

Leaf flavonoids in Chinese sea-buckthorn (*Hippophae rhamnoides* subsp. *sinensis* Rousi) and their response to environmental gradients across northern China

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ABSTRACT: Changes to leaf flavonoids along environmental gradients are often related to the self-protection of plants, reflecting the latter's adaptability to their external environment. Yet few studies have investigated the relationship between flavonoid and environmental factors. Here, we determined the concentrations of quercetin (QUE), isorhamnetin (ISO), and kaempferol (KAE) in leaves of Chinese sea-buckthorn at 37 sites across northern China, and explored the relation of these flavonoids to the environment (using Generalized Additive Modeling, collinearity of hierarchical partitioning, and structural equation modeling). The QUE component exhibited significant changes along the environmental gradients, while ISO changed significantly along the gradients of mean annual temperature (MAT), altitude (ALT), soil available nitrogen (AN), and the ratio of soil available nitrogen to soil available phosphorus (AN/AP) ($p < 0.05$). However, KAE changed significantly along fewer gradients, only those of mean annual precipitation (MAP), AP and soil available potassium (AK) ($p < 0.05$). After removing environmental collinearity, for the three flavonoids' total content the order of environmental factors' influence was ALT > MAT > AN/AP > AK > AN > MAP > AP. Considered separately, for QUE the order was ALT > MAT > AN/AP > AN > MAP; that for ISO was MAT > ALT > AN/AP > AN > MAP; and that for KAE is AK > AP. Chinese sea-buckthorn adapts to harsh habitats by increasing QUE and ISO, with KAE responding to specific nutrients, offering a theoretical basis for improved cultivation and economic value.

KEYWORDS: flavonoids, environmental gradients, ecological adaptation, Chinese sea-buckthorn

INTRODUCTION

Chinese sea-buckthorn is drought-, cold-, saline-alkali-resistant woody plant species that has ecological functions in barren sites, such as wind and sand erosion control as well as water conservation [1]. This shrub plant is also rich in a variety of nutrients and bioactive substances, with the ascorbic acid content of its fresh fruit being 2–8 times that of kiwi, earning it the moniker 'King of Vitamin C' [2], while the oil content of its dried fruit is about 25%, hence the latter's 'green gold' reputation [3]. Chinese sea-buckthorn has high edible and medicinal value, placing it among the top ten raw materials for health products according to the World Health Organization, being widely used in food, cosmetics, and medicine [4]. Chinese sea-buckthorn branches and leaves are rich in flavonoids, amino acids, tannins, ursolic acid, and mineral elements, all of which are excellent raw materials for the production of animal feed [5]. In sum, Chinese sea-buckthorn combines a wealth of ecological functions and economic values.

Plant secondary metabolism is derived from primary metabolic pathways and synthesizes small-molecule organic compounds not essential for plant

growth and development which are called secondary metabolites [6]. These mainly include flavonoids, phenols, and lignans [7]. Research has shown that flavonoids have pronounced anti-tumor, anti-inflammatory, anti-aging, and anti-myocardial ischemia properties as well beneficial effects for heart health [8]. The total flavonoids of Chinese sea-buckthorn mostly consist of dozens of flavonoid glycosides, with quercetin, kaempferol, and isorhamnetin as the main aglycones [9].

Habitat is the ecological environment that a species or a group of species depends upon, which may be characterized by such abiotic factors as altitude, temperature, and precipitation [10, 11]. Secondary substances have evolved via long-term interactions between plants and their environment, and these products serve as the material basis for environmental adaptation, which in turn generates a unique diversity of chemical [12]. Flavonoids play a very important role in the cold-, salt-, and drought-resistance/tolerance traits of Chinese sea-buckthorn, which ought to have correspondence and relevance for its habitat range [13]. Excessive solar radiation can have substantial effects on flavonoids in plants. For example, Tattini et al [14] found significant differences in the phenylpropanoid

content of *Phillyrea latifolia* between its heliophilous and shade leaves, with the former having a higher flavonoid content. Excessive temperature can inhibit flavonoid biosynthesis, leading to the degradation of flavonoid compounds [15]. Although long-term drought stress can decrease the content of flavonoids in plants, an early but brief onset of drought stress can increase that content [16].

Studying relationship between flavonoids in plants and their environment is beneficial to the development and efficient use of wild. To date, research on the secondary products of Chinese sea-buckthorn, such as its flavonoids, has mainly aimed at improving their extraction process. Regarding Chinese sea-buckthorn's adaption to the environment, most studies have focused chiefly on its morphological characteristics and photosynthesis, leaving few papers addressing the response of its flavonoid to changes in the environment. In this study, the leaves of Chinese sea-buckthorn from 37 sampling sites (populations) across northern China were used as research materials. Using reverse phase high-performance liquid chromatography (RP-HPLC) detection technology, the leaf contents of three main flavonoids (isorhamnetin, quercetin, and kaempferol) were determined. We had three objective questions. (1) To examine the correlation between leaf flavonoids' content and the environment (climate and soil factors) and how these secondary products vary along environmental gradients. (2) To determine whether the content of flavonoids in Chinese sea-buckthorn changes consistently with environmental gradients. (3) To analyze the percentage contribution of single environmental factors to the changed contents of isorhamnetin, quercetin, and kaempferol. An overarching goal was to reveal the mechanism underpinning Chinese sea-buckthorn leaves' adaption along altitudes, and to provide basic data for the further development and utilization of this shrub species in an applied context.

MATERIALS AND METHODS

Study sites

This research was conducted at 37 sites across northern China, spanning a wide geographical range in terms of latitude (34°–40°N), longitude (100°–114°E), and altitude (1024–3209 m a.s.l.) (Fig. 1).

Sample collection

Leaf samples of Chinese sea-buckthorn were collected from 37 natural populations during the growing season (August) in 2019. Only 20 fully-expanded and healthy mature leaves were collected from 30 independent plants at each sampling site.

Determination of flavonoids

The collected fresh Chinese sea-buckthorn leaves were first dried in an oven (NBS Test Oven NBS-1010, China) at 60 °C for 48 h. Next, the dried leaves were

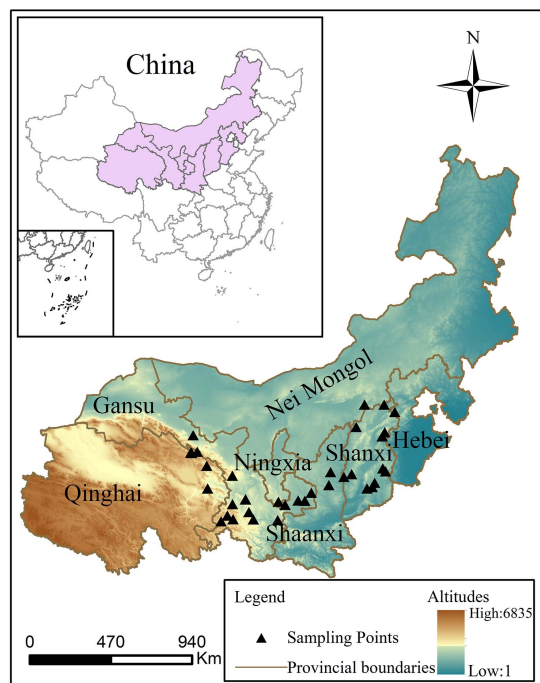


Fig. 1 Map of northern China showing the locations of the 37 sampling sites (plots).

ground into powder using a mortar and pestle. Each powdered sample of leaves was mixed with ethanol, and then sonicated for 30 min to extract the flavonoids. The extracted solution was filtered and concentrated under reduced pressure. The concentrated extract was diluted with methanol, and filtered again. Then the filtered solution was injected into a HPLC system (Agilent 1100 USA) equipped with a UV detector (Thermo Scientific UV-Vis Detector, USA). The HPLC column used was a C18 column with a particle size of 5 μm (Agilent 1100). The mobile phase consisted of a mixture of acetonitrile and water, with a gradient program applied to separate the flavonoids. The flow rate was set to 1.0 ml/min, with the maintained column temperature at 25 °C. The UV detector was set to a wavelength of 254 nm, at which the absorbance of flavonoids measured. The peak area or peak height was used to calculate the concentration of each flavonoid [17]. 10 mg quercetin, 2.5 mg kaempferin, 10 mg isorhamnetin and 1 mg myricetin were weighed in a beaker, dissolved with methanol and transferred to a 50 ml volumetric bottle at a constant volume to obtain 0.2, 0.05, 0.2, 0.02 mg/ml reference solution, respectively. Then the concentration gradient dilution is performed. The standard curve was drawn with the injection concentration (mg/ml) as the horizontal coordinate and the peak area as the vertical coordinate. The concentration of each flavonoid was calculated according to a standard curve equation that was obtained

from the calibration curve. The percentage content of each flavonoid in the Chinese sea-buckthorn leaves was then expressed on a per sample weight basis.

Determination of soil chemical properties

From each sampling site's plot, 0–60 cm soil samples were collected via the five-point method. These collected soil samples were taken to the laboratory to remove plant residues and other debris, and then dried and crushed. After natural air-drying, each soil sample was passed through a 1-mm screen; then about 50 g was taken per sample for further grinding, and these subsamples packed into sealed bags for later use. Soil available nitrogen (AN) concentrations were measured by the alkaline hydrolysis diffusion method [18]. For soil available phosphorus (AP) concentrations, 2.5 g of air-dried soil was extracted with 50 ml of a 0.5 mol/l NaHCO₃ solution. Soil available potassium (AK) in soil was determined by atomic absorption spectrophotometry (visible spectrophotometer 721G, China).

Climate data

All the climate data came from the World Climate website (<http://worldclim.org>). That online dataset provides almost 50 years of global monthly average weather data at a spatial resolution of 1 km × 1 km. Using that downloaded monthly data, we calculated the average annual precipitation (MAP) and mean annual temperature (MAT) at each sampling site.

Data analysis

The MS Excel 2019 program was used for preliminary data sorting. Origin 2019b software was used to obtain statistics for the flavonoid content and to analyze trends in changes to each flavonoid's content. A Spearman correlation analysis was applied to the flavonoid to examine their relationships. The 'mgcv' package in R software (Version 4.1.0, R Core Team) was used for the Generalized Additive Modeling (GAM) fitting of changes in the flavonoid along seven environmental gradients; these fits would better convey the real change in the data since it is not constrained by an a priori assumed form for the relationship [19]. Finally, a structural equation model (SEM) was fitted by implementing the 'lavaan' package in R.

RESULTS

Content of flavonoids and their relationship in Chinese sea-buckthorn

The mean values of the three flavonoids were ranked as follows: QUE (1.37 mg/g) > ISO (1.27 mg/g) > KAE (1.09 mg/g) (Fig. 2A). The distribution range of QUE was 0.73–2.47 mg/g (Fig. 2B), that of KAE was 0.47–2.41 mg/g (Fig. 2C), and that of ISO was 0.49–3.65 mg/g (Fig. 2D).

Among them, the coefficient of variation (CV%) = (standard deviation/mean) × 100 of QUE exceeded that of the other two flavonoids, and its boxplot's lower quartile extended further. These differences indicated that QUE's leaf content changed greatly. Yet the skewness for all three flavonoids' content was greater than zero, showing an obvious right tail. Among the three flavonoids (Fig. 3), only QUE and ISO were significantly positively correlated ($p < 0.05$).

Response of flavonoid contents to environmental factor gradients

The QUE concentration significantly decreased in a linear way as the MAT and MAP increased (Fig. 4A,B). However, at sites where AN > 0.2, the values of QUE changed little (Fig. 4C). With an increasing AN/AP, QUE showed a significant linear trend of increasing (Fig. 4E). Further, QUE also displayed significant non-linear relationships with the gradients of other environmental factors: QUE initially decreased and then increased as AP or AK increased (Fig. 4D,F), but that rate of decrease surpassed the corresponding rate of increase. When AN < 0.2, QUE increased with an increasing AN. However, QUE did not change significantly across latitude ($p > 0.05$; Fig. 4G).

For ISO, it decreased significantly along the increasing MAT gradient (Fig. 5A), whereas it significantly increased with rising ALT (Fig. 5G). For AN < 0.2, ISO increased in tandem with AN, but at values of AN > 0.2, the concentration of ISO decreased slightly (Fig. 5C). In response to AN/AP gradient, leaf concentration of ISO exhibited 'bimodal' change (Fig. 5E). ISO showed no significant trends along the other three environmental gradients (Fig. 5B,D,F).

For KAE, its concentration increased significantly and linearly with MAP, AP, and AK (Fig. 6B,D,F). Yet KAE did not show any significant trends along the other four environmental gradients (Fig. 6A,C,E,G).

Effects of single environmental factors on the three leaf flavonoids

The influence of various environmental factors on the flavonoid content was meticulously investigated using the hierarchical partitioning method. In terms of their pooled concentrations, the environmental factors had the following descending order of impact: ALT > MAT > AN/AP > AK > AN > MAP > AP (Fig. 7A). For QUE, the influence of those environmental factors took this ranking: ALT > MAT > AN/AP > AN > MAP (Fig. 7B). In the case of ISO, the rank order differed slightly: MAT > ALT > AN/AP > AN > MAP (Fig. 7C). In stark contrast, for KAE, only AK stood out strongly as the most influential factor, followed by AP (Fig. 7D).

After removing collinearity among environmental factors, the adaptive model for Chinese sea-buckthorn was optimized to reveal the paths regulating its altitude–climate–soil–metabolites interactions,

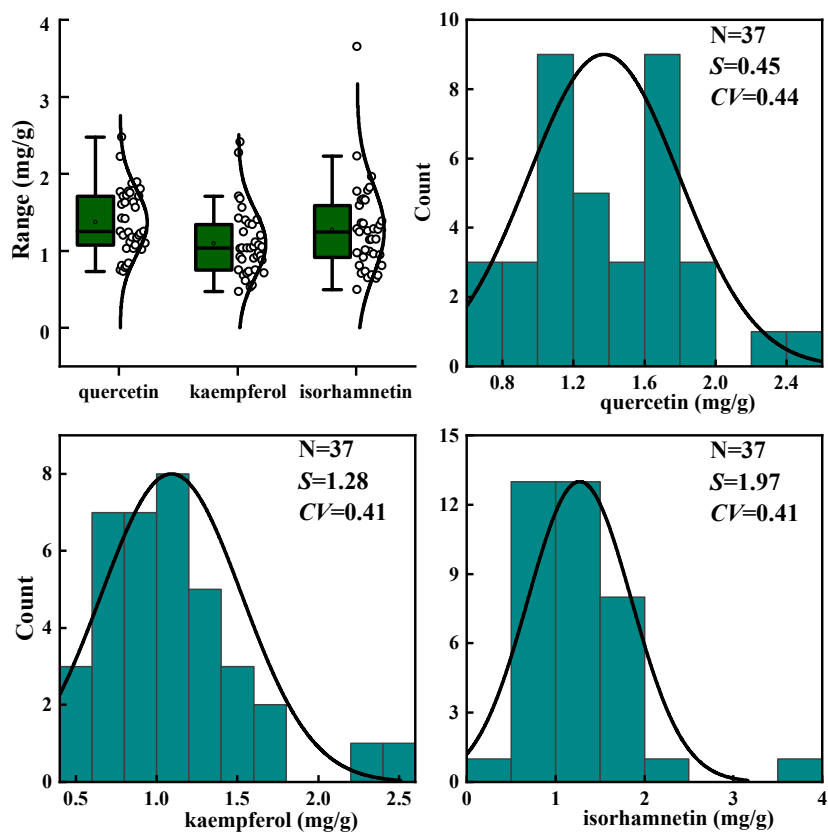


Fig. 2 Distribution of three flavonoid in leaves of Chinese sea-buckthorn. (A) Boxplots and actual values. Data for (B) quercetin, (C) kaempferol, and (D) isorhamnetin. N is the number of sampled plots; S is skewness; CV is the coefficient of variation.

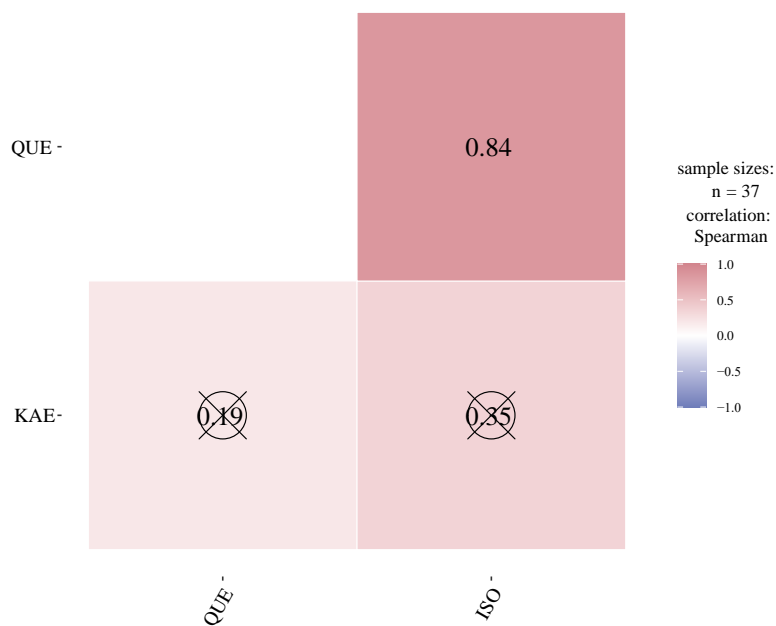


Fig. 3 Relationships between the three flavonoids. The “⊗” denotes correlations were non-significant

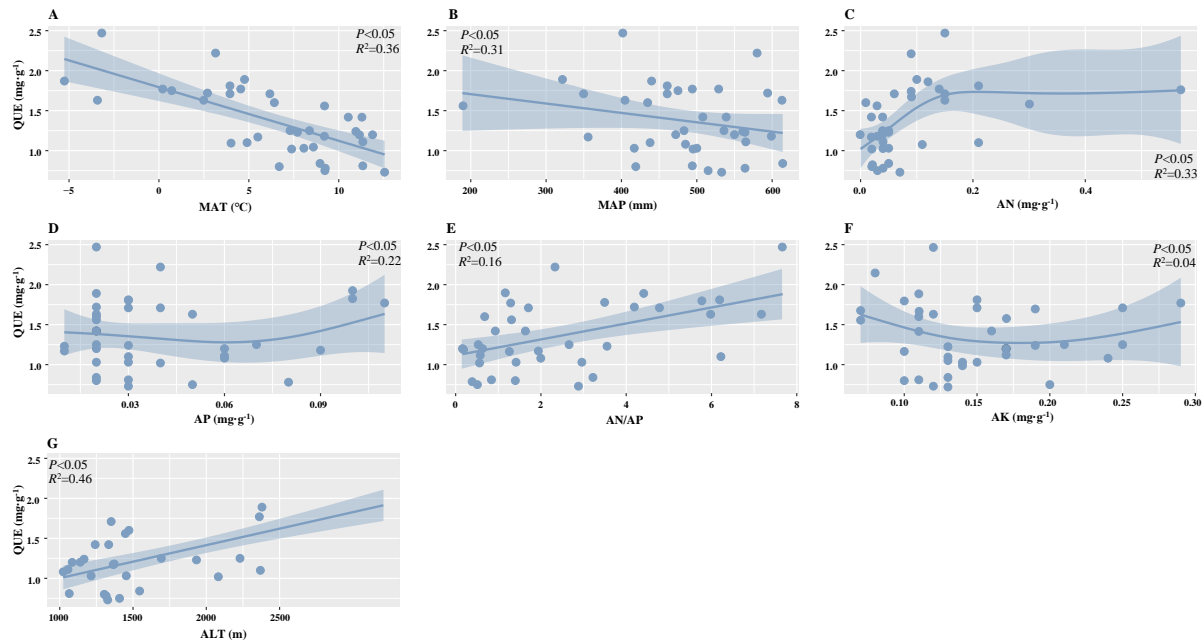


Fig. 4 Variation in QUE along seven environmental gradients. Notes: mean annual temperature (MAT); mean annual precipitation (MAP); soil available nitrogen (AN); soil available phosphorus (AP); the ratio of soil available nitrogen and soil available phosphorus (AN/AP); soil available potassium (AK); altitude (ALT). Abbreviations are the same for Figs. 5–7.

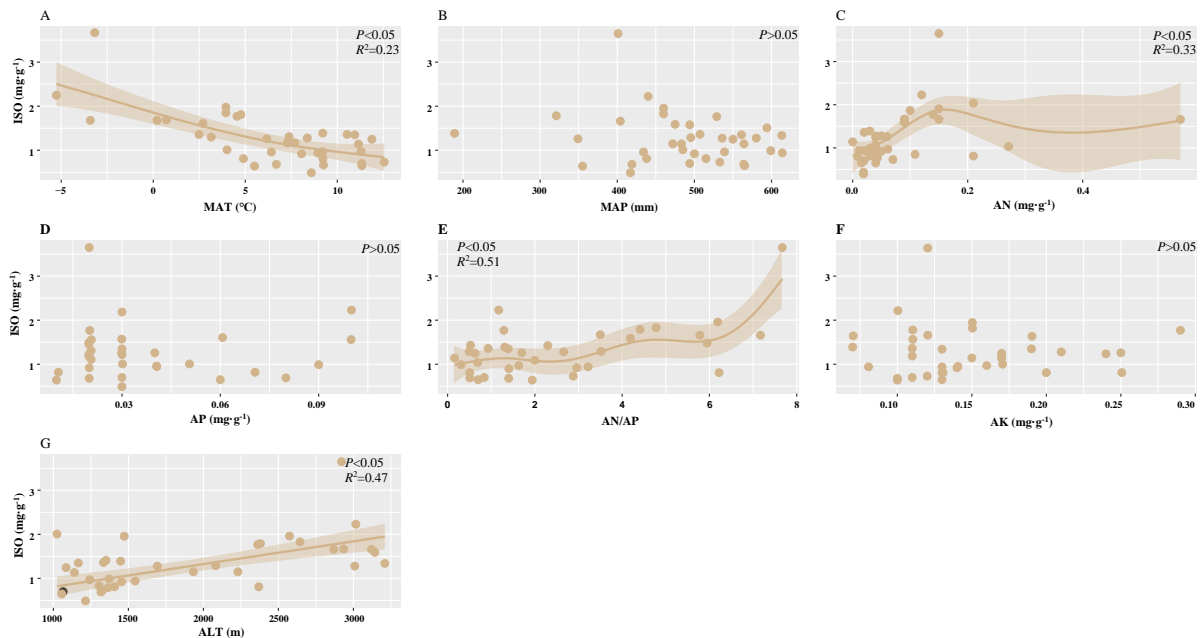


Fig. 5 Variation in ISO along the seven environmental gradients.

through an SEM construction (Fig. 7E,F). These two models had DF (degree of freedom) values of 3.41 and 3.52 (< 5.0), GFI (goodness of fit index) values of 1.2 and RMSEA (root mean square error of approximation) values of 0.05 (< 0.08), all of which indicated a

basically sound model fit that could meet our research needs. The effect on QUE or ISO from ALT arose mainly by it affecting the average annual temperature, which in turn affects soil available nitrogen. Notably, this regulation path influenced QUE more than ISO.

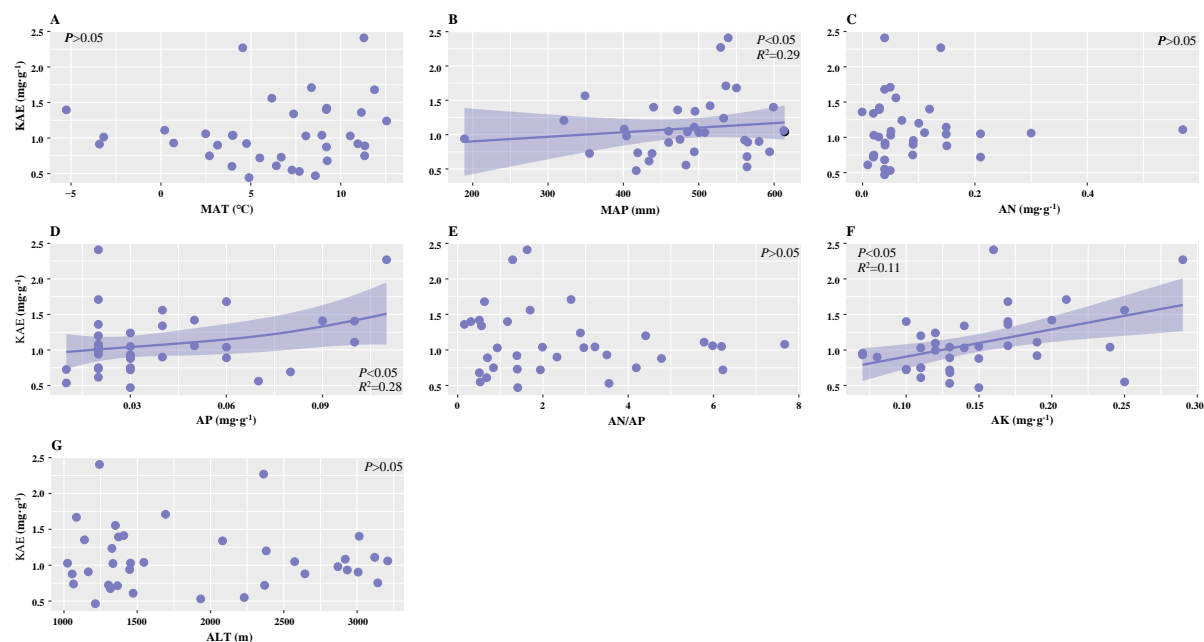


Fig. 6 Variation in KAE along the seven environmental gradients.

DISCUSSION

Content characteristics of the three flavonoids

Flavonoid compounds are known to play an important role in anti-cardio-cerebrovascular, anti-tumor, free radical scavenging, and antioxidant functions. There are few studies, however, of the different flavonoid present in leaves of sea-buckthorn plant species. In our study, the collected Chinese sea-buckthorn leaves all contained the three flavonoids of interest, for which QUE content is the highest, followed by ISO, with KAE content being the lowest. This result differed from Zhang's [20] determination of flavonoids' content of sea-buckthorn leaves to be ISO > QUE > KAE, perhaps due to differences in the studied species, sampling location, and sampling time. QUE not only had the highest content but also the highest CV (coefficient of variation), indicating this flavonoid was more susceptible to environmental effects than the other two flavonoids. So far, many studies have shown that stress can induce a higher concentration of total flavonoids in Chinese sea-buckthorn [21, 22], but studies of single secondary metabolites' yield are still scarce. We found a significant positive correlation between QUE and ISO ($p < 0.05$), thus indicating these two flavonoids responded similarly to the environment across northern China.

Relation between flavonoids in Chinese sea-buckthorn and environmental factors

The adaptation of plant species to the environment manifests in many aspects, such as their morphol-

ogy and structure, physiology, and biochemistry, etc. Among them, changes in the content and composition of leaf flavonoids constitute a material basis for plant adaptation to the environment (i.e., biosynthesis of flavonoids to mitigate effects of stress), which plays a pivotal role in plants' self-protection and environmental adaptation. Our results show that, both QUE and ISO in leaves of Chinese sea-buckthorn increased with rising altitude. This result is consistent with that of Ma et al [23]. Altitude encompasses a comprehensive suite of environmental factors. At higher altitudes, temperature decreases and radiation increases, and so forth; hence along that gradient, the habitat tends to become increasingly unfavorable for the growth of plants. When Chinese sea-buckthorn is exposed to cold, ultraviolet radiation, drought, and other stresses, its defense system springs into action, especially the metabolism of phenylpropane. Once this is activated it becomes possible to catalyze the synthesis of a variety of secondary metabolites with known defense functions in plants [24]. The greater activity of phenylalanine ammonia-lyase (PAL), cinnamyl alcohol dehydrogenase (C4H), and 4-coumarate CoA ligase (4CL), the key enzymes of the phenylpropanoid metabolic pathway, significantly affects the accumulation of secondary metabolites such as flavonoids in Chinese sea-buckthorn [25]. The energy of solar radiation is so high that even a small increase is sufficient to have serious effects on plants. Work by Schreiner et al [26] showed that the flavonoids' accumulation in plants is increased by exposure to UV-B radiation. In addition to being the main absorbers of UV-B radiation, flavonoids can effec-

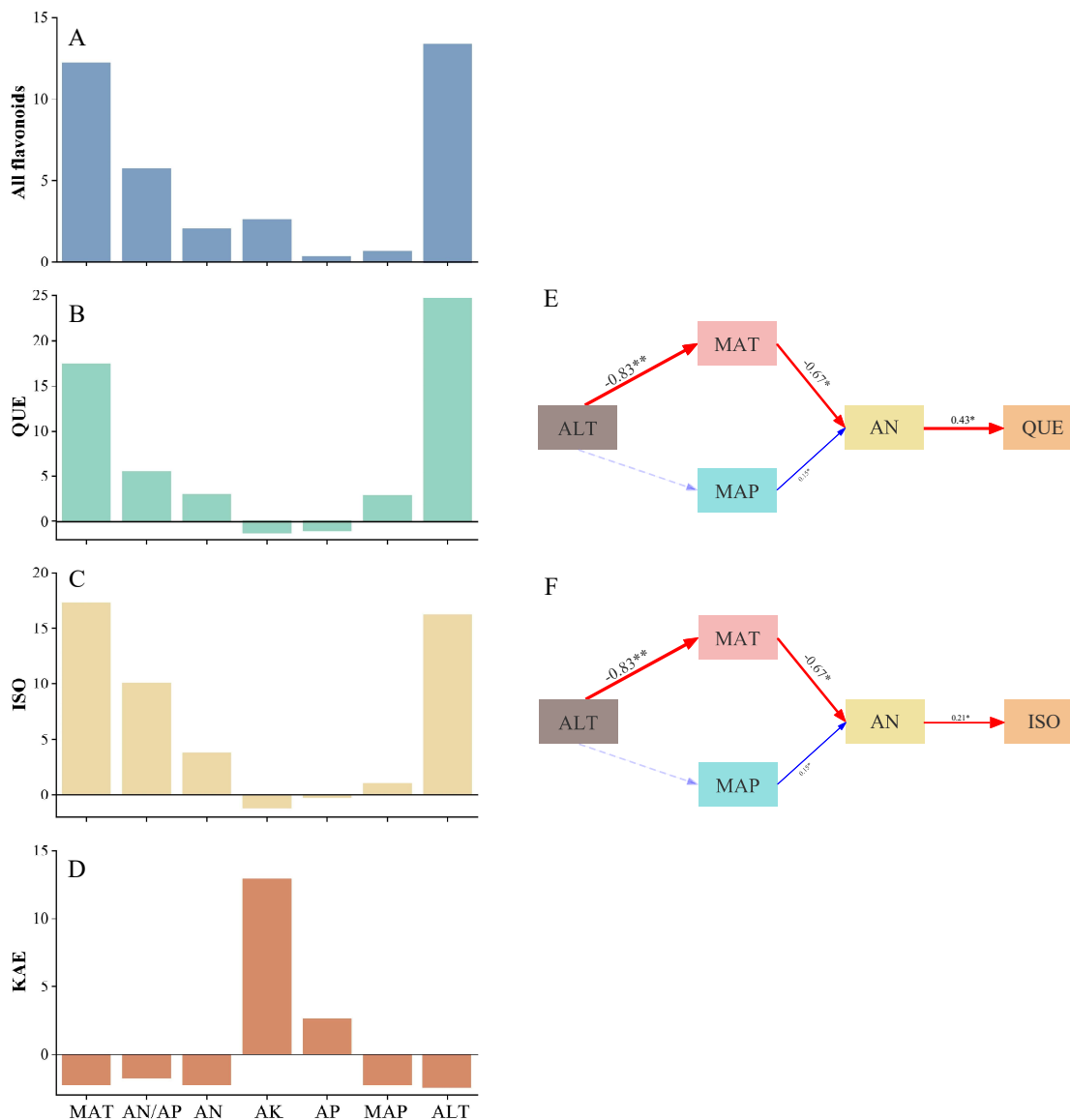


Fig. 7 The influence of seven environmental factors on the content of three flavonoids in leaves of Chinese sea-buckthorn. *Notes:* Panels (A–D) show the analyzed influence of environmental factors on the flavonoid by hierarchical segmentation. Panels (E, F) present the corresponding structural equation model (SEM) showing the respective influence of environmental factors after removing collinearity among them.

tively reduce the transmittance of UV-B radiation in the epidermal layer by accumulating in aboveground plant parts. In the present study, Chinese sea-buckthorn was found distributed from east to west in the Loess Plateau up to the eastern margin of the Qinghai-Tibet Plateau, at altitudes spanning 800 to 3600 m. Accordingly, radiation increased along that gradient, and the contents of three flavonoid compounds increased as well. This is likely due to the accumulation of flavonoid compounds that act as enzymes and antioxidants to

alleviate the toxicity of reactive oxygen species (ROS) produced by plants in response to high radiation [27]. In another study, Zhou et al [28] found that Chinese sea-buckthorn had a stronger tolerance of radiation than did *Hippophae neurocarpa*, which was related to the faster electron transport rate in leaves of Chinese sea-buckthorn.

Temperature is one of the most important factors affecting plant secondary metabolism, and it can affect plant life processes in different ways [29]. As

our results show, QUE and ISO decline along the average annual temperature gradients. In order to protect their leaves from damage due to stress, such as low temperatures, Chinese sea-buckthorn plants would have synthesized and accumulated flavonoids in the upper epidermis of leaves. Many studies have confirmed that flavonoids play a key protective role in the growth and development of plants and their stress resistance [30]. In addition, low temperatures can induce the accumulation and biosynthesis of PAL and chalcone synthase (CHS), which would promote the accumulation of flavonoids in leaves [31]. Albert et al [32] studied changes in the altitude gradient of the flavonoid matter, finding them not related to radiation but instead to temperature. Furthermore, with an increase in temperature, the ratio of QUE to KAE decreased, a trend consistent with the results of our paper.

Water plays a fundamental role in plant life activities, which can affect photosynthesis and the absorption and transport of nutrients in plants. The effects of drought stress on the leaf content of flavonoids have been well studied, but the findings are inconsistent. For example, Zhang et al [33] showed that moderate drought stress was beneficial for the synthesis of flavonoids in leaves; however, the accumulation of flavonoids in leaves of *Ligustrum vulgare* changed negligibly under drought stress. In our study, with a greater average annual precipitation, the QUE content increased significantly ($p < 0.05$) while the KAE content decreased significantly ($p < 0.05$), yet the ISO content of leaves was not significantly changed ($p > 0.05$). These results show that water stress has the greatest effect on content of QUE, followed by KAE, with no apparent effect on ISO. Altered water availability can affect plant enzymes that produce secondary metabolites, as found by Zhang [20] when studying the leaves of *H. neurocarpa* test-tube seedlings. The PAL, C4H, and 4CL enzymes were sensitive to drought stress, and their activities were significantly augmented by greater imposed polyethylene glycol-6000 (PEG-6000) stress. Therefore, we speculate that with more water available, the enzyme responsible for QUE synthesis decreased while that for KAE increased, resulting in a reduced QUE content and bolstered KAE content in Chinese sea-buckthorn. It is also possible that a greater amount of QUE could consume excess energy and ROS, to reduce the cellular oxidative damage and membrane structural damage caused by drought stress [34]. The insignificant change of ISO may be related to the use of intermediate metabolites such as lignin in the biosynthesis of secondary metabolites [35].

Soil provides a nutritious growth environment for the roots of plants. The carbon/nutrient balance hypothesis predicts that the content of carbon as flavonoids is negatively correlated with the content of

nitrogen in plants. Since soil is the main source of nutrients absorbed by plants, an appropriate level of nitrogen stress will be more conducive to the generation of flavonoids. Yet we found that QUE and ISO both increased with soil nitrogen content, a result inconsistent with the carbon/nutrient balance hypothesis. The soil conditions suitable for the growth and development of medicinal materials were likely not conducive to the accumulation of secondary metabolites. When soil nutrients are sufficient, plant growth is vigorous and biomass increases, but the content of flavonoid secondary metabolites tends to decrease [36]. In our study, however, as soil nutrients increased, the three flavonoid secondary metabolites all significantly increased as well ($p < 0.05$). This positive response could be explained by the fact that our 37 experimental population plots were mainly located in areas with relatively poor soil status, leaving unavailable those nutrients suitable for the growth of Chinese sea-buckthorn [37]. Thus, we suggest the greater flavonoid content as soil nutrients increase reflects Chinese sea-buckthorn's poor habitat adaptation.

Relationships among flavonoids and environmental factors

We examined how flavonoid secondary metabolites are related to environmental factors. These results show that for the pooled content of all three flavonoids, the environmental factors' influence could be ranked as follows: ALT > MAT > AN/AP > AK > AN > MAP > AP. Their order with respect to QUE is ALT > MAT > AN/AP > AN > MAP, and for ISO it is MAT > ALT > AN/AP > AN > MAP. These ranking results are similarly to those reported by Su et al [38], who found that altitude was the main factor affecting the content changes of QUE as well as ISO. According to our hierarchical segmentation results, AK and AP of soil factors have little effect on QUE and ISO contents, but they have strong effects on the KAE content. Li et al [39] believed that available phosphorus considerably affects flavonoid accumulation in *Flaveria bidentis*, which is consistent with our results here. Throughout the current analysis, QUE and ISO were found to be very similar in both their content and response to environmental factors, whereas KAE differed markedly from either flavonoid. The reasons for that divergence among the three leaf flavonoids could be related to the involved enzymes, metabolic pathways, structure and properties that drive their synthesis, which needs to be analyzed in further experiments.

CONCLUSION

We studied the relation between the content of flavonoids and environmental factors in the leaves of Chinese sea-buckthorn across northern China. The results clearly show that QUE, ISO, and KAE differ in their environmental responses. In addition, when

the habitat tends to be unfavorable, Chinese seabuckthorn is capable of adapting to that environment by increasing the content of its flavonoids. Average annual temperature, altitude, and the ratio of available nitrogen to available phosphorus are the main environmental factors affecting the content of flavonoids in this shrub species. After removing the collinearity of environmental factors, we find that QUE and ISO actually feature very similar relationships with the environmental factors, but KAE only responds to available potassium and available phosphorus. Further experiments are now needed to uncover the reasons for that disparity.

Our study clarified the environmental gradient-induced changes of leaf flavonoids in Chinese seabuckthorn in China, thus providing a theoretical basis for the cultivation, management, development, and utilization of this shrub species, as well as ways to enhance its economic and ecological value.

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