield of steroid saponins is the major obstacle to industrial production. Previous studies indicated that the content of diosgenin was unstable when using different batches of *D. zingiberensis* rhizome from different locations (Zhang et al., 2018a, 2018b). Meanwhile, huge volumes of acidic wastewater are produced during the extraction process (Shen et al., 2020). Even if many clean production techniques are established, the fluctuating contents of specialized metabolites would hold back their industrial application (Shen et al., 2020; Wei et al., 2013). Consequently, fluctuating yields of specialized metabolites may have a negative impact on the industrial production of *D. zingiberensis*, which is not conducive to their further development. Whereas, researches on *D. zingiberensis* paid more attention on the isolation of functional compounds, pharmacological activities and analysis of saponin biosynthesis (Zheng et al., 2014; Zhang et al., 2014; Song et al., 2019). Hence, there is an urgent need to find out the environmental influences on the steroid saponins in *D. zingiberensis*.

Phytosterols are intermediates in the steroid saponin biosynthetic pathway and previous studies showed that the accumulation of phytosterols had a negative correlation with fatty acids in plants (Moses et al., 2014; Liang et al., 2019). Steroid saponins are biosynthesized by the mevalonate acid (MVA) pathway in *D. zingiberensis* (Li et al., 2018;

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Patel et al., 2012). Acetyl coenzyme A is the initial substrate to biosynthesize steroid saponins in MVA pathway, and then is successively converted into phytosterols, diosgenin and steroid saponins by a series of complex metabolic steps (Ciura et al., 2017b). Li et al. (2019) and Miao et al. (2011) reported that fatty acids were negatively correlated with specialized metabolites in the MVA pathway, which suggested that fatty acids may have important effects on steroid saponins in *D. zingiberensis*. In addition, fatty acids are often co-extracted during the industrial production of diosgenin, which negatively affects the value and purity of diosgenin (Chen et al., 2005; Olvera-García et al., 2015). Meanwhile, many signaling molecules are derivatives of fatty acids, and exert indispensable impacts on the plant growth (Shi et al., 2019). Nevertheless, little is known about the correlation of steroid saponins with fatty acids in *D. zingiberensis*. Several studies have shown that the content of fatty acids in plants exhibited a strong correlation with environment (Ma et al., 2020; Hou et al., 2019). In consequence, it would provide valuable information for comprehensive utilization in *D. zingiberensis* via conducting research on the correlation between fatty acids and environmental conditions.

In this study, we investigated the rhizomes of *D. zingiberensis* widely distributed from northwestern to southwestern China. Steroid saponins, diosgenin, phytosterols and fatty acids of *D. zingiberensis*'s rhizomes were determined. The objectives were to explore the variability of those bioactive components and investigate the relationships between specialized metabolites and environment. The results obtained will provide scientific basis for enhancing plant environmental adaptability and selecting the optimum growth region for large-scale cultivation.

2. Materials and methods

2.1. Plant materials

Rhizomes of *D. zingiberensis* were collected from plantations at 20 different localities covering most of the *D. zingiberensis* distribution area in China in October 2020 (latitudinal range 25°31′ to 34°33′ and longitudinal range 104°25′ to 112°51′). With the support of local research institutions, rhizomes were collected from seven provinces or direct-controlled municipalities (Chongqing, Sichuan, Hubei, Gansu, Shaanxi, Henan and Hunan) (Fig. 1). *D. zingiberensis* plants, grown from seeds, are full productive age (4 years old). All rhizomes reached natural maturation stage with several capsules cracking and were sampled at habitats with minimal human intervention. Surface contaminants on rhizomes were removed by deionized water. Samples were then freeze-dried to constant weight at -80 °C and ground to powders with a tissue lyser.

2.2. Standards and chemical reagents

Ethanol, n-hexane, diosgenin and β-sitosterol were purchased from Macklin Biochemical Co., Ltd (China). Methanol, acetonitrile and chloroform were purchased from Thermo Fisher Scientific Inc (USA). Dioscin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (China). Methyl ester mixture standards (C4-C24) were purchased from Merck KGaA (Germany). Stigmasterol, campesterol and N-methyl-N-(trimethylsilyl) trifluoroacetamide were purchased from Sigma-Aldrich (Germany). Parvifloside and protodeltonin were purchased from Kunming Institution of Botany Cas (China). All chemical reagents listed above were of HPLC grade.

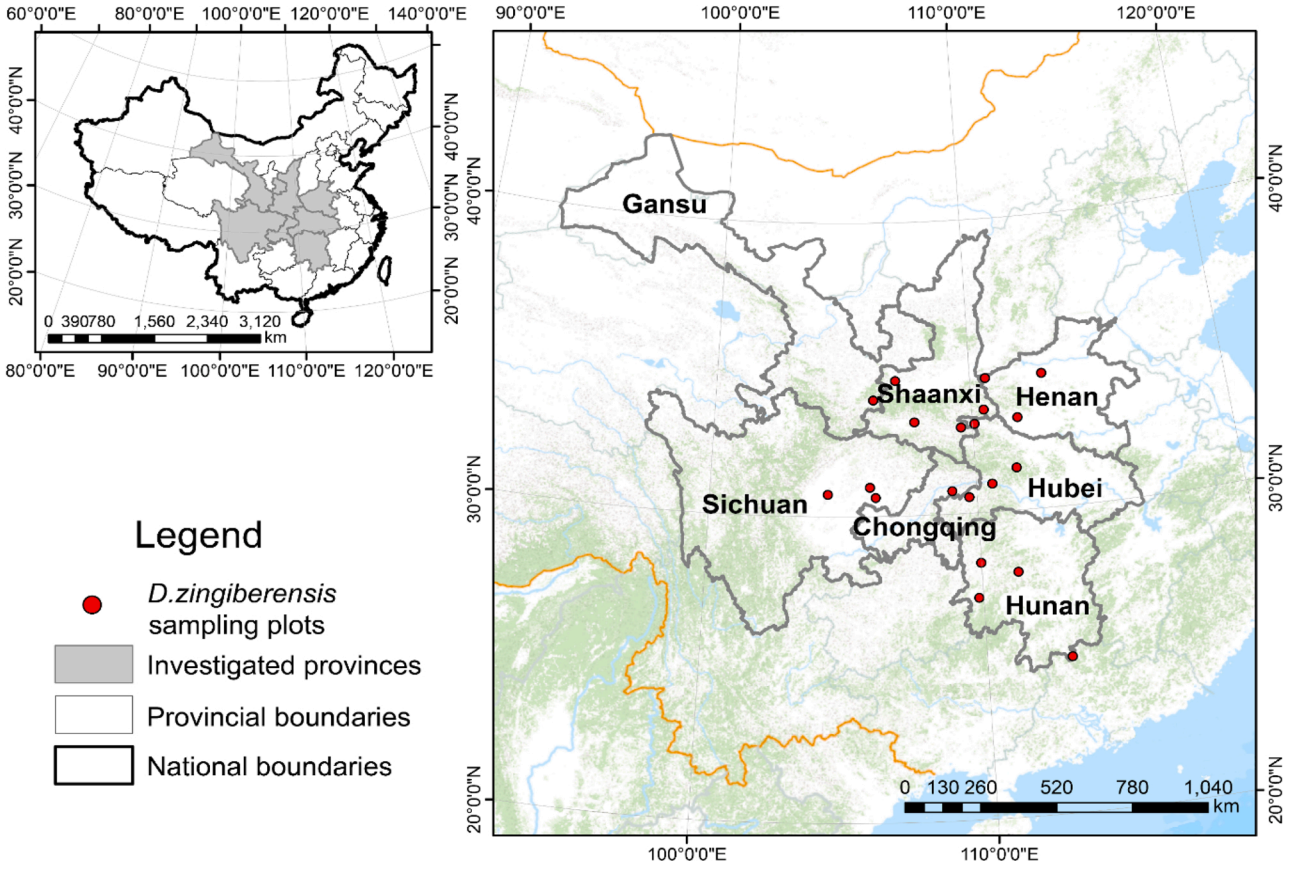

Fig. 1. Sample locations for *D. zingiberensis*.

2.3. Analysis of phytosterols

About 50 mg freeze-dried rhizomes powder was dissolved in 2 mL 2 mol/L KOH-ethanol. The mixture was then heated in the water bath for one hour at 80 °C. After cooling, 2 mL Hexane was added and the phytosterols were soluble in hexane in the top layer. The supernatant was filtered by a membrane solution filter (0.22 μm), after which the solvent was evaporated under a vacuum at 25 °C. Subsequently, dried sample was dissolved in 50 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide and derivatized in room temperature for 30 min, then 150 μL n-hexane was added for GC–MS analysis (Boulom et al., 2014).

The mixture was analyzed by Thermo Trace GC gas chromatograph equipped with TG-5 ms column (30 m × 0.25 mm ×0.25 μm) under the following temperature conditions: 80 °C for 1 min, followed by increases of 15 ℃/min to 290 ℃, and then maintenance at 290 ℃ for 10 min. The transfer line and ion resource temperatures were set to 290 ℃.

2.4. Analysis of diosgenin

About 50 mg freeze-dried rhizomes powder was dissolved in 1 mL mixture solvent (2 chloroform and 1 methanol), shaken vigorously by ultrasonication for 30 min, followed by centrifugation at 12,000×*g* for 10 min. The supernatant was filtered through a membrane solution filter (0.22 μm) and subjected to GC–MS analysis using the same equipment and column described above (Cheng et al., 2021).

The conditions were as follows: 240 °C for 1 min, followed by increases of 10 °C/min to 300 °C, and then maintenance at 300 °C for 10 min. The transfer line and ion resource temperatures were set to 290 °C. The GC–MS data were collected in full scan mode (*m/z* 50–500) and selected ion monitoring mode with diagnostic ions monitored at *m/z* 139, *m/z* 282 and *m/z* 414.

2.5. Analysis of fatty acids

According to previous researches, chloroform-methanol solvent (2:1, v:v) was used for extraction of fatty acid and sulfuric acid-methanol solvent (1:99, v:v) was added for methyl esterification. Then, 2 mL n-hexane was added to extract the fatty acid methyl esters (Jordi Folch and Sloane Stanley, 1956; Christie, 1993; Ma et al., 2020). The supernatant was filtered using a membrane solution filter (0.22 μm) and then analyzed by GC–MS under the following temperature conditions: 80 °C for 1 min, increases of 50 °C/min to 175 °C, maintenance at 175 °C for 10 min, increases of 5 °C/min to 200 °C, maintenance at 200 °C for 1 min, increases of 3 °C/min to 230 °C, and maintenance at 230 °C for 5 min. The MS transfer line and ion resource temperatures were set to 250 °C.

2.6. Analysis of steroid saponins

About 50 mg freeze-dried rhizomes powder was dissolved in 1 mL 80 % ethanol and then extracted by ultrasonication for 30 min. The mixture was at 12,000×g for 10 min and supernatant was filtered using a membrane solution filter (0.22 μm) prior to ultra-performance liquid chromatography tandem MS (UPLC-MS/MS) (Del Hierro et al., 2018). The ultra-performance liquid chromatography equipment was connected to the Q Exactive hybrid quadrupole mass spectrometer (Thermo Scientific). Steroid saponins were separated using a reverse phase C18 column (Thermo, 100 mm × 2.1 mm, 3 μm).

Mobile phases A and B contained 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile, respectively. The gradient program was as follows: 0–2 min, isocratic 12 % B; 2–8 min, linear gradient of 12%–45% B; 8–10 min, isocratic 45 % B; 10–15 min, linear gradient of 45–95% B; 15–19 min, isocratic 95 % B; 19–19.1 min, linear gradient of 95 to 12 % B; 19.1–21 min, isocratic 12 % B. Chromatography column temperature was 40 °C and flow rate was 0.3 mL/min.

2.7. Environmental factors

The dataset of geographical variables, including longitude, latitude and elevation, was collected via a global positioning system (GPS; G120BD, Jisibao Co., China). The dataset of climate influences was acquired by using closest-proximity values from the National Meteorological Data of China (http://www.escience.gov.cn/metdata/page/index.html). The climate dataset contained mean atmospheric pressure, annual maximum and minimum atmospheric pressure, mean sunshine duration, annual average temperature, annual temperature difference, annual maximum and minimum temperature, daily temperature difference, relative humidity, mean annual precipitation, annual maximum precipitation and annual minimum precipitation.

2.8. Statistical analysis

Phytosterols, diosgenin, fatty acids and steroid saponins were identified from their mass spectra and quantified by calculating the peak areas of external standards (Table 1). The fatty acids and environmental data were preprocessed via stepwise regression using the Akaike information criterion (AIC) to filter variables. Spearman analysis was carried out to explore the relationship between fatty acids and steroid saponin biosynthesis. A de-trended correspondence analysis (DCA) was carried out on the contents of diosgenin, steroid saponins, phytosterols and fatty acids, which were then applied to calculate the lengths of the gradient axes (LGA). Environmental vector fitting (envfit) and variance inflation factor (VIF) were used to simplify the correlation analysis model and test co-linearity, respectively. The significance of all screened environmental variables was assessed using the Monte-Carlo permutation test (permutation = 999). The above analyses were performed using the R packages car and vegan in R.4.0.2.

3. Results and discussion

3.1. Steroid saponin, phytosterol, diosgenin and fatty acid compositions

As expected, the contents of functional metabolites and fatty acids varied among 20 *D. zingiberensis* accessions. The statistical results for the natural diosgenin, three steroid saponins, four phytosterols and 13 fatty acids was listed in Table 2. The steroid saponins (parvifloside, protodeltonin and dioscin) were the most abundant in all 20 *D. zingiberensis* accessions. Similar contents of diosgenin and phytosterols were detected in all samples (< 1 mg/g). The fatty acids with higher content were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). Fatty acids with lower content were myristic acid (C14:0), pentadecanoic acid (C15:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), arachidic acid (C20:0), behenic acid (C22:0), tricosanoic acid (C23:0) and lignoceric acid (C24:0).

The mean value of diosgenin was 0.262 mg/g, ranging from 0.05 to 0.61 mg/g. In steroid saponins, the content of parvifloside ranged from 4.3 to 38.5 mg/g, whereas protodeltonin ranged from 2.5 to 46.9 mg/g; the mean value of dioscin was 1.869 and was in a range of 0.7–3.62 mg/g. *β*-sitosterol was the most abundant phytosterol with a mean value of 0.073 mg/g, while cholesterol was the least abundant phytosterol with a mean value of 0.012 mg/g. The contents of campesterol and stigmasterol were similar and their mean values were 0.030 and 0.020 mg/g, respectively. By comparing with external standards and the mass spectra library, a total of 13 fatty acids were identified. Among these, saturated fatty acids (SFA) were the most abundant in *D. zingiberensis* and ranged from 2.5 to 4.7 mg/g. Polyunsaturated fatty acids (PUFA) were the second abundant, ranging from 1.1 to 3.6 mg/g, while the content of monounsaturated fatty acids (MUFA) were the lowest, ranging from 0.3 to 1.8 mg/g. There were only two types of fatty acids belonging to PUFA in *D. zingiberensis*. C18:2 was the highest PUFA and ranged from 0.7 to 3.1 mg/g. However, the content of C18:3 was in a range of 0.1–1.4 mg/g. In agreement with PUFA, monounsaturated fatty acids also contained

Table 1
Quantitative determinations of each specialized metabolites.

Compound name	Regression equation	r^2	Compound name	Regression equation	r^2
Diosgenin	$y = 5*10^{-7}x+1.6219$	0.9937	C16:1	$y = 5*10^{-8}x+3.8557$	0.9953
Parvifloside	$y = 8*10^{-8}x$-47.3690	0.9991	C17:0	$y = 6*10^{-8}x+7.8032$	0.9973
Protodeltonin	$y = 8*10^{-8}x$-61.2890	0.9972	C18:0	$y = 4*10^{-8}x+4.6727$	0.9978
Dioscin	$y = 4*10^{-8}x$-49.3620	0.9906	C18:1	$y = 5*10^{-8}x+2.2633$	0.9981
Cholesterol	$y = 8*10^{-8}x+0.6829$	0.9960	C18:2	$y = 5*10^{-8}x+6.7618$	0.9976
Campesterol	$y = 6*10^{-8}x+2.0574$	0.9965	C18:3	$y = 5*10^{-8}x+2.3478$	0.9979
Stigmasterol	$y = 3*10^{-8}x+0.9850$	0.9977	C20:0	$y = 4*10^{-8}x+3.2273$	0.9989
β-sitosterol	$y = 4*10^{-8}x+1.7157$	0.9987	C22:0	$y = 4*10^{-8}x+3.4387$	0.9991
C14:0	$y = 6*10^{-8}x+5.7259$	0.9961	C23:0	$y = 4*10^{-8}x+2.7717$	0.9977
C15:0	$y = 4*10^{-8}x+2.8680$	0.9984	C24:0	$y = 4*10^{-8}x+3.4494$	0.9990
C16:0	$y = 7*10^{-8}x$-4.5798	0.9929	–	–	–

y, the content of specialized metabolites; x, peak area; r^2, regression coefficient.

Table 2
Diosgenin, steroid Saponins, phytosterols and fatty acids composition (mg/g rhizome) in *D. zingiberensis*.

Compound name	Mean	Min	Max	Compound name	Mean	Min	Max
Diosgenin	0.262	0.070	0.606	C17:0	0.145	0.117	0.198
Parvifloside	16.189	4.357	38.427	C18:0	0.768	0.508	1.096
Protodeltonin	19.905	2.608	46.837	C18:1	0.953	0.236	1.652
Dioscin	1.869	0.755	3.611	C18:2	1.843	0.760	3.068
Cholesterol	0.012	0.005	0.030	C18:3	0.528	0.150	1.388
Campesterol	0.030	0.011	0.088	C20:0	0.057	0.049	0.070
Stigmasterol	0.020	0.006	0.063	C22:0	0.077	0.058	0.115
β-sitosterol	0.073	0.031	0.193	C23:0	0.066	0.043	0.082
C14:0	0.123	0.095	0.153	C24:0	0.081	0.057	0.109
C15:0	0.050	0.045	0.065	∑SFA	3.627	2.504	4.669
C16:0	2.259	1.476	3.035	∑MUFA	1.063	0.313	1.774
C16:1	0.110	0.071	0.242	∑PUFA	2.372	1.126	3.594

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

two types of fatty acids: C18:1 and C16:1. The content of C18:1 was between 0.2 and 1.7 mg/g, whereas the content of C16:1 was in the range of 0.07 to 0.25 mg/g. Among SFA, C16:0 was not only the highest saturated fatty acid but also the most abundant fatty acid in *D. zingiberensis*, and its mean value was 2.259 mg/g. C18:0 was the second abundant SFA, it ranged from 0.5 to 1.1 mg/g. The rest of saturated fatty acids including C14:0, C15:0, C20:0, C22:0, C23:0, C24:0 were in the range of 0.04 to 0.16 mg/g. Table 2 showed that *D. zingiberensis* was not abundant in fatty acids, which was in consistence with other *Dioscorea* plants (Padhan et al., 2020).

So far, the utilization of *D. zingiberensis* has focused mainly on four functional chemical compounds: diosgenin, parvifloside, protodeltonin and dioscin. It is necessary to perform a comprehensive evaluation of functional compounds produced by rhizomes from various locations due to climate-driven fluctuations in the production of specialized metabolites (Xue et al., 2021). In this study, the four main metabolites mentioned above were used as explanatory variables and then were used to calculate the comprehensive contents of rhizomes from 20 locations. Results were shown in the Fig. 2 and 20 *D. zingiberensis* accessions could be divided into four groups. A total of four *D. zingiberensis* accessions were categorized into group I whose comprehensive content exceeded 50 mg/g. The rhizome collected from Hubei Shiyan exhibited the highest content of 60.48 mg/g, followed by the rhizomes from Shaanxin Ankang and Sichuan Chengdu (approximately 55 mg/g). The rhizomes from Shaanxi Baoji exhibited the lowest content of 52.76 mg/g in group I. There were four accessions in group II, and the content range of which

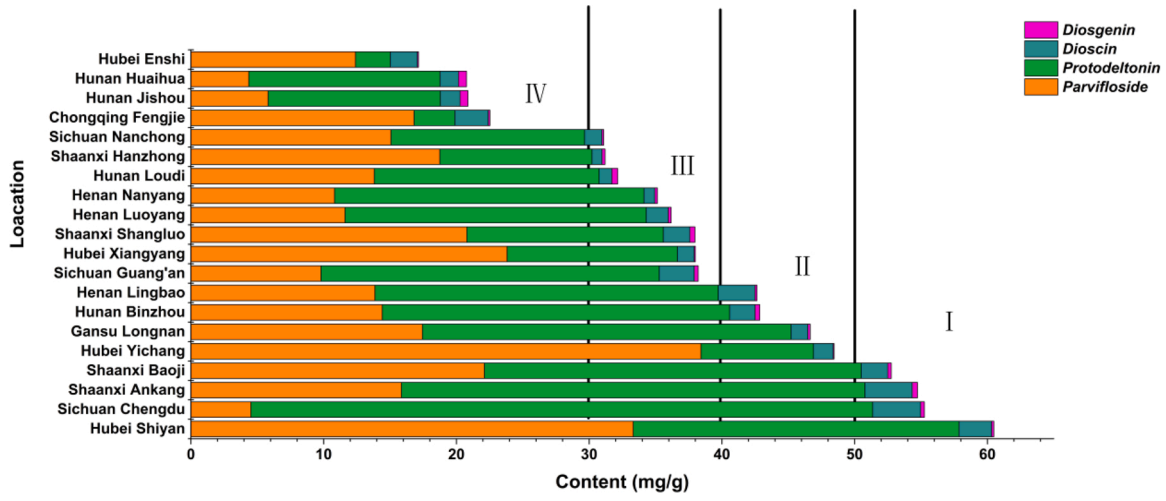

Fig. 2. Comprehensive evaluation of *D. zingiberensis* accessions.

was 40–50 mg/g. The content of rhizomes from Hubei Yichang was the highest in group II, and the rest of accessions were in the range of 42–47 mg/g. Group III comprised accessions from eight locations. The content of rhizomes from Sichuan Guang'an was 38.21 mg/g, while other accessions in group III ranged from 31 to 38 mg/g. A total of four accessions were categorized into group IV, whose comprehensive content was relatively low and in a range of 17–23 mg/g.

Previous studies suggested that *D. zingiberensis* rhizomes collected from Shaanxi Ankang and Hubei Shiyan were of higher quality and produce higher quantities of specialized metabolites compared with those grown in other locations, because *D. zingiberensis* is native to these regions (Zhang et al., 2018b). This was consistent with our results, as we observed large variations in the specialized metabolite contents of *D. zingiberensis* rhizomes collected in different locations. In view of the enormous difference in those specialized metabolites between different locations, the qualities of rhizomes from different areas could be considered distinct. Energy and resource consumption during the production of diosgenin from steroid saponins may vary with different metabolic compositions for different steroid saponins contained various aglycone structures (Jiang et al., 2021; Moses et al., 2014). Moreover, to achieve pure diosgenin extracts, different microbial treatments are required to remove metabolites such as fatty acids and phytosterols; fluctuations in the metabolic components of *D. zingiberensis* rhizomes impede the application of the clean extraction methods in industry (Wei et al., 2013). *D. zingiberensis* steroid saponins and their monomers exhibit various bioactivities, such as decreasing the risk of cardiovascular diseases, inhibiting the proliferation of many types of cancer cells and ameliorating the severity of hyperlipidemia (Tang et al., 2015a, 2015b; Hashidume et al., 2018). As shown in Fig. 2, the contents of steroid saponins differed among rhizomes from diverse habitats, and the unstable content of specialized metabolites may have a negative effect on the pharmacological application of *D. zingiberensis*. To satisfy medicinal requirements, it is necessary to blend rhizomes from different habitats at various proportions and to comprehensively evaluate the pharmacological activities of the blends. Meanwhile, because of regional variations in bioactive compounds, changing habitats of *D. zingiberensis* may enhance the accumulation of steroid saponins. Therefore, the correlation between environmental factors and specialized metabolites should be investigated to enhance the environmental adaptability of *D. zingiberensis*.

3.2. Correlations among fatty acids, phytosterols, diosgenin and steroid saponins

Variable screening using the AIC revealed that eight fatty acids had strong correlations with phytosterols, diosgenin and steroid saponins. As shown in Fig. 3, the majority of fatty acids were negatively associated with specialized metabolites in the MVA pathway. C16:1 and C17:1 were negatively associated with dioscin, protodeltonin and parvifloside but positively associated with diosgenin and other phytosterols. C18:0 was negatively associated with diosgenin and protodeltonin but positively associated with dioscin and phytosterols. C18:1 had positive correlations with cholesterol, diosgenin and protodeltonin but negative correlations with campesterol, stigmasterol, parvifloside and dioscin. C18:2 was negatively correlated with diosgenin and parvifloside but positively correlated with the rest of specialized metabolites. Likewise, C20:0 had negative correlations with parvifloside and protodeltonin but positive correlations with other metabolites. Meanwhile, C22:0 was negatively associated with β-sitosterol, diosgenin and parvifloside but positively associated with dioscin, protodeltonin and other phytosterols. Moreover, C23:0 was negatively associated with dioscin, protodelton and diosgenin but positively associated with parvifloside and phytosterols.

In view of Fig. 3, fatty acids were profoundly associated with the biosynthesis of steroid saponins. In *Nannochloropsis oceanica*, inhibiting the biosynthesis of phytosterols results in the accumulation of fatty acids (Lu et al., 2014). Likewise, in *Schizochytrium*, the fluconazole treatment inhibits phytosterol biosynthesis, leading to an increase of total fatty acids (Li et al., 2019). These studies suggested that there would be a feedback regulation between fatty acids and MVA pathway, which might be one of the reasons why fatty acids had strong correlations with steroid saponins in *D. zingiberensis*. More importantly, the expression of genes related to the degradation of some fatty acids was positively correlated with the accumulation of steroid saponins, indicating that

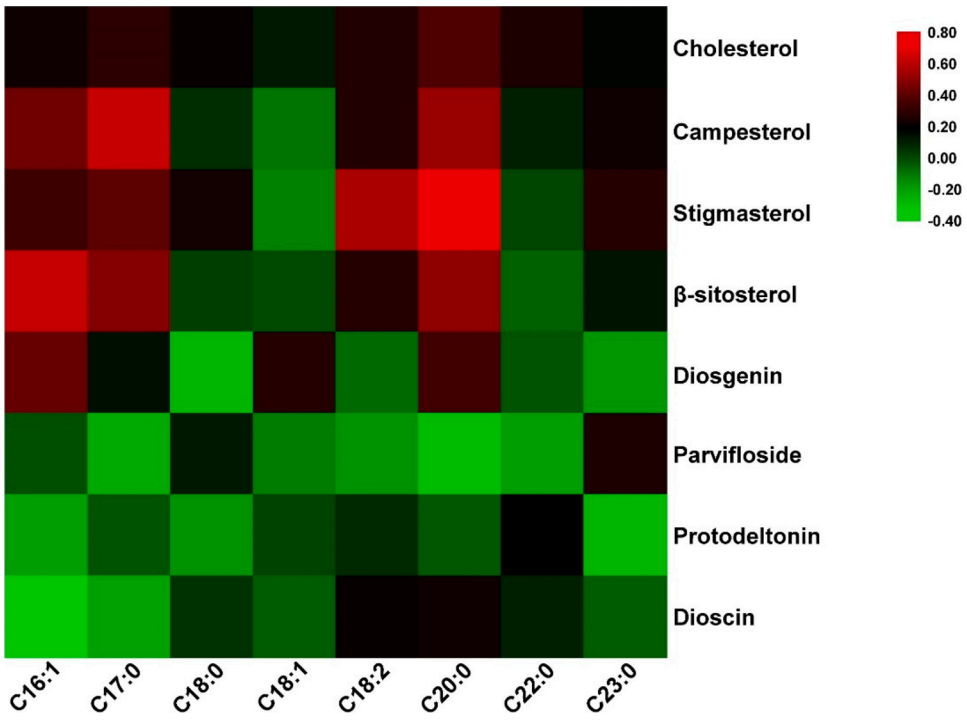

Fig. 3. Spearman analysis of fatty acids, phytosterols, diosgenin and steroid Saponins in *D. zingiberensis*.

fatty acids play a crucial role in the biosynthesis of steroid saponins (Ciura et al., 2017a). Moreover, some fatty acids also exerted positive impacts on the biosynthesis of steroid saponins (Fig. 3). Phytosterols and fatty acids are vital components for cell membranes, so the biosynthesis of both groups of compounds is necessary for normal function of the cell in plant, which might be one of the reasons why some fatty acids were positively correlated with the biosynthesis of steroid saponins (Georgopapadakou et al., 1987). In addition, fatty acids can also synthesize many signaling molecules, which have indispensable impacts on the accumulation of bioactive compounds in plants (Shi et al., 2019). Many phytohormones, such as jasmonic acid and methyl dihydrojasmonate, are derivatives of fatty acids. Meanwhile, it is reported that such phytohormones exerted a positive effect on enhancing saponins (Li et al., 2017; León and Sánchez-Serrano, 1999; Song et al., 2019). Thus, there would be a considerably complex relationship between fatty acids and steroid saponins in *D. zingiberensis*. The advancement of high-throughput sequencing and bioinformatics analysis technologies have enabled analysis of molecular mechanism underpinning the relationships among various specialized metabolites (Won et al., 2010). Further studies should be conducted to explore the feedback and correlation between fatty acids and steroid saponins for improving the production of steroid saponins.

3.3. Environmental factors affecting variability in fatty acids and steroid saponins biosynthesis

After the DCA was performed, the maximum LGA values of steroid saponins biosynthesis and fatty acids were both below two. According to previous studies, redundancy analysis can be deemed fit for subsequent analyses if the LGA values are below three (Hill and Gauch, 1980). In RDA algorithm, the constrained ability can be limited by including a large number of environmental variables. Therefore, the environmental factors were screened using the AIC algorithm with stepwise regression, and a total of nine environmental variables were applied as explanatory variables in our RDA model. The environmental variables that affected steroid saponins were the same as those that affected fatty acids (Table 3). The results of the RDA analysis involving all screened environmental variables were summarized in Table 4. These nine screened environmental variables constrained 89.64 % of the variance in fatty acids but only 64.67 % of the variance in steroid saponins biosynthesis (including phytosterols, diosgenin and steroid saponins). For steroid saponins biosynthesis, the first two axes of the RDA model constrained 36.05 % and 15.23 % of the total variance, suggesting that this RDA model represented the total constrained proportion. Likewise, the cumulative constrained proportion of the first two axes in the RDA model of fatty acids was 84.75 %, which revealed the main correlation between fatty acids and environment variables. About 43 % of the variance of the phytosterols, diosgenin and steroid saponins could be constrained by climatic variables, while only 15 % was constrained by geographical variables. In contrast, approximately 32 % of the variance of fatty acids could be constrained by geographical variables, however, only 7.62 % was constrained by climatic variables. With respect to comprehensive influences between geographical and climatic variables, their constrained percentage was 6.19 % in steroid saponins biosynthesis but 50.61 % in fatty acids. In regard to specialized metabolites in steroid saponins biosynthesis, the first RDA axis was positively associated with annual maximum precipitation and annual minimum precipitation, but negatively associated with latitude. The second RDA axis was negatively associated with elevation, annual maximum pressure and mean annual temperature, but positively associated with sunshine duration and

Table 4
Results of redundancy analysis of steroid saponins biosynthesis and fatty acids in *D. zingiberensis*.

Statistics	Steroid saponins biosynthesis		Fatty acids	
	RDA1	RDA2	RDA1	RDA2
Eigenvalue	116.57	49.26	30.04	0.25
Proportion explained	0.3605	0.1523	0.8405	0.0070
Cumulative proportion	0.3605	0.5128	0.8405	0.8475
Total inertia (variance explained %)	209.12(64.67 %)		35.74(89.64 %)	
Explained variance of A	15.17 %		31.86 %	
Explained variance of B	43.31 %		7.62 %	
Explained variance of C	6.19 %		50.61 %	
Latitude [a]	−0.9689	−0.2475	0.8295	−0.5585
Elevation [a]	0.2732	−0.9620	0.2385	−0.9711
Sunshine duration [b]	−0.3613	0.9324	0.9669	−0.2550
Annual maximum pressure [b]	0.2575	−0.9663	−0.8538	0.5206
Mean annual temperature [b]	0.3653	−0.9309	0.8623	−0.5064
Annual temperature difference [b]	−0.1387	0.9903	0.8623	−0.5064
Relative humidity [b]	0.8294	−0.5587	−0.9054	0.4246
Annual maximum precipitation [b]	0.9168	−0.3994	−0.9970	0.0773
Annual minimum precipitation [b]	0.9380	−0.3467	−0.9919	−0.1271

A and [a], geographical variables; B and [b], climatic variables; C, geographical variables × climatic variables.

Table 3
Selected environmental factors at each sample locations.

Location	Latitude (°N)	Elevation (m)	SD (h)	AAP (hPa)	MAT (°C)	ATD (°C)	RH (%)	MAP (mm)	MIP (mm)
Hubei Enshi	30°32′15″	744	1300.3	969.3	15.4	21.2	80	2131.5	943.6
Hubei Xiangyang	31°25′23″	922	1509.5	1028.8	15.6	24.7	76	1297.5	662.9
Hubei Yichang	30°56′26″	507	1271.1	1029.3	17.1	22.7	75	1807.5	768.3
Hubei Shiyan	32°59′37″	633	1944.4	1015.1	15.3	24.4	74	1070.9	566.4
Sichuan Chengdu	30°44′08″	572	1239.1	989.1	16.8	20.1	79	1217.1	405.9
Sichuan Guang'an	30°37′15″	313	1139.4	994.3	16.9	20.6	84	1307.7	657.2
Sichuan Nanchong	30°58′06″	407	1369.1	996.3	16.8	20.7	83	1442.6	459.8
Hunan Jishou	28°19′36″	318	1429.6	1017.8	16.9	22.2	79	1774.5	978.8
Hunan Binzhou	25°31′47″	269	1432.5	1012.8	18.5	21.2	79	1897.3	1002.0
Hunan Huaihua	27°10′24″	213	1415.9	1021.8	17.2	22.4	81	1695.2	863.7
Hunan Loudi	27°56′42″	457	1417.4	1017.5	17.1	23.2	78	2009.9	1071.9
Shaanxi Shangluo	33°27′29″	585	2056.7	976.0	13.7	23.9	67	1035.7	483.1
Shaanxi Ankang	32°53′39″	574	2057.9	990.2	15.2	22.8	70	1020.0	467.3
Shaanxi Baoji	34°33′44″	906	1913.9	975.0	13.5	25.4	66	951.0	378.3
Shaanxi Hanzhong	33°08′24″	611	1752.2	982.9	14.7	22.8	79	1462.8	519.1
Henan Luoyang	34°32′37″	300	2248.3	1024.3	14.8	26.9	67	931.8	342.6
Henan Nanyang	33°06′54″	193	2120.9	1027.0	15.1	25.3	74	1079.0	468.3
Henan Lingbao	34°31′15″	591	2254.7	990.1	13.5	27.4	66	857.0	318.7
Chongqing Fengjie	30°46′04″	955	1639.8	968.2	16.3	21.2	72	1636.3	759.9
Gansu Longnan	33°54′44″	947	1726.5	934.6	12.1	23.4	75	967.1	458.8

SD, sunshine duration; AAP, annual maximum pressure; MAT, mean annual temperature; ATD, annual temperature difference; RH, relative humidity; MAP, annual maximum precipitation; MIP, annual minimum precipitation.

annual temperature difference. However, for fatty acids, the second axis was negatively associated with elevation. The first axis was positively associated with sunshine duration, but negatively associated with relative humidity, annual maximum precipitation and annual minimum precipitation.

Previous researches reported that the composition of specialized metabolites could be influenced by abiotic factors, such as soil types and properties (Zhang et al., 2021). But geographical and climatic variables profoundly affect soil conditions, hence these two factors should be primarily considered in the investigations into the correlation between bioactive compounds and environment (Zhong et al., 2018; Wang et al., 2017; Kilpeläinen et al., 2020). According to previous researches, the content of phytosterols in plants could be significantly influenced by the fluctuation of environmental variables (Blits and Gallagher, 1990). Similarly, some studies illustrated that the fatty acids content was associated with climatic variables (Ma et al., 2020; Sun et al., 2017). In this study, the variance constrained by environmental variables was lower for steroid saponins biosynthesis than for fatty acids, which suggested that the biosynthesis of phytosterols, diosgenin and steroid saponins might be more likely to be influenced by genetic interactions and polygene effects. Phytosterols, especially cholesterol, had a minimum content but exerted crucial effects on diosgenin biosynthesis. Cholesterol is a biosynthetic precursor to diosgenin (Cheng et al., 2021). However, some studies also suggested that high cholesterol could negatively influence plant growth (Diener et al., 2000). Thus, cholesterol likely participates in diosgenin biosynthesis at a low content but is influenced by other genetic effects via unknown mechanisms.

3.4. Environmental factors in relation to the variation of steroid saponin biosynthesis and fatty acids

Following screening and evaluation of environmental variables, we applied envfit and VIF programs to check for co-linearity among the variables. The r^2 value represents the contribution of each environmental variable to the RDA model (Table 5). Latitude contributed strongly to the RDA model of steroid saponins, while mean annual temperature and sunshine duration contributed significantly to the RDA model of fatty acids. In the envif analysis, three environmental variables contributed to steroid saponins biosynthesis (latitude, mean annual temperature and annual maximum precipitation), while five environmental contributed to fatty acids (elevation, sunshine duration, mean annual temperature, annual temperature difference and relative humidity). In the meantime, VIF was applied to examine the co-linearity among the environmental variables. If the VIF value exceeds ten, there is multi-collinearity among environmental variables, which would affect the accuracy of the built model (Kelava et al., 2008; Hou et al., 2019). As shown in Table 5, the VIF values of the screened variables ranged from two to nine, thus supporting the RDA model. These variables were then applied to simplify the RDA model.

Table 5
Results of key environmental factors in the redundancy analysis model.

Environmental factors	Steroid saponins biosynthesis			Fatty acids		
	r^2	$Pr(>r)$	VIF	r^2	$Pr(>r)$	VIF
Latitude	0.6070	0.001	2.8248	0.1781	0.184	–
Elevation	0.0385	0.700	–	0.3331	0.042	2.6247
Sunshine duration	0.1147	0.388	–	0.4734	0.005	7.3530
Annual maximum pressure	0.0792	0.514	–	0.2357	0.115	–
Mean annual temperature	0.3586	0.028	8.9727	0.5062	0.001	3.8993
Annual temperature difference	0.0187	0.852	–	0.3913	0.018	3.5418
Relative humidity	0.0402	0.712	–	0.3800	0.020	5.3999
Annual maximum precipitation	0.3214	0.049	6.4128	0.2171	0.114	–
Annual minimum precipitation	0.2532	0.088	–	0.1713	0.193	–

As shown in Fig. 4, elevation was negatively associated with C16:1 and C17:0 but positively associated with those of the other 11 fatty acids. Sunshine duration had positive correlations with C14:0, C16:0, C18:1 and C18:2 but negative correlations with the other nine fatty acids. Mean annual temperature was positively correlated with C16:0, C17:0, C18:3, C20:0, C22:0 and C23:0 but negatively correlated with the rest fatty acids. Annual temperature difference had positive associations with C14:0, C16:0, C18:1, C18:2 and C18:3 but negative associations with other eight fatty acids. Relative humidity was negatively correlated with C14:0, C18:1, C18:2 and C18:3 but was positively corelated with the other ten fatty acids. In terms of steroid saponins biosynthesis, latitude was positively associated with three steroid saponins (parvifloside, protodeltonin and dioscin) but negatively associated with diosgenin and four phytosterols (cholesterol, campesterol, stigmasterol and β-sitosterol). Mean annual temperature had positive correlations with campesterol, β-sitosterol and diosgenin but negative correlations with cholesterol and three steroid saponins. Notably, stigmasterol exhibited no correlation with the mean annual temperature. Annual maximum precipitation was positively correlated with three steroid saponins but negatively correlated with diosgenin and four phytosterols.

In our study, the final simplified RDA model contained five environmental variables affecting fatty acids but only three environmental variables affecting steroid saponins biosynthesis, suggesting that fatty acids are more susceptible to the environment. Some investigations indicated that *D. zingiberensis* was chiefly distributed in the south of the Qinling Mountains–Huaihe River line, and its distribution is strongly related to temperature and moisture, which was consistent with our results (Zhang et al., 2018b). Previous researches illustrated that medicinal plants were often vulnerable to abiotic factors, especially temperature, because they play key roles in the regulation of cellular functions, membrane structures and activities of key enzymes involved in the biosynthesis of specialized metabolites (Kumar et al., 2020). In *Salvia miltiorrhiza*, temperature affects the accumulation of tanshinones, indicating that mean annual temperature can have important effects on the biosynthesis of plant's bioactive compounds (Zhao et al., 2020). Furthermore, phytosterols are major components of membranes and have sharp responses to environmental stresses (Kopischke et al., 2013). In poplars, leaves accumulate a large amount of phytosterols in response to heat (Behnke et al., 2013). Likewise, the phytosterols of *Corchorus depresus* were higher in winter than in summer, which is suggestive of a relationship between phytosterols and temperature and may explain why mean annual temperature was strongly related to steroid saponins biosynthesis (Mathur, 2012). Moreover, precipitation can represent an abiotic stress to plants that triggers phytosterol biosynthesis, corresponding to our results our results (Zhang et al., 2020). However, the steroid saponins were negatively correlated with maximum annual precipitation. The saponins in *Panax notoginseng* show a decreasing trend with increasing soil moisture, which agrees with our results (Zheng et al., 2021). In addition, high osmotic pressure can increase water absorption from the soil, although too much water in plant tissues can increase the accumulation of toxic components, leading to growth inhibition and even death (Komatsu et al., 2015). Steroid saponins are water soluble and can increase osmotic pressure; maximum annual precipitation may have a negative correlation with steroid saponins because the plants are less able to accumulate water-soluble metabolites under high moisture conditions.

Temperature, relative humidity and precipitation all exert important influences on the fatty acids of *Sapindus* and *Zanthoxylum* plants, which is consistent with our study (Sun et al., 2017; Hou et al., 2019). Latitude played a key role in the biosynthesis of steroidal saponins. Nevertheless, sunshine duration was nearly as influential as mean annual temperature on fatty acids (Table 5). Approximately 90 % of variance in fatty acids could be constrained by environmental variables (Table 4), suggesting

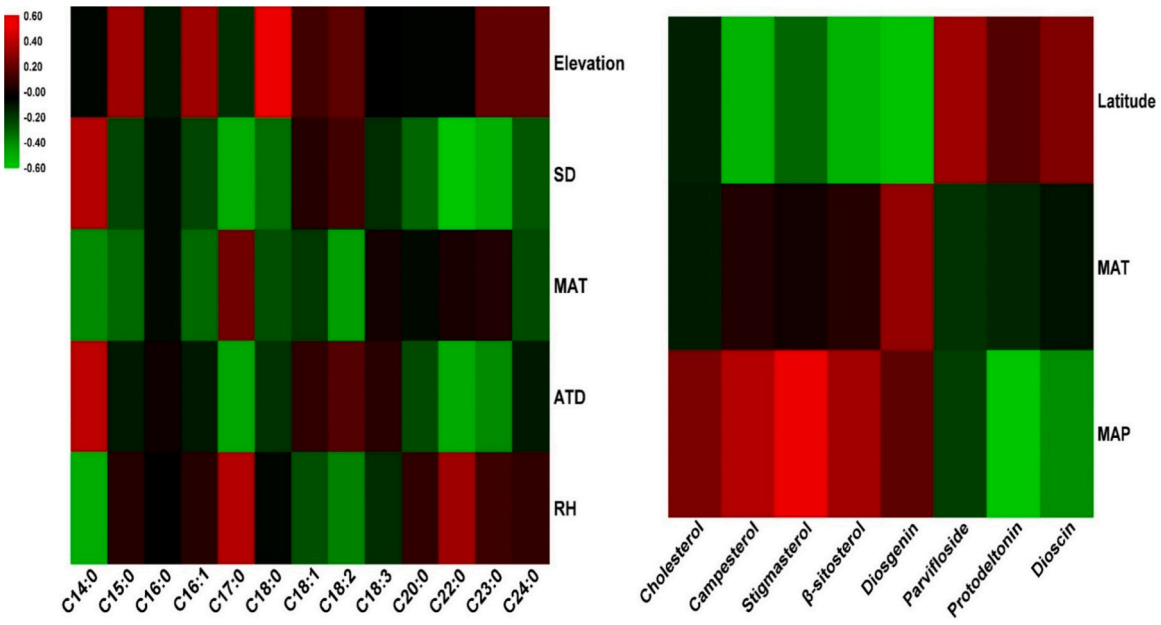

Fig. 4. The Correlation between Environmental Factors and Specialized Metabolites in *D. zingiberensis*.
SD, sunshine duration; MAT, mean annual temperature; ATD, annual temperature difference; RH, relative humidity; MAP, annual maximum precipitation.

that fatty acids could be largely influenced by climatic and geographical factors in *D. zingiberensis*. Sunshine duration and temperature were reported that exerted important impacts on the expression of key genes in the biosynthesis of fatty acids, and these two climatic factors also exerted considerable influences on the fatty acids of soybean, which was consistent with our study (Song et al., 2016).

In addition to geographical and climatic factors, other variables, such as microorganism and genetic factor, may also affect the composition of bioactive compounds, hence further studies should be conducted to investigate the correlation between specialized metabolites and more variables. Furthermore, our study showed that there were significant differences between the influential environmental factors of fatty acids and steroid saponins biosynthesis. Most fatty acids were strongly correlated with metabolites in the biosynthesis of steroid saponins, while fatty acids in *D. zingiberensis* tended to be influenced by environment. Hence, changing location of *D. zingiberensis* cultivation may enhance the accumulation of steroid saponins. Such results would provide valuable information about *D. zingiberensis* and could aid the selection of promising lines able to produce useful steroid saponins.

4. Conclusion

We found that the contents of steroid saponins, diosgenin, phytosterols and fatty acids in *D. zingiberensis* rhizomes varied by region. The rhizomes of the 20 collected *D. zingiberensis* accessions could be categorized into four groups based on diosgenin and steroid saponins. The determined 13 fatty acids exerted a profound effect on phytosterols, diosgenin and steroid saponins. The RDA analysis revealed that environmental factors constrained 64.67 % of the total variance for steroid saponin biosynthesis (phytosterols, diosgenin and steroid saponins) and 89.64 % for fatty acids. Latitude, mean annual temperature and annual maximum precipitation were profoundly associated with steroid saponin biosynthesis. Sunshine duration, mean annual temperature, annual temperature difference and relative humidity had strong correlations with fatty acids. The results of this study provide useful information for selecting breeding lines and optimal cultivation.

Author statement

The work described in manuscript has not been submitted elsewhere for publication in whole. The relevant contents and data of the paper meet the requirements of integrity. All authors have contributed and read the manuscript, and agree to its submission.

Declaration of Competing Interest

The authors report no declarations of interest.

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