

The effects of nano-iron on volatile compounds and phytoestrogens in the Flowering Process of *Trifolium pratense*

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Research Article

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Abstract

Fe fertilizer is usually used to accelerate the growth and development of plants and trigger the accumulation of specific secondary metabolites to promote resistance and/or immunity of plants. Flowering is a critical process for plant growth and species continuation in angiosperms. However, little is known about the effect of Fe fertilizer on the flowering process. The volatile organic compounds (VOCs) and phytoestrogens in five flowering stages of *Trifolium pratense* with three Fe treatments (0, 1 and 2 g/mu) were evaluated in the study, which is a very important leguminous forage worldwide. The results showed that Fe contents in flowers dramatically increased with Fe concentrations. It was interesting that the highest Fe content of the flowers was in the senescent stage with 1 g/mu Fe treatment. The control group (CK) in the bud stage had the lowest amount of volatile organic compounds, but nano Fe significantly increased the amount of volatile organic compounds in the bud stage with various aromas. Alkane had the highest relative abundance among VOCs, accounting for more than 45–81%, and gradually increased in pace with the flowering process. Furthermore, nano Fe fertilizer promoted the accumulation of phytoestrogens. The main secondary metabolites exhibited obvious continuity throughout the whole flowering process. The results of the partial least squares discriminant analysis model illustrated that nano Fe fertilizer significantly changed the highly related VOCs and phytoestrogens in different flowering stages. The concentration of 1 g/mu was considered as a cost-effective agronomic measure to promote effectively reproduction and improve the contents of secondary metabolite in seed.

1 Introduction

Flowering is an integral biological process in life cycle of angiosperms and is critical for plant growth and species continuation, in addition to its economic importance (González-Suárez et al. 2020). Plants display different shapes, colors and odors to serve as visual and olfactory cues to attract biological pollinators during the process of flowering, which is a marker of flowering plants entering the reproductive phase (the 'floral transition') (Wang et al. 2020). In particular, the odors of flowers were used to ward off herbivores, transfer information, and interact with the environment (Zhou et al. 2020). Flowers from budding to senescence are affected by carbohydrates and hormone levels as well as by light, temperature, and mineral nutrients. The volatile organic compounds (VOCs) extracted from flowers, as the main source of plant aromas, play a critical role in floral transition, such as pollinator attraction, plant defense, transmission of information, and interaction with the environment (Brosset and Blande 2021), and are widely used in perfumes, food, flavoring agents and cosmetics (Du et al. 2019).

Fe fertilization has positive effects on flower development and production in different species. For example, Fe fertilization could increase the diameter of flower stems, flowering time, quality and yield in roses (Vigani et al. 2013; Wang et al. 2020). As an essential micronutrient for plants, iron is involved in electron transfer, nitrogen fixation, synthesis of genetic information, synthesis of hormones and chlorophyll, and many other related metabolites in plant tissues (Herlihy et al. 2020; Khalid et al. 2021). But iron in its natural state is biologically inactive because it is usually bound in oxidized and insoluble ferric forms, although iron is abundant in the Earth's crust (Kobayashi et al. 2019; Herlihy et al. 2020).

Recently, nanotechnology has been extensively used to boost plant growth, improve nutrient utilization efficiency and reduce environmental pollution caused by excessive use of traditional fertilizer (Giraldo et al. 2019). Nano Fe fertilization has been reported to contribute to photosynthesis and synthesis of plant hormones (Singh et al. 2018), alleviate iron deficiency symptoms, and regulate that anabolism (Zhang et al. 2009; Tavallali et al. 2019), protein formation and plant volatile compounds in leaves (Sánchez et al. 2022). However, the effects of Fe fertilizer on the flowering stages remain rather limited.

Red clover (*Trifolium pratense* L.), belonging to the genus *Trifolium*, is an important high-quality perennial feed legume grown in temperate climate regions of the world (Marshall et al. 2017). Red clover is rich in phytoestrogens and utilized as a food additive with medical value, such as antiaging and anticancer properties, regulating human hormone balance, treating menopausal syndrome, and preventing osteoporosis and antioxidation (Marshall et al. 2017; Gómez-Zorita et al. 2020). Phytoestrogens may be a defense mechanism in plants to control the reproduction of herbivores and protect plant populations (Jeong et al. 2018).

To evaluate VOC emission and phytoestrogen synthesis during the flowering process by applying Fe fertilizer, we applied combined omics technologies, including gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), in different flowering periods of red clover to provide a new strategy for research on plant interactions with the environment.

2 Materials And Methods

2.1 Plant materials

Red clover (*T. pratense* cv. Minshan) was planted at experimental sites for six years in Hezheng county, Gansu, China (36°3'25" N, 103°15'20" E, 2050.0 m above sea level). Three concentrations of nano Fe fertilizer (0, 1 and 2 g per mu) were sprayed twice in an at intervals of 7 days on leaves of red clover at the early flowering stage. Nano-Fe got from Guoshuo Nanong Agricultural Technology Co., Ltd. The flowers were collected in five stages: bud stage (1), half-open stage (2), blooming stage (3), mature stage (4) and senescent stage (5) (see Supplementary Fig. S1). The flower samples were transferred at low temperature and freeze-dried in a lyophilizer (Labocene Scandinavian by Design, ScanVac CollSafe 110-4, Denmark).

2.2 Determination of Fe content

The method described by Kumar et al. (2016) was used with slight modifications. Freeze-dried flower tissue of red clover was ground to a fine powder using a ball mill instrument (Verder Scientific, MM400, Shanghai, China) to avoid metal contamination. The grain powder (1 g) was digested under a fume hood with a 10 mL acid mixture (concentrated nitric acid: concentrated sulfuric acid: perchloric acid = 10:1:1) until a clear white residue was obtained. After cold digestion, the samples were digested at 150°C for 1 h, followed by 3 h of digestion at 280°C. The digestion solutions were diluted to 100 mL after completion of the digestion process and were analyzed using an atomic absorption spectrophotometer (Thermo Fisher Scientific, MKII M6, MA, USA). Each sample was measured thrice, and the means of these triplicate

observations were used to represent the sample's Fe contents. The Fe concentrations in different samples were calculated by plotting OD values against a standard curve made with FeCl_3 .

2.3 Analysis of volatile compounds

n-Hexane was used to extract VOCs from red clover flowers in different flowering stages to obtain abundant volatile components of lower volatility and longer chain fatty acid esters (Noushini et al. 2020). The extractions were analyzed by GC–MS (Shimadzu GCMS-QP2020 system, Shimadzu Co., Ltd, Kyoto, Japan). One gram of flower powder was weighed into a headspace vial, 5 mL of n-hexane was added, and the vial was sealed after fully mixing (Li et al. 2022).

The method conditions of GC–MS were as described by Li et al. (2019) with some modifications. The headspace vial solutions were immediately moved to the GC–MS injection port. The chromatographic conditions were as follows: DB-5ms capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm , J&W Scientific, Folsom, USA); programmed ramp-up procedure: 40°C for 3 min, ramped up to 160°C at 10°C/min and held for 2 min, ramped up to 200°C at 5°C/min and held for 1 min, ramped up to 240°C at 20°C/min and held for 2 min, ramped up to 280°C at 5°C/min and held for 5 min, ramped up to 300°C at 10°C/min and held for 5 min; carrier gas (He, 99.999%) flow rate 1.0 mL/min; and inlet temperature 280°C. The sample was injected in splitless mode. The mass spectrometry conditions were as follows: EI ion source (70 eV), temperature 250°C, and mass scan range m/z 50–650. The quantities of volatiles were calculated by available volatile standard curves. For unavailable standards, tentative identification of the volatiles was achieved by comparing their mass spectrometric information of the chromatographic peak with a mass spectra library (similarity > 75%), and manual analysis was used to identify the structure of the compound for qualitative analysis (Li et al. 2022). All of the mass spectra data were analyzed and quantified by using a Shimadzu GC–MS solution workstation with NIST 05 and Wiley 6 mass spectrometry databases (Hewlett-Packard Co., Palo Alto, USA).

2.4 Determination of phytoestrogens

1.5 mL of 80% methanol (LC–MS grade, Merck) was added to 120 mg red clover flower powder, and ultrasonically extracted at 30°C for 10 min. After storage overnight at -18°C, the sample was thawed at room temperature and then centrifuged at 12,000 rpm for 10 min. The supernatant was collected and filtered with a 0.2 μm syringeless filter for UHPLC–MS/MS analysis (Förster et al. 2021).

Chromatographic separation was achieved on UHPLC system (Agilent 1290 Infinity II, Agilent Technologies Inc., Cal, USA) equipped with a reversed-phase column (ZORBAX RRHD HILIC Plus, 2.1 $\times$ 100 mm, 1.8 μm , Agilent Technologies Inc., California, USA) using ultrapure water and methanol as mobile phases A and B, respectively. The injection volume was 2 μL for extracts of red clover flowers, the column temperature was maintained at 35°C, and the flow rate was 0.2 mL/min. The following gradient was used for the separation of phytoestrogens: 0–0.5 min, 10% B; 0.5–1.5 min 25% B; 1.5–2.5 min 40% B; 2.5–4 min 70% B; 4–4.5 min 80% B; 4.5–6 min 90% B; and 6–7 min 100% B. The UHPLC system was coupled to a quadrupole time-of-flight mass spectrometer (6550 iFunnel Q-TOF LC/MS, Agilent Technologies Inc., Cal, USA) equipped with the ESI ion source operated in positive ionization mode (50–

1000 m/z) for the analysis of phytoestrogens. The parameters were set as follows: sheath gas temperature and flow rate: 380°C and 12 L/min, respectively; nozzle voltage: 500 V; nebulizer pressure: 27 psi; capillary voltage: 3750 V; and Frag voltage: 120 V. Data Acquisition 7.0 software was used for data acquisition. In addition, the software of Qualitative Analysis 7.0 and Q-TOF Quantitative Analysis 7.0 were used for data analysis.

2.5 Statistical analysis

The z-score algorithm was used to standardize the content of the identified volatiles in the heatmap. Analysis results of compounds were processed by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (Tukey HSD) test and statistical significance set at $P < 0.05$ using SPSS Statistics 20.0 software (IBM Watson Analytics, Beijing, China). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used to evaluate the aroma difference of samples by SIMCA 14.1 (MKS Umetrics AB, Malmo, Switzerland).

3 Results

3.1 Effects on the types of VOCs in red clover flowers

A total of 170 volatile components from the process of flowering of red clover detected by GC–MS were analyzed via petal diagram and Venn diagram (Fig. 2A), which included 63 alkanes, 35 alcohols, 34 fatty acids and their derivatives, 12 aldehydes, 11 aromatic hydrocarbons, 4 olefins, 3 acids, 2 ketones and 6 others (see Supplementary Table S3). We found that 15 VOCs existed in five flower stages of all treatments. Among these 15 compounds, heptane, 1-octen-3-ol, phytone, phytol and heptadecane were reported to have various aromas, such as sweet, fruity and jasmine (Chung TY et al.1993; Nakaya S et al. 2015; Feng T et al. 2020). There were the least types ($P < 0.05$) of VOCs in the bud stage of CK (Fig. 2A), but nano Fe fertilizer caused obvious increases in the stage. However, the varieties of VOCs were reduced with the increase of Fe concentrations in Fe treatment groups. Fe-1g groups were more than Fe-2g except for the blooming stage.

As shown in Fig. 2B, alkane, alcohol and aromatic compounds were dominant as the main types of VOCs in red clover flowers, the sum of which accounted for more than 85% of the total VOCs (85.07–95.92%). The sums in the Fe treatments were higher than CK. Among them, the most kinds of alkanes were found in CK-2, followed by Fe-2g-3 and Fe-1g-4, and CK-1 had the least kinds of alkanes. It was worth noting that 63 alkanes were detected in GC-MS, accounting for 63.9% of the content of VOCs. Among them, 8 identical alkanes were found in all treatments, including tetracontane, heptane, 2,6,10-trimethyltridecane, eicosane, dotriacontane, hexadecane, heneicosane and heptadecane (listed in order of content). It was interesting that tetracontane and heptane, the most two abundant alkanes, decreased in half-open stages, and increased in blooming and mature stages compared with CK, while 2,6,10-trimethyltridecane was increased in all stages, especially in the blooming stages. In addition, the types of alcohols and aromatics showed an increasing trend during flower growth, indicating that the main aromatic compounds gradually accumulated during the flowering process. A decreasing trend was observed in the senescent

stage. As fragrant compounds were reduced, the aroma of the flower faded. Overall, there were the most types of VOCs detected in Fe-1g-1, while Fe-2g-2 had the lowest ($P < 0.05$). Compared with other samples, alcohols, esters and aromatics in Fe-1g-1 were obviously more, which indicated a rich aroma at this stage. Furthermore, the change of VOCs' types was completely different from those in CK. Among five stages of Fe fertilization, the types of aroma and alcohol compounds in the bud stage were the highest, which were thought to be useful to attract pollinators and resist Fe fertilization (Zhou et al. 2018; Rachersberger et al. 2019).

The clustering relationship between the relative abundance values of different compounds was analyzed to evaluate the differences in VOC anabolism in the process of flowering of red clover under different treatments (Fig. 2C). Among them, alkanes made up the largest proportion in the relative abundance of VOCs, accounting for 45–81% of VOCs, and exhibited gradually increasing trend with the advancement of flowering stages.

Ketones might be an important precursor for other compound types. High proportions of ketones appeared in the first stage, of which CK-1 had the highest proportion. Moreover, the clustering analysis showed obvious differences in five stages of the flowering process for CK treatment. The clustering relationships of the first and second stages were close for Fe treatments, indicating that appropriate nano Fe fertilizer accelerated the synthesis of secondary metabolites.

3.2 Effects on the contents of VOCs in red clover flowers

A heatmap was used to analyze the contents of 77 VOCs in different flowering stages and different concentrations of nano Fe (Fig. 3), in which 15 treatments were clustered into two distinct groups.

Fifteen compounds, such as 1-octane-3-ol, phytone, eicosane and chlorophyll, were found in all fifteen treatment samples. Fe-2g-5 was clearly distinct from the other treatments due to the high level of esters and acid compounds (n-hexadecanoic acid, linoleic acid ethyl ester, octacosyl acetate, oleyl acetate, etc.). The remaining 14 treatments belonged to two groups (A and B). All flowering stages of CK were located in Group B. The classification of groups A and B were consistent with the application of nano Fe fertilizer. The cluster analysis results clearly illustrated that the influence of Fe on VOCs was very significant. Although it has been mentioned that an increase of Fe concentration reduced the variety of VOCs in the previous analysis (Fig. 2), it was interesting that the content of the same compound in different stages increased with the application of Fe fertilizer. Then groups A and B were divided into 4 subgroups (A1, A2, B1 and B2) according to the correlation. The flowering stages CK1, CK2 and CK3 were in B2, while the end of flowering stages CK4 and CK5 were in B1. The distinctions between subgroups B1 and B2 were caused by the contents of 1-hentetracontanol, fucosterol, 1-triacontanol, (Z)-3-hexen-1-ol, heptanal, diethyl phthalate, tetracontane, octadecane and antioxidant 168, which may also be used to distinguish the blooming condition.

There were some specific compounds only found in the Fe treatments, such as 16-hentriacontanol, octacosan-14-ol, cholesterol, benzyl alcohol, butyl-2-methylvalerate, ethyl linoleate and cis-3-hexenyl

acetate. Benzyl alcohol provided floral and rose aromas; ethyl linoleate exuded floral, fruity and chamomile aromas; cis-3-hexenyl acetate provide sweet, fruity, banana and apple aromas. These compounds with rich fruity and floral aromas likely attract pollinators, which may be beneficial to promote pollination, improve seed setting rates and increase economic potential (El-Sayed et al. 2018; Rachersberger et al. 2019; Bohman et al., 2020). These results indicated that Fe fertilizer was useful for promoting the metabolism of VOCs and improving the pollination efficiency of red clover.

Principal component analysis (PCA) was used to analyze the abundance of 77 compounds (covariance matrix) to assess the total variation in different treatment groups based on major VOC components (Fig. 4B). The first two principal components (PCs) explained 89.9% of the observed variation in VOCs, in which PC1 and PC2 explained 53.17% and 36.73%, respectively. The flowers of red clover were clearly classified into a nonfertilized group (CK group) and an Fe-fertilized group. The red clover flowers in the CK group were distributed throughout the flowering process, which demonstrated the excellent reproducibility of the analytical method. In addition, the Fe treatments were further divided into group A (Fe-1g-1, Fe-1g-2, Fe-1g-3, Fe-1g-4, Fe-1g-5, Fe-2g -1 and Fe-2g-3) and group B (Fe-2g-2, Fe-2g-4 and Fe-2g-5). The results of different types of compounds and different treatments were visualized via a chord diagram (Fig. 4A) to express the dynamic changes in each VOC content under different treatments. It was also observed that alkanes were the most abundant VOCs in each sample group and increased with the flowering stage, followed closely by alcohols and aromatics. The red clover flowers in the blooming stage contained the highest content of VOCs in CK. It is worth mentioning that Fe could delay VOCs release. Fe fertilizer was conducive to the enrichment of VOCs in the mature stage and senescent stage.

3.3 The effects on phytoestrogens in red clover flowers

To study the metabolism of phytoestrogens in red clover flowers, 30 known phytoestrogens were identified by LC–MS in the 15 treatments based on the accurate mass ($\Delta m/z \leq 5$ ppm) (Fig. 5). Among treatments, the CK and Fe-2g groups had high phytoestrogen contents in the half-open stage and senescent stages, while significantly high contents were exhibited in mature stage for Fe-1g ($P < 0.05$). As reported in previous studies, Fe treatment could increase the content of phytoestrogens (polyphenols and isoflavones), especially total anthocyanins and flavonoids (Gryszczynska et al. 2018; Farhadi et al. 2020). A notable cause for concern is that some phytoestrogens were only present in early blooming stages (bud, half-open and blooming stages), such as (-)-epicatechin, cianidanol, astragalin, 6-methoxykaempferol 3-O-glycoside and isorhamnetin 3,7-O-di-beta-D-glucopyranoside. However, some phytoestrogens could be activated in advance in the half-open stage of Fe treatments, such as astragalin and 6-methoxykaempferol 3-O-glycoside. At the same time, several phytohormonal compounds were found to exist throughout the flowering process of Fe treatments, such as proanthocyanidin A2, 6-methoxykaempferol 3-O-glycoside and 3-hydroxycoumarin.

3.4 The characteristic compounds of red clover flowers

Multivariate statistical analysis via the PLS-DA model was performed to evaluate the characteristic compounds from 77 VOCs and 30 phytoestrogens in red clover flowers (Belmonte-Sánchez et al. 2019; Song et al. 2020). The relationship among the map of loadings and the scores was simultaneously displayed on the biplot via k-fold cross-validation (Fig. 6A). Through the PLS-DA biplot of red clover flower samples, it was observed that red clover flowers of Fe-1g-1, Fe-2g-1, Fe-2g-2 and Fe-2g-5 were significantly affected by Fe fertilizer. At the same time, the CK groups were clustered in the same quadrant, which also showed that this model could well distinguish CK and Fe fertilizer groups.

The PLS-DA model was also used to analyze the relationship between Fe fertilizer and secondary metabolites of red clover flowers. There were differences in the VOCs and phytoestrogens among red clover flowers of different treatments, especially Fe fertilizer, which completely changed the correlations of VOCs and phytoestrogens in different stages (Fig. 6A). The compounds with a strong correlation in CK-1 stage were (3R)-4'-methoxy-2',3,7-trihydroxyisoflavanone, diethyl phthalate and triacontyl acetate (Supplementary Table S4). Two alcohol compounds, 1-hexacosanol and (E)-2-hexen-1-ol, were closely related to CK-3, CK-4 and CK-5. The highly correlated compounds of CK-3 and CK-5 were the same. Although the related compound anthricin in CK-4 was different from 2,2',5,5'-tetramethyl-1,1'-biphenyl and genistein in CK-3 and CK-5, the correlations between their structures were high. The same phenomena were also observed in the Fe treatment groups. For example, the same related compounds were found in Fe-1g-3 and Fe-1g-4 or Fe-2g-1 and Fe-2g-2. It was interesting that the related compounds 6-methoxykaempferol 3-O-glycoside in Fe-1g-1, isorhamnetin 3,7-O-di-beta-D-glucopyranoside in Fe-1g-2, and isoquercitrin in Fe-1g-3 and Fe-1g-4 contained to the same flavone carbon skeleton, 3,4,7-trihydroxyflavone. These results indicated that the main secondary metabolites have obvious continuity during the flowering process, although nano Fe fertilizer significantly changed the highly related VOCs and phytoestrogens in different flowering stages. Moreover, the same related compounds were also in Fe-1g-2 and Fe-2g-3, which may be because the accumulation mechanisms of secondary metabolites in the plant flowering process were consistent for different Fe treatments, and a high concentration of Fe evidently delayed the accumulation of secondary metabolites.

The characteristic components of red clover flowers from different treatments were screened by the VIP method (Fig. 6B). Except 10 phytoestrogens (3-hydroxycoumarin, aloe-emodin, 3'-methoxydaidzein, biochanin A, 5-methoxy-6,7-methylenedioxyflavone, coumafuryl, anthricin, 6-methoxykaempferol 3-O-glycoside, (-)-olivil-4"-O-beta-D-glucopyranoside, and daturametelin E), 25 volatile compounds were screened out, including 8 alcohols (1-hexacosanol, 1-hexanol, campesterol, 2-methyl-1-butanol, 2-ethyl-1-hexanol, (E)-2-hexen-1-ol, and (E)-3-hexen-1-ol, octacosanal), 2 aldehydes (2-hexenal and heptanal), 1 ester (palmitic acid ethyl ester), 10 alkanes (tetrapentacontane, heptane, 2,2,3,3-Tetramethyl-butane, pentacosane, dotriacontane, heptadecane, 2-methylhexacosane, 2,4-dimethyl-undecane, 7-acetoxyeicosane, and 4,6-dimethyl-dodecane), and 3 aromatics ((3,3-dimethyldecyl) benzene, 2,3,6-trimethylnaphthalene, and 2,3-dimethylnaphthalene). Among them, (E)-3-hexen-1-ol has the highest importance in VIP values, and the VIP values of 3-hydroxycoumarin and (-)-olivil-4"-O-beta-D-glucopyranoside in phytoestrogens are also significantly high ($P < 0.05$). It is worth noting that palmitic

acid ethyl ester and (E)-3-hexen-1-ol with high VIP values were reported to be indicative of an adaptation to butterfly pollination (Andreas 2004).

4 Discussion

Fertilizers are often used to improve the nutrient environment of plants, enhance phytoremediation, and improve crop quality. Iron (Fe) fertilizers can promote various metabolic processes during plant growth and development and increase pollinator abundance and plant immunity to cope with variable environmental conditions over their lifetimes (Kovacs-Hostyanszki et al., 2011; Khalid et al., 2021;). Nano-iron particles can effectively reduce plant oxidative stress and increase plant biomass (Wu et al. 2021; Baragaño et al. 2022;). Studies have reported that magnetite nanoparticles (Fe_3O_4 -NPs) significantly augment the growth rate constant and extracellular polysaccharides of soybean and alfalfa microsymbionts, which are beneficial to the growth of legumes (Iannone et al. 2021; Groppa et al. 2022;). In the present study, the effects of nano iron on flowers of *T. pratense* were discussed for the first time.

Nano iron, as a fertilizer, is reported to promote photosynthesis and make plants synthesize more hydrocarbons (Rui M et al. 2016). The VOC and phytoestrogen metabolisms of plants were found to vary greatly depending on developmental stages (Mujiono et al. 2020). The VOCs and phytoestrogens detected in five flowering stages of red clover in the study served as context-related modulators of possible ontogenesis. Our results showed nano-iron fertilizer significantly increased the VOCs' types of the bud stage and promote phytoestrogen accumulation in red clover flowers, which was beneficial for enhancing pollinator attraction, plant autoimmunity and plant resistance through plant metabolism.

Plants grow in complex multispecies communities and multi-ecological environment rather than monocultures. Therefore, plants need to compete with other organisms in the ecological environment by releasing VOCs and nonvolatile components (Kigathi et al. 2019). It is well documented that plant volatile chemicals, together with nonvolatile specialized chemicals, play vital roles in both direct and indirect adaptations of plants to their environments (Wei et al. 2016; Rachersberger et al. 2019). It was found that the VOCs (e.g., 1-hexacosanol, (E)-3-hexen-1-ol, 2,2,3,3-tetramethyl-butane and pentacosane were closely related to Fe content via PLA-DA analysis (Fig. 6). It was reported that 1-hexacosanol and (E)-3-hexen-1-ol have potential biological activities and present grass, floral and fruity aromas (de Melo et al. 2020; Ni et al. 2021). Female beetles showed attraction to pentacosane, which could be used as a short-range attractant and spawning stimulator for *Lema praeusta* (Das et al. 2019). In addition, the metabolism of phytoestrogens such as 3-hydroxycoumarin, (-)-olivil-4"-O-beta-D-glucopyranoside and aloe-emodin in red clover was closely related to the Fe content. 3-Hydroxycoumarin has excellent antibacterial and antifungal activity and is a promising photoprotective drug (de Araujo Leite et al. 2015).

Overall, nano Fe fertilizer at a concentration of 1 g/mu was most conducive to the enrichment of Fe in red clover flowers and significantly improved the richness and contents of VOCs and phytoestrogens during the flowering process. The results showed that reasonable and appropriate use of iron fertilizer, especially

nano iron, would be beneficial to enhance the accumulation of VOCs and phytoestrogens and promote the reproduction of the plant.

5 Conclusions

For the first time the research on metabolic characteristics of VOC and phytoestrogens in flowering stages of red clover and influences of nano-iron fertilizer. We found that the concentration of nano-iron and the enrichment of Fe content in red clover flowers did not show gradient dependence, and 1 g/mu was more conducive to the enrichment of iron in red clover flowers. volatile organic compounds (VOCs) and phytoestrogens in five flower stages at three Fe concentrations (0, 1 and 2 g/mu) were evaluated in red clover. And iron fertilizer is the main factor to promote the metabolism of the main volatile compounds and phytoestrogens in red clover, it will delay the release of VOCs, which is beneficial to the enrichment of VOCs in the mature stage and senescent stage. Therefore, nano-iron fertilizers may be suitable for regulating plant volatile components. Our results also provide data support for iron fertilization to promote phytoestrogen accumulation and phytoestrogen compounds that are highly correlated with iron fertilization at different flowering stages of red clover. That is, we report for the first time the effect of nano-iron fertilizer on the content and types of VOCs in red clover flowers through metabolomics technology, and it is beneficial to the enrichment of plant active components such as phytoestrogens.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by RL and YL. XR conducted experiments and provide data; YL conducted experimental data processing; HY conducted field experiments; QL designed experiments and wrote the paper. The first draft of the manuscript was written by RL. All authors read and approved the final manuscript.

Data availability statement

All data supporting the findings of this study are available within the paper and within its supplementary data published online or through contacting the authors of this paper.

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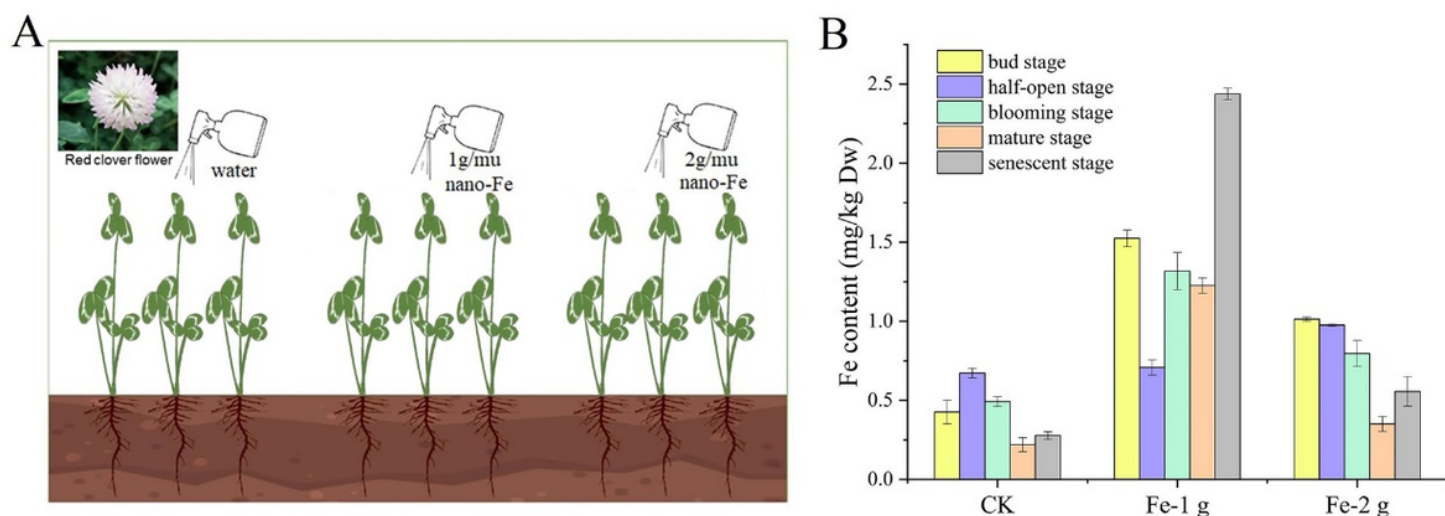
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Figures



Field test conditions of red clover (A) and Fe content in red clover flowers of different experimental groups (B). Different letters (a–i) indicate significant differences ($P < 0.05$) between different flower samples determined separately for each group (control or WOS) by one-way ANOVA followed by Tukey HSD test. Dw, dry weight; n=3; error bars represent SE.

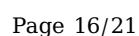


Figure 2

Venn diagram of the types of volatile compounds (VOCs) in red clover flowers of different experimental groups (A), radar map of VOCs different types (B), and relative abundance tree map of VOCs different types (C).

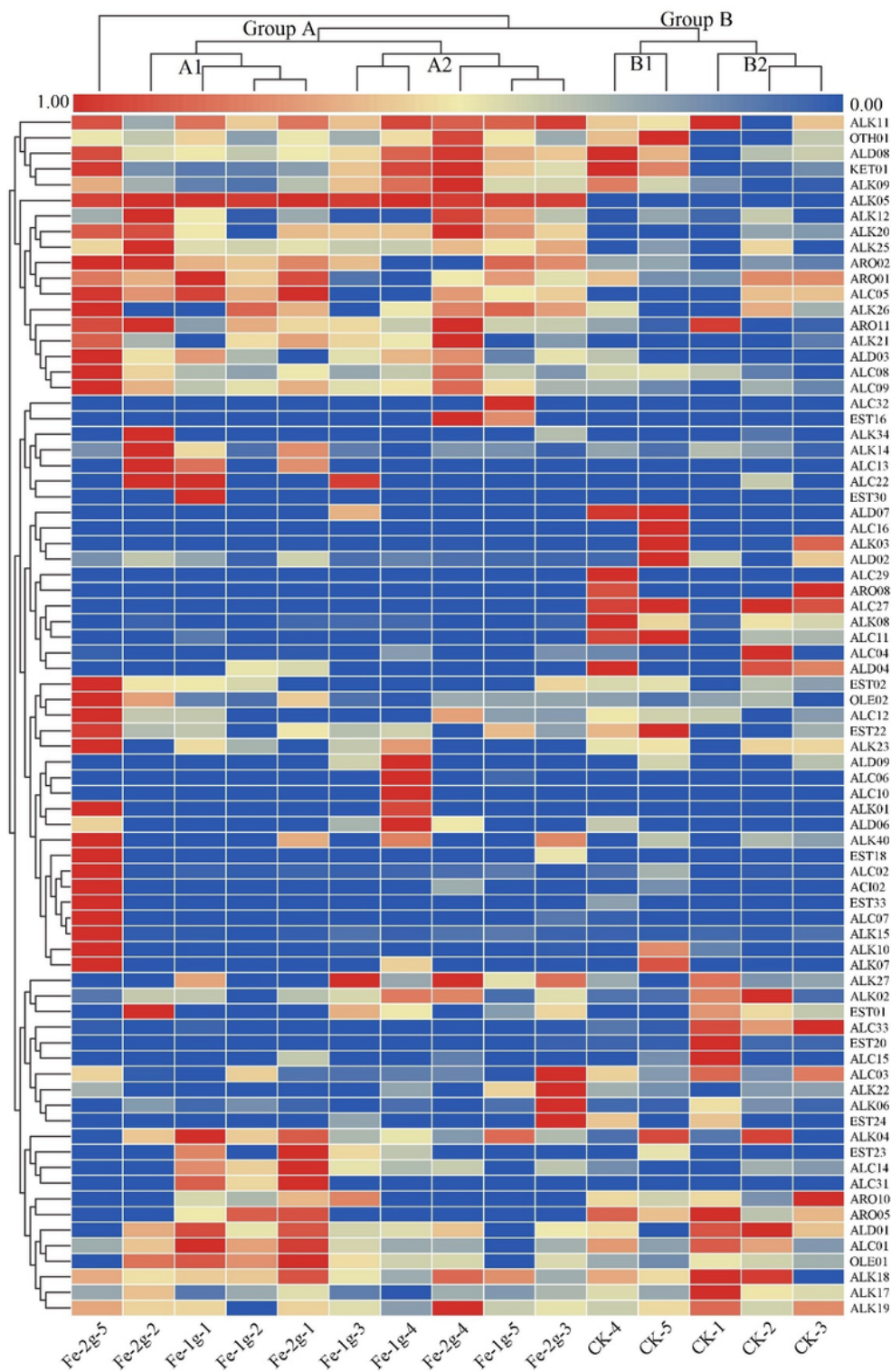


Figure 3

Heat map of the VOCs in red clover flowers of different experimental groups. The redder colors indicate higher content of the compounds, while bluer colors indicate lower the level of the compound. Each data point is the average of three biological replicates. Compound abbreviations and odor are reported in supplementary table S1.

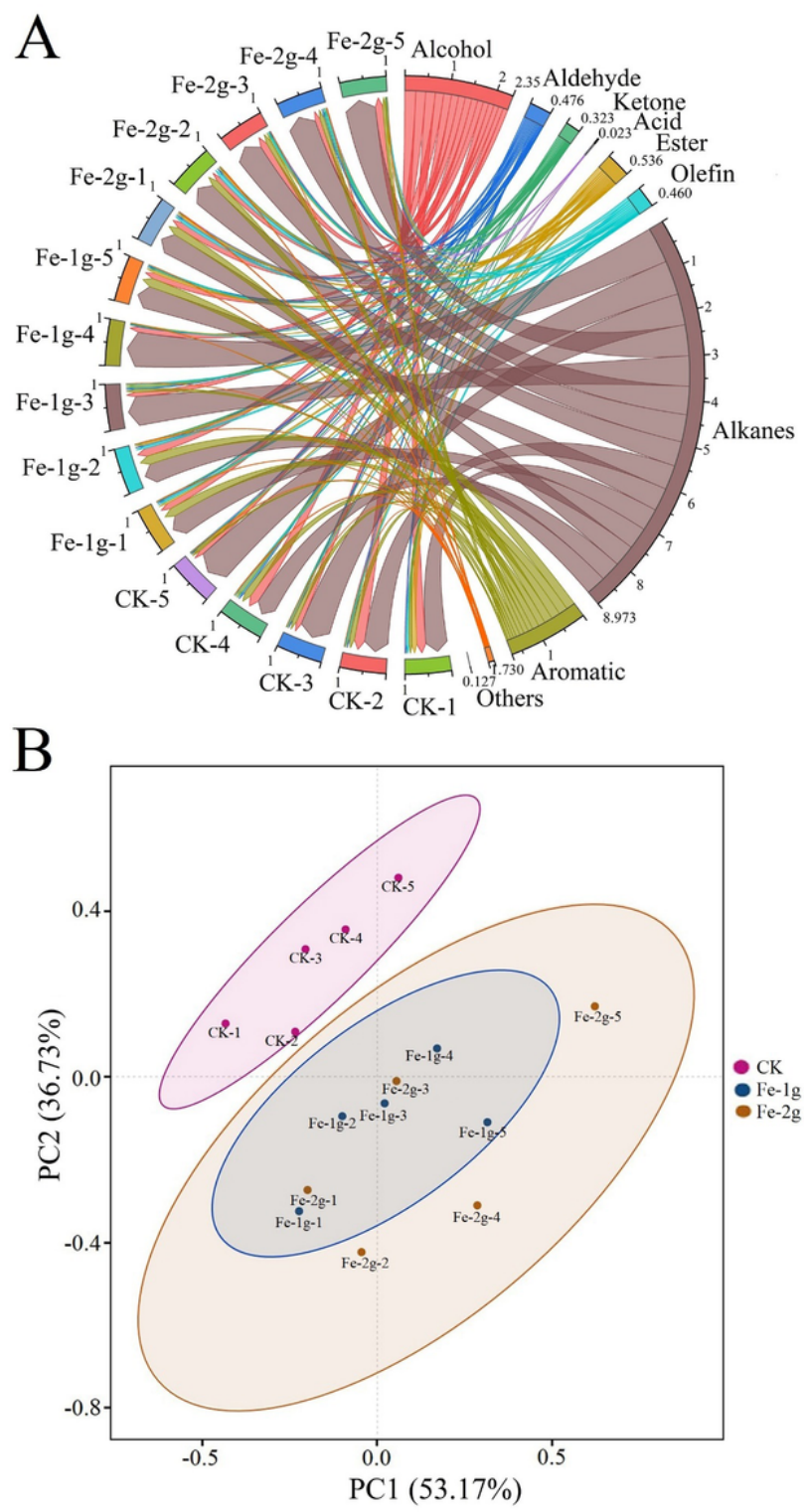


Figure 4

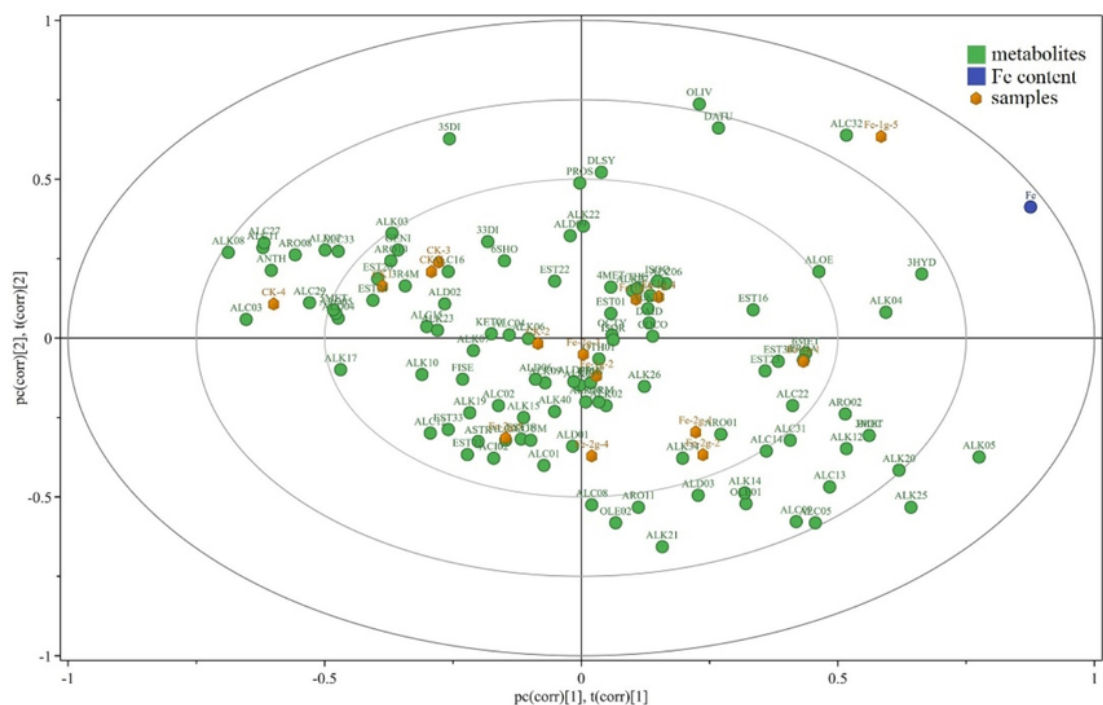
Distribution of VOCs content in red clover flowers of different experimental groups (A) and principal component analysis (PCA) between different samples of red clover flowers (B). Each data point is the average of three biological replicates.



Figure 5

The effect of nano-Fe fertilizer on phytoestrogens in red clover flowers. Mean relative abundances are shown via heat map ($n = 3$). The redder colors indicate higher relative abundances, while bluer colors indicate lower the level of relative abundances.

A



B

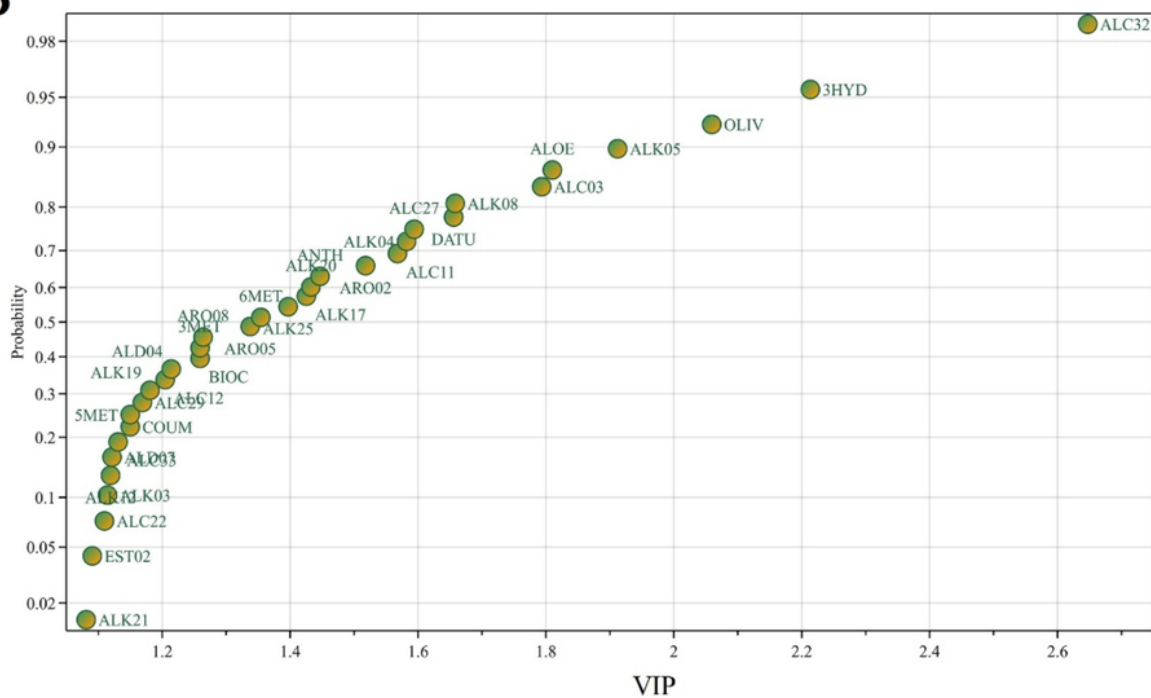


Figure 6

PLS-DA biplot of flower samples, Fe concentration and metabolites on the basis of normalized peak areas of 77 VOCs and 30 phytoestrogens (A); VIP scores plot of compounds in different red clover samples (B). Compound abbreviations were reported in supplementary table S1 and S2.

Supplementary Files

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