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•Research article•

Plant metabolomics for studying the effect of two insecticides on comprehensive constituents of Lonicerae Japonicae Flos

PAN Hui-Qin[1, 2], ZHOU Heng[1], MIAO Shui[1], GUO De-An[2], ZHANG Xiao-Li[3], HU Qing[1], MAO Xiu-Hong[1], JI Shen[1*]

[1] *NMPA Key Laboratory for Quality Control of Traditional Chinese Medicine, Shanghai Institute for Food and Drug Control, Shanghai 201203, China;*
[2] *Shanghai Research Center for Modernization of Traditional Chinese Medicine, National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China;*
[3] *Shanghai Kaibao Pharmaceutical Co., Ltd., Shanghai 201401, China*

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[ABSTRACT] Pesticides' overuse and misuse have been reported to induce ingredient variations in herbal medicine, which is now gaining attention in the medicinal field as a form of alternative medicine. To date, available studies on pesticide-induced ingredient variations of herbal medicine are limited only on a few compounds and remain most others unexamined. In this study, a plant metabolomics-based strategy was performed to systematically explore the effects of two frequently used insecticides on the comprehensive constituents of Lonicerae Japonicae Flos (LJF), the flower buds of *Lonicera japonica* Thunb.. Field trials were designed on a cultivating plot of *L. japonica* with controls and treatments of imidacloprid (IMI) and compound flonicamid and acetamiprid (CFA). Unbiased metabolite profiling was conducted by ultra-high performance liquid chromatography/quadrupole-Orbitrap mass spectrometer. After data pretreatment by automatic extraction and screening, a data matrix of metabolite features was submitted for statistical analyses. Consequently, 29 metabolic markers, including chlorogenic acids, iridoids and organic acid-glucosides were obtained and characterized. The relative quantitative assay was subsequently performed to monitor their variations across flowering developments. This is the first study that systematically explored the insecticide-induced metabolite variations of LJF while taking into account the inherent variability of flowering development. The results were beneficial for holistic quality assessment of LJF and significant for guiding scientific use of pesticides in the large-scale cultivation.

[KEY WORDS] Plant metabolomics; Insecticide; Metabolite variation; Lonicerae Japonicae Flos
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Introduction

Lonicera japonica Thunb., also known as Japanese honeysuckle, is commonly planted as an ornamental groundcover in East Asia [1]. Its flowers are extensively used as medicines, cosmetics, functional tea, among others [1]. The 2015 edition of Chinese Pharmacopoeia has officially recorded the dried flower buds of *L. japonica* Thunb. as Lonicerae Japonicae Flos (LJF), which is widely used as a traditional Chinese medicine called Jin-Yin-Hua. LJF is the main ingredient in several clinical preparations and is commonly used for the treatment of exopathogenic wind-heat or epidemic febrile disease, sores, carbuncles, furuncles, swellings, headache, and respiratory infection [2-3]. The extensive therapeutic effects of LJF are mainly attributed to its diverse bioactive compounds, such as chlorogenic acids, flavonoids, and iridoids in LJF, which reportedly contain anti-inflammatory, antiviral, antibacterial, antioxidative, hepatoprotective, anti-tumor, and antipregnant activities [1].

The developmental phase of *L. japonica* flower can be divided into six morphological stages: the juvenile bud stage, the third green stage, the second white stage, the complete white stage, the silver flowering stage, and the gold flowering stage [1]. Among the six stages, the flower buds at the third green stage (TGS), the second white stage (SWS), and the complete white stage (CWS) are used traditionally as

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[*]**Corresponding author]** Tel: 86-21-3883-0990, Fax: 86-21-5079-8195, E-mail: jishen2013@163.com
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sources of LJF. The flowers in the silver flowering stage and the gold flowering stage are considered to have low therapeutic effects. As an agricultural product for medicinal materials, *L. japonica* has a relatively short harvest time but its large-scale cultivation can easily suffer from insect pests and plant diseases. To ensure the yield and quality of LJF, different types of pesticides are applied to control these natural damages [4]. Indeed, many studies on pesticide residues of LJF revealed the frequent utilization of insecticides and fungicides, such as acetamiprid, carbendazim, chlorantraniliprole, emamectin B1a benzoate, hexa-conazole, imidacloprid, and so on [5-6].

Pesticides play a vital role in protecting plants from diseases and pests, as well as guaranteeing the yield and quality of medicinal agriproducts. However, given that most pesticides usually possess a certain degree of toxicity, their residues in soil, atmosphere, and plant-derived medicines can cause potential harm to the environment and human health. In particular, individuals that are already weak or sick may have an increased risk of pesticide toxicity even at a residual level. Meanwhile, the use of pesticides, including herbicides, fungicides, insecticides, and plant growth regulators, could also affect the secondary metabolism of plants [7-8]. Since the secondary metabolites are the main bioactive ingredients known to have various therapeutic effects, the recent trend in herbal medicine research addresses whether pesticides could induce variations on the level of plant secondary metabolites. Systematic evaluation of these variations would understand the relationship between action mechanism of pesticides and the metabolism of secondary metabolites thereby guiding the scientific use of pesticides in large-scale cultivation of medicinal plants [7]. Moreover, knowledge of such effects from pesticides, especially those negative effects on bioactive constituents, is beneficial for the holistic quality assessment of herbal medicines. Thus far, available studies on the effects of pesticides on constituent metabolism are limited only on a few compounds where targeted quantitative analysis method was employed [9-10].

Insecticides and fungicides are two types of frequently used pesticides in the large-scale cultivation of *L. japonica*. With regard to ingredient variations of LJF caused by the application of these pesticides, some published studies only focused on the chlorogenic acid and luteolin, which are known markers for its quality control, but other compounds still remain unexamined [4, 11-12]. Moreover, in these studies, inherent metabolic variability across the flowering development of *L. japonica* was also not taken into consideration. As a systematic program for quality assessment, our recent study has tentatively characterized 537 components from LJF, including 37% organic acids, 29% iridoids, 9% flavonoids, and 25% others, based on two-dimensional ultra-high performance liquid chromatography along with Q-Orbitrap mass spectrometry (2D UPLC/Q-Orbitrap MS) [13]. In the aspect of herbal medicines with the "multi-component and multi-target" property, there is still a lack of a systematic evaluation of the metabolite variations of LJF induced by commonly-used pesticides. Therefore, an untargeted method is necessary to cover its global chemical constituents.

In plant metabolomics, the integration of the unbiased acquisition of high resolution mass spectrometric (HRMS) or nuclear magnetic resonance (NMR) data and multivariate statistical analysis, has been proven to be a powerful tool for global metabolites assessment and monitoring metabolite variations [14]. This method has been successfully applied to resolve plant system issues, including species or geographical origin discriminations [15], comparison of cultivated plants with their processed types [16], investigations of influence on metabolic profiles from external or internal environmental stimuli [17], and so on. The workflow of plant metabolomics-based analysis commonly includes sample collection and preparation, unbiased metabolite profiling, data pretreatment and multivariate statistical analysis, and qualitative and quantitative analyses of metabolic markers [14]. Among comprehensive metabolite profiling, liquid chromatography-mass spectrometry (LC-MS) outperforms gas chromatography tandem mass spectrometry (GC-MS) and NMR, due to its powerful separation capacity in resolving a complex plant matrix, high sensitivity, and mass accuracy for a chemical structural elucidation [18]. Pattern recognition analyses, including unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discrimination analysis (OPLS-DA), are effective tools that are extensively used for discrimination and discovery of potential contributing markers [19].

To evaluate the effects of pesticides on the comprehensive constituents of LJF, the present study proposed an untargeted plant metabolomics-based strategy, as illustrated in Fig. 1. Two commercial insecticides, imidacloprid (IMI) and compound flonicamid and acetamiprid (CFA), which are frequently used in the cultivation of *L. japonica* were investigated. First, field trials were conducted on a cultivating field of *L. japonica*, with controls and treatments of IMI and CFA. The flower buds at the third green stage (TGS), the second white stage (SWS), and the complete white stage (CWS) were separately collected. Unbiased metabolite profiling was then performed by an untargeted UPLC/Q-Orbitrap-full scan method and the data were preprocessed by automatic extraction and screening. Furthermore, the generated data matrix was submitted for statistical analyses to evaluate the metabolite profiles and discover potential metabolic markers. Lastly, qualitative elucidation and relative quantification of these markers were conducted. To our knowledge, this is the first study that systematically evaluates the influence of insecticides on the comprehensive constituents of LJF using on untargeted plant metabolomics that simultaneously takes into account the inherent metabolic variability of the flowering development.

Materials and Methods

Chemicals and reagents

Commercial 70% imidacloprid (IMI) and 35% com-

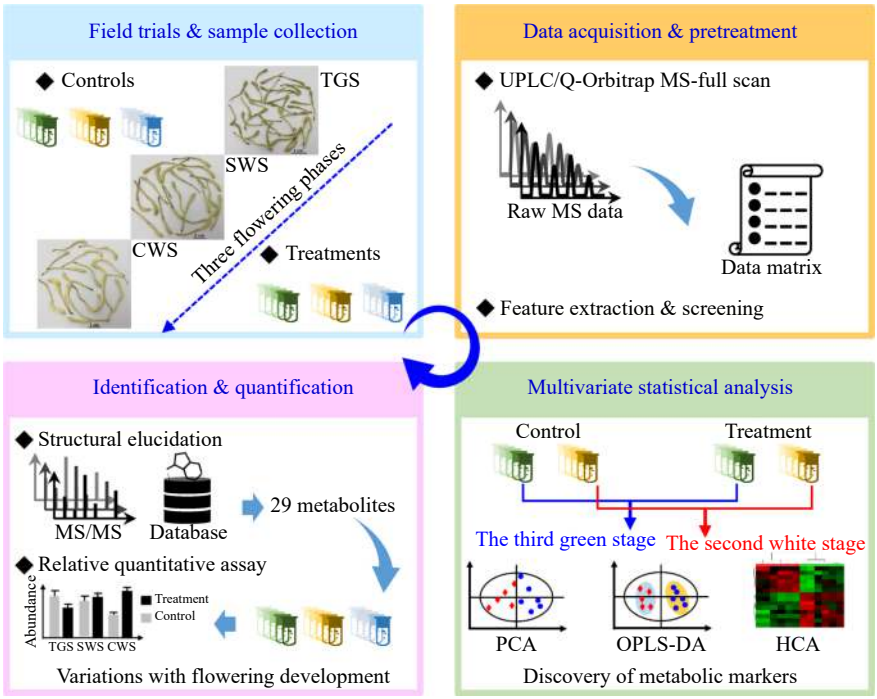

Fig. 1 Workflow of plant metabolomics-based study on the effect of insecticides on the comprehensive constituents in the flower buds of *Lonicera japonica*

pound preparation of flonicamid and acetamiprid (CFA) were purchased from Shenzhen Noposion Agrochemicals Co., Ltd. (Shenzhen, China) and Shanxi Hengtian Biological Agriculture Co., Ltd. (Shanxi, China), respectively. Both IMI and CFA insecticides were water-soluble granules. As reference standards, nine compounds were purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China) and Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China), namely, chlorogenic acid, isochlorogenic acid A, isochlorogenic acid C, sweroside, morroniside, loganin, loganic acid, secoxyloganin, and secologanic acid. Their structures are shown in Fig. S1.

As for other reagents, the LC/MS-grade water, methanol, and acetonitrile that were used for chromatographic separation were purchased from Merck KGaA (Darmstadt, Germany). The LC/MS-grade formic acid (FA), a chromatographic additive reagent, was purchased from Thermo Fisher Scientific (Shanghai, China).

Field trials and sample collections

Field trials were conducted on an uncovered cultivating plot of *L. japonica* in Fengqiu County, Henan Province, China, in June 2019. The three-year-old plants grown under uncontrolled environmental conditions were chosen for this experiment, which was consistent with the actual planting situation of LJF. The relative humidity and temperature recorded during the whole field trials were separately 35%−70% and 38/21 °C (day/night). There were 11 rows (Row 1–Row 11) on the trial plot and each row contained 12 plants. Each row and plant was separated by 2 meter- and 1.8 meter-distance, respectively. As illustrated in Fig. S2, plants in Row 1–Row 3 and Row 9–Row 11 were treated with IMI and CFA, respectively. Both commercial insecticides were sprayed once at three times the recommended dosages before the flowering phase (on June 6, 2019). The spraying concentrations of IMI and CFA solutions were 180 g a.i. hm^{-2} (three times the recommended dosages: 90−180 g a.i. hm^{-2}) and 450 g a.i. hm^{-2}, respectively. As for the control, plants in Row 5–Row 7 were maintained without the application of the two insecticides. Of note, the cultivating conditions of controls and treatments were kept consistent. Row 4 and Row 8 were used as guards between the control and the two treatments. The flower buds at the third green stage (TGS), the second white stage (SWS), and the complete white stage (CWS) were collected separately using an equidistant sampling method (Fig. S2). The selected fresh samples were immediately dried at 35 °C. Lastly, 17 batches of flower buds were collected from the control plot, 17 batches from the IMI-treated plot, and 18 batches from the CFA-treated plot.

Sample preparation

An aliquot of 0.5 g fine powder of each batch sample was weighed and immersed in 10 mL 50% aqueous methanol (*V*/*V*). The ultrasonic extraction on a water bath at 30 °C was subsequently operated for one hour and the obtained extract was centrifuged at 14 000 $r \cdot min^{-1}$ for 10 min. Then, the supernatant was diluted by ten folds with a 50% aqueous methanol. After another centrifugation at 14 000 $r \cdot min^{-1}$ for 10 min, the test solution was obtained and stored at 4 °C prior to analysis. To evaluate the stability of the analytical system, a quality control (QC) sample was yielded by pooling the equal volumes of the test solution from each batch material.

Metabolite profiling by UPLC/Q-Orbitrap-Full MS

An AQUITY UPLC system (Waters, Miliford, MA, USA) and an Agilent Poroshell EC-C18 column (3.0 mm × 150 mm, 2.6 μm) maintained at 35 °C were employed for the chromatographic separation. The elution solvent consisted of water containing 0.1% formic acid (*V/V*, A) and acetonitrile (B). The gradient elution was performed as follows: 0–1 min, 5% (B); 1–10 min, 5%–18% (B); 10–15 min, 18%–25% (B); 15–16 min, 25%–65% (B); 16–22 min, 65%–95% (B); 22–25 min, 95% (B). The flow rate was set as 0.4 mL·min^{-1} and the injection volume was 1 μL.

A Q-Exactive^{TM} hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with heated electrospray ionization (ESI) source was applied to acquire the MS data in the negative mode. The source parameters were the same as those in our previous work [13]. A full MS scan method was established to record data over a mass range of *m/z* 100–1500. A resolution of 70 000 (FWHM defined at *m/z* 200) was set for the Orbitrap analyzer. AGC target and maximum injection time were 3e6 and 100 ms, respectively. An inclusion-list-induced data-dependent MS^2 scan method (IL-ddMS^2) was developed to acquire MS/MS information. AGC target and maximum injection time were 1e5 and 50 ms, respectively. The isolation window was 3.0 (*m/z*). The scan resolution was 17 500. The stepped normalized collision energies (NCE) were used at the settings of 10%, 30%, and 50%. Minimum AGC target and apex trigger were respectively defined as 8e3 and 2–6 s. An Xcalibur 3.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control data acquisition.

Data analysis

A Progenesis QI 2.1 software (Waters, Milford, CT, USA) was applied for the automatic extraction. The retention time range was set over 0.1–17 min. A series of additive forms, including $[M - H]^-$, $[M + HCOO-]^-$, $[2M - H]^-$, $[3M - H]^-$, $[M + Na^+ - 2H]^-$, $[M + NO^{3-}]^-$, $[2M + HCOO^- - H]^-$, $[M + NO^{3-} + Na^+ - H]^-$, $[3M - 2H]^{2-}$, and $[M + HCOO^- + Na^+ - H]^-$ were selected or newly edited for ion fusions. Minimum absolute ion intensity for peak picking was defined as 5e4. The abundance of picked ions was normalized to all compounds. A SIMCA v14.1 software (Umetrics, Umea, Sweden) was applied for the multivariate statistical analysis. Principal component analysis (PCA) and orthogonal partial least squares discrimination analysis (OPLS-DA) models were developed to monitor the metabolite variations. *S*-plot and variable importance in projection (VIP) were applied to discover potential metabolic markers. The Graphpad Prism software was used to perform the Student's *t*-test.

Results and Discussion

Design of field trials

In a plant metabolomics study, correct cultivation and sample collection of plant materials of interest are essential for the evaluation of metabolite variations caused by a specific stimulus [20]. Since plant metabolites generally differ within developmental stages and are susceptible to environmental factors, such as soil, illumination, and temperature, it is essential to ensure a controlled cultivating condition to minimize any unwanted variations induced by these factors. In addition, collecting a large number of samples at the same developing stages is also beneficial to discover metabolic markers that are significantly correlated to a given effect [14]. To investigate the effect of two commonly used commercial insecticides on the comprehensive constituents of LJF, field trials were initially performed on an uncovered cultivating plot of *L. japonica*. The design of the field trial comprised of two groups independently treated with IMI and CFA and one control group without any insecticide treatment. In order to minimize unwanted impact from environmental factors, a complete and independent plot was selected to carry out the field trials. Consistent cultivating conditions and processes with the actual planting situation of LJF were maintained across experimental conditions. Moreover, blank rows without any insecticide application were designed as guards between the control and the two treatments. Following the practical operations, both treatments were performed once before the flowering phase, about ten days before the harvest. To mimic the overuse of pesticides [5], the spraying concentrations of two insecticides were at three times the recommended dosages. Afterward, flower buds at three stages, TGS, SWS and CWS, were separately collected. Taking the limited production into consideration, an equidistant sampling method was used. It was worthy to note that, although repeat trials and collecting a large number of samples are certainly beneficial for further increasing the credibility of the experimental results, the sample size obtained in this study could basically meet the requirements of statistical analysis.

Unbiased metabolite profiling and data pretreatment

The untargeted full scan method based on UPLC/Q-Orbitrap MS was developed for unbiased chemical profiling of constituents in LJF. The chromatographic separation conditions and source parameters were kept consistently optimized as described previously in our recent work [13]. To assess system stability, a continuous acquisition was applied and the QC sample was analyzed after every six injections of the test solutions.

To perform the multivariate statistical analysis, data pretreatment was necessary to generate a data matrix involving meaningful metabolite features. First, the acquired raw data were imported into the Progenesis QI software. Peak alignment, ion fusion, deconvolution, peak picking, and normalization were successively performed by following its automatic processing workflow. Under the present ionization parameters, the chemical constituents in LJF, especially the major ones in the negative mode, tended to form multiple degenerate features, such as $[M - H]^-$, $[M + HCOO^-]^-$, $[2M - H]^-$, $[3M - H]^-$, $[M + Na^+ - 2H]^-$, $[M + NO^{3-}]^-$, $[2M + HCOO^- - H]^-$, $[M + NO^{3-} + Na^+ - H]^-$, $[3M - 2H]^{2-}$, and $[M + HCOO^- + Na^+ - H]^-$. To simplify the data, an application of in-source collision energy was tried to decrease the formation of these

complex adduct ions [21]. However, an obvious improvement was not observed with the increase of in-source collision energies. Thus, these adduct forms were set in the Progenesis QI platform for ion fusion and automatic deconvolution. As a result, a total of 2358 metabolite features and their normalized abundance were extracted in a csv file. Next, these metabolite features were further screened using a four-step filtering strategy to remove those metabolism-unrelated interference ions. In step I, a total of 119 unstable features were removed according to a 30% rule. Namely, the relative standard derivation (RSD) of the normalized abundance of these ions in all QC injections was more than 30% [22]. In step II, a total of 2228 metabolite features were retained after removing those with non-zero values of normalized abundance in < 80% test samples [23]. In step III, a mass defect filtering (MDF) method developed in our recent work was applied to eliminate metabolism-unrelated interference ions [13]. As illustrated in Figure S3, ions located within the two MDF windows which were pre-defined according to the constituents isolated from *L. japonica* were considered as meaningful metabolites. According to a series of "IF" functions on the Microsoft Excel platform, 1490 metabolite features in blue were obtained by deleting approximately 33% ions in black. In step IV, the blank matrix interferences (setting an abundance ratio in blank and QC samples above 20%), two-charge ions, and complex adduct ions of the major components were further eliminated. Consequently, a data matrix composed of 1317 metabolite features and their normalized abundance was generated. The missing values were input with minimum metabolite abundance across all features.

Multivariate statistical analysis

After data extraction and screening, the obtained data matrix was imported into the SIMCA software for multivariate statistical analysis. In total, 1317 metabolite features were defined as variables and scaled by Pareto (Par). PCA and OPLS-DA models were developed to explore the metabolite variations of LJF, which was induced by the commercial insecticides IMI and CFA. Taking into consideration the inherent metabolic variability of the flowering development, the effect of chemical constituents of the flower buds at TGS and SWS of *L. japonica* was examined. Given that the morphological features of flower buds at CWS are likely to change by further processing, the metabolic markers induced by two insecticides were not included in the analysis.

Metabolite variations of LJF induced by IMI

A total of six batches of IMI-treated flower buds and six controls collected at TGS were first investigated. To get an overview of the sample distributions of the two groups, a PCA model was developed in which three autofitted model dimensions that resulted in $R^2X = 0.95$ and $Q^2 = 0.85$ were applied to reduce the data dimensionality. As depicted in Fig. 2A, the score plot of PCA showed that the two groups were completely separated by the first principal components. The first two principal components indicated the 89.7% of the variations in the original dataset [$R^2X(cum) = 0.897$]. No outliers were detected within 95% Hotelling T^2 ellipse. It demonstrated that the usage of IMI caused changes in the global metabolic profiles of flower buds at TGS. To determine the affected metabolites, a supervised OPLS-DA model was built for further classification. Acceptable fitness and predictability of the model was represented by the values of R^2X (0.95) and Q^2 (0.92), respectively. Moreover, the Root Mean Square Error of Estimation (RMSEE) value at 0.108 also indicated the excellent fitness of the OPLS-DA model. From its score plot (Fig. 2B), a clear separation of the two groups was also observed. Afterward, the *S*-plot and VIP plot were constructed to determine the contributing metabolic markers. As shown in Figs. 2C and 2D, a total of 27 metabolite features, which are highlighted in red in the *S*-plot, were obtained by defining the VIP threshold value at 2. Furthermore, Student's *t*-test was performed to evaluate the abundance variations of these features between the two groups. After eliminating 14 features with *P* values > 0.05 and one adduct ion, 12 potential markers were finally disclosed. For intuitive visualization of the variations, a dataset embodying these 12 components and their abundance was submitted into Metaboanalyst. For hierarchical cluster analysis, distance measure of Euclidean and Ward clustering algorithm were selected. As shown in the heatmap (Fig. 2E), the contents of 12 compounds in the flower buds of TGS were obviously different between the two groups, presenting a decrease of two components and an increase of the other ten after the IMI treatment.

In the same way, the IMI-treated and the control groups of the flower buds collected at SWS were submitted for statistical analysis (Fig. S4). From the score plot of the PCA model, the two groups were clearly segregated. The first two principal components described the 87.5% of the variations [$R^2X(cum) = 0.875$] implying that the model could well represent the variations of the original dataset and the different metabolite profiles of the two conditions. For further classification, the OPLS-DA model with good fitness ($R^2X = 0.95$; RMSEE = 0.037) and predictability ($Q^2 = 0.99$) was constructed in which the score plot presented a complete separation of two groups. Next, the *S*-plot and the VIP plot were established to give 24 differential variables with VIP values > 2, which were significantly responsible for the classification. Using the Student's *t*-test, a total of 9 components with *p*-values > 0.05 were finally considered to be the potential metabolic markers. As shown in the heatmap, the contents of the six components increased and the contents of the three decreased after IMI application, all of which occurred in the flower buds of SWS (Fig. S5).

Metabolite variations of LJF induced by CFA

Using a similar approach described above, the chemical constituents in the flower buds of *L. japonica* were compared between CFA-treated samples and controls through multivariate statistical analysis (Fig. S6). The score plot of the PCA model for the flower buds at TGS showed that the CFA-treated samples were well-separated with the controls. The

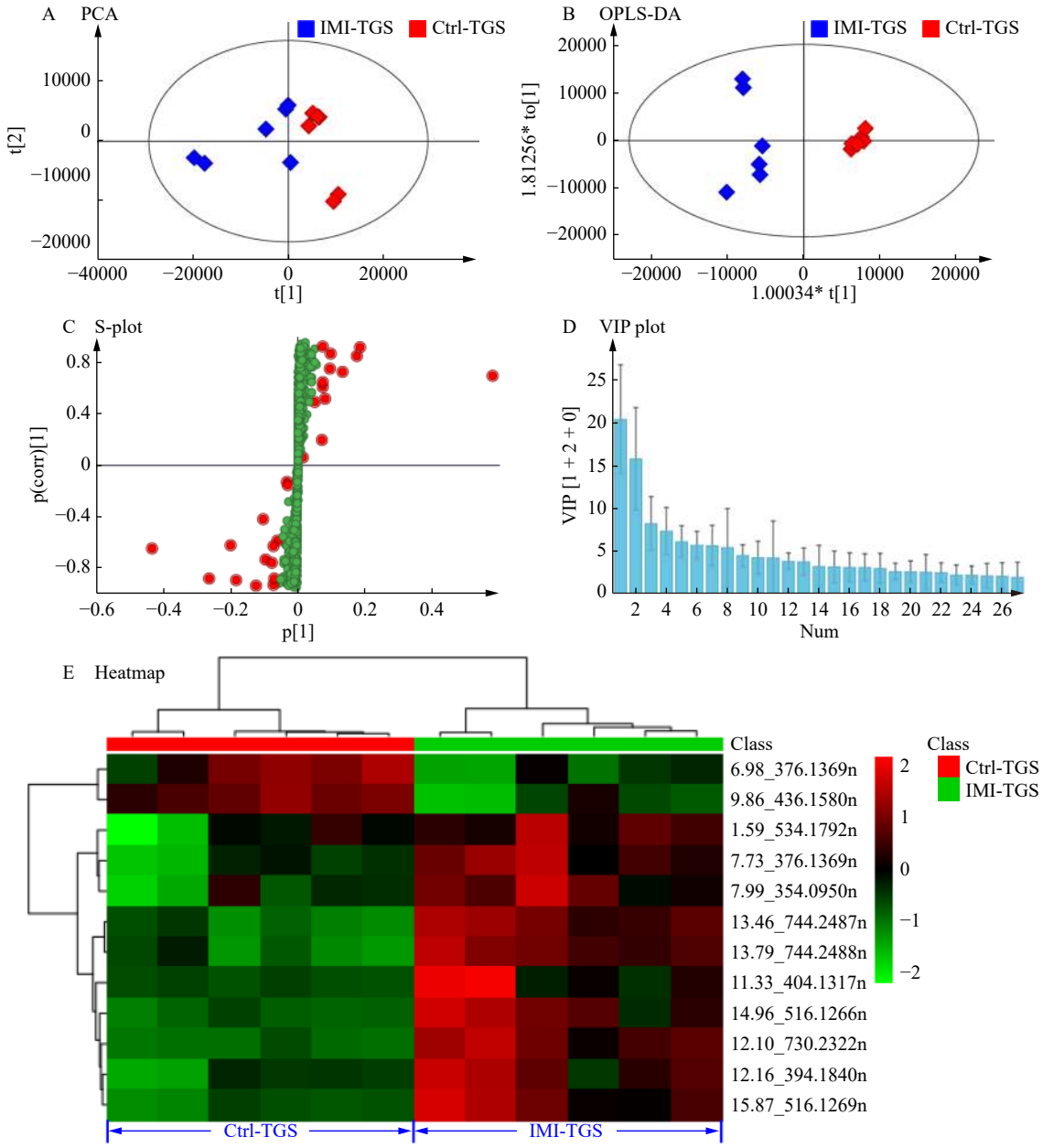

Fig. 2 Multivariate statistical analysis for monitoring the metabolic variations of the flower buds at third green stage (TGS) induced by imidacloprid (IMI). (A) The score plot of PCA, (B) the score plot of orthogonal partial least squares discrimination analysis (OPLS-DA), (C) the *S*-plot, (D) the variable importance in projection (VIP) plot, and (E) the heatmap, based on the abundance of 12 potential metabolic markers

first two principal components contributed 84.4% to total variations [$R^2X(cum) = 0.844$] in the original data and no outliers were observed. Furthermore, the OPLS-DA model integrated with the *S*-plot and VIP plot was employed to determine which variables contributed to the classification of two groups. Good fitness and predictability of the OPLS-DA model were represented by the values of R^2X (0.84) and RMSEE (0.073), and Q^2 (0.97), respectively. Based on the VIP values (> 2) and *t*-test ($P < 0.05$), a total of 13 metabolite features were considered as potential markers. The relative content distributions of these components in the two groups are shown in the heatmap. Among these, seven components displayed relatively higher contents in the CFA-treated samples while the others presented relatively higher contents in the controls.

The dataset of flower buds at SWS was also submitted for a systematic comparison of the CFA-treated group and the control (Fig. S7). A PCA model was developed to visualize the distributions of the two groups and to detect potential outliers. From its score plot, two groups were clearly separated by the first principal component and the contribution of the first two principal components to total variance in the origin-

al data was 80.8% [$R^2X(cum) = 0.808$]. For further classification, an OPLS-DA model was autofitted to obtain good fitness (R^2X = 0.80; RMSEE = 0.162) and predictability (Q^2 = 0.87). Twenty-six features with VIP values > 2 are highlighted in red in the *S*-plot. Coupled with the Student's *t*-test, a total of 8 potential markers with $P < 0.05$ were screened. As shown in the heatmap, the use of CFA resulted in a decrease in the contents of the two compounds and an increase in the contents of the other compounds in the flower buds of SWS.

Qualitative characterization of metabolic markers

A total of 29 metabolic markers were determined using multivariate statistical analysis. To further elucidate their structures, a targeted inclusion-list-induced $ddMS^2$ method was developed based on UPLC/Q-Orbitrap MS to acquire the MS/MS information. The inclusion list was composed of accurate *m/z* and retention time windows of the 29 compounds. According to the obtained high-resolution MS and M/MS information, these compounds were identified and tentatively characterized as shown in Table 1. Among these, nine compounds were unambiguously identified by comparing the retention time and MS/MS spectra with reference standards. Here, compound **17** was described as an example of how other compounds should be interpreted. In the negative mode, the compound easily presented as the formate anion at *m/z* 743.2262, which was evidenced by its deprotonated ion of *m/z* 697.2195 in the MS/MS spectrum (Fig. S8). Thus, its molecular formula was speculated as $C_{28}H_{42}O_{20}$ (1.4 ppm). Through further analysis of its fragmentation ways, compound **17** produced a pair of complementary ions at *m/z* 341.11 and *m/z* 355.10. In combination with the diagnostic ion of *m/z* 179.06 ($[C_6H_{11}O_6]^-$) and the neutral losses of $C_6H_{10}O_5$ and $C_6H_{12}O_6$, it was suggested that the product ion at *m/z* 341.11 was a residue of diglucose. Based on the diagnostic ion of feruloyl at *m/z* 193.05 was generated from a product ion of *m/z* 355.10 through a neutral elimination of $C_6H_{10}O_5$, which implied the presence of another residue, feruloyl glucoside. Therefore, compound **17** was tentatively characterized as a feruloyl triglucoside. Unfortunately, it was difficult to confirm the connections between feruloyl and sugars by MS data alone.

Relative quantification of the metabolic markers

The structural elucidation revealed that organic acids, iridoids, sugars, and organic acid-glucosides were among the 29 compounds discovered. To describe the metabolite variations induced by IMI and CFA, relative quantification analyses of these compounds in the flower buds at three flowering stages (TGS, SWS, and CWS) were performed based on the raw abundance data.

Effects of IMI on the chemical constituents of LJF

A total of 19 compounds, which comprised of 3 chlorogenic acid isomers, 10 iridoids, glucose, quinic acid, and 4 organic acid glucosides in *L. japonica*, showed substantial variations after the IMI treatment. Results of the relative quantitative assay (Fig. 3) showed that (1) the contents of the three identified chlorogenic acid isomers, chlorogenic acid, isochlorogenic acid A, and isochlorogenic acid C, tended to decrease across the flowering development in the IMI-treated samples compared to the controls. A remarkable increase of the three compounds was detected in the flower buds of TGS. (2) To some extent, the contents of the four iridoids, loganin, secologanin, morroniside, and loganic acid, showed a decreased amount after IMI treatment, of which significant decrease of loganin at TGS and CWS, secologanin at SWS and CWS, morroniside at SWS, and loganic acid at TGS were observed. (3) The contents of secologanic acid, secoxyloganin, and two tentatively characterized strychoside isomers, secologanioside and euphroside, were relatively higher in the IMI-treated samples than the control group (Fig. S9). (4) As shown in Figure S10, in the flower buds of SWS and CWS, the content of glucose remarkably decreased while the content of quinic acid significantly increased after the IMI treatment. Moreover, four characterized organic acid glucosides presented a relatively higher content in the IMI-treated samples.

Chlorogenic acids are the main bioactive ingredients of LJF. Its significant antimicrobial, anti-inflammatory, and antiviral activities facilitate the wide usage of LJF in the treatment of flu and severe acute respiratory syndrome (SARS). Based on our findings, the proper utilization of IMI in the cultivation of *L. japonica* would not affect the quality of LJF due to the positive effect of IMI on the three chlorogenic acids. However, the results of the untargeted strategy suggested that the application of IMI can cause variations in iridoids, including both negative and positive influences. Of note, iridoids contain anti-flammatory, anti-tumor, neuroprotective, and hepatoprotective activities. The negative affect in iridoids suggested that it is essential to take precautions in using this type of insecticides. Furthermore, these decreased iridoids induced by the application of IMI could also be used as quality markers for assessing the quality consistency of LJF.

Effect of CFA on the chemical constituents of LJF

CFA-induced variations were observed in 17 components including 10 iridoids, diglucose, citric acid, 4 feruloyl-triglucoside isomers, and 1 unknown glucoside. The relative quantitative analyses of these compounds across the flowering development were summarized in Fig. 4. (1) The contents of three iridoids, secoxyloganin, secologanioside A, and *L*-phenylalaninosecologanin, showed a significant decrease in the flower buds of TGS while these compounds showed an increasing trend at SWS and CWS. (2) The contents of the identified morroniside, lamalbide, and secologanin decreased to varying extents at three flowering stages, whereas (3) secologanioside and sweroside remarkably increased at three flowering stages. (4) The level of euphroside at SWS and CWS, and secologanic acid at SWS were significantly increased. (5) As shown in Figure S11, the citric acid contents decreased substantially at TGS. Four characterized feruloyl-triglucoside isomers were remarkably increased at the three

Table 1 The detailed information of 29 varied metabolites induced by IMI and CFA

No.	Compound ID	t_R/min	*m/z*	Mass error/ppm	Adducts	Formula	MS/MS information	Characterization	Treatments
1	1.48_225.0615*m/z*	1.48	225.0615	−0.491	$[M + HCOO^-]^-$	$C_6H_{12}O_6$	179.06, 161.04, 87.01	glucose	IMI
2	1.56_342.1161n	1.56	387.1143	−0.346	$[M + HCOO^-]^-$	$C_{12}H_{22}O_{11}$	341.11, 179.06, 161.04	diglucose	CFA
3	1.59_534.1792n	1.59	533.1720	−0.699	$[M - H + H_2O]^-$	$C_{19}H_{32}O_{16}$	191.06, 173.04	qunic acid-diglucose	IMI
4	1.60_192.0632n	1.60	383.1193	−0.416	$[2M - H]^-$	$C_7H_{12}O_6$	191.06, 173.04, 127.04	quinic acid	IMI
5	2.09_191.0197*m/z*	2.09	191.0197	−0.292	$[M - H]^-$	$C_6H_8O_7$	111.01, 87.01, 129.02, 173.00	citric acid	CFA
6	4.54_390.1164n	4.54	389.1091	0.399	$[M - H]^-$	$C_{16}H_{22}O_{11}$	227.06, 209.05, 183.07, 165.05, 121.06	secologanoside	IMI, CFA
7	6.07_422.1426n	6.07	421.1353	0.405	$[M - H]^-$	$C_{17}H_{26}O_{12}$	241.07, 389.11, 197.04, 179.06, 139.00	lamalbide	CFA
8*	6.98_376.1369n	6.98	375.1296	−0.160	$[M - H]^-$	$C_{16}H_{24}O_{10}$	213.08, 169.09, 113.02, 151.08	loganic acid	IMI
9	6.98_698.2260n	6.98	743.2257	0.719	$[M + HCOO^-]^-$	$C_{28}H_{42}O_{20}$	341.11, 697.22, 355.10, 179.06, 193.05	feruloyl-triglucoside	CFA
10*	7.67_406.1473n	7.67	451.1457	0.013	$[M + HCOO^-]^-$	$C_{17}H_{26}O_{11}$	405.14, 243.09, 179.06, 155.03, 141.05	morroniside	IMI, CFA
11	7.73_376.1369n	7.73	375.1296	−0.213	$[M - H]^-$	$C_{16}H_{24}O_{10}$	151.08, 195.07, 169.09, 119.03	euphroside	IMI, CFA
12*	7.99_354.0950n	7.99	707.1830	0.159	$[2M - H]^-$	$C_{16}H_{18}O_9$	191.06	chlorogenic acid	IMI
13	8.20_698.2266n	8.20	743.2269	2.320	$[M + HCOO^-]^-$	$C_{28}H_{42}O_{20}$	341.11, 697.22, 355.10, 179.06, 193.05	feruloyl-triglucoside	CFA
14*	8.60_374.1210n	8.60	747.2357	0.540	$[2M - H]^-$	$C_{16}H_{22}O_{10}$	193.05, 149.06, 167.07, 119.03, 97.03	secologanic acid	IMI, CFA
15	8.63_508.1788n	8.63	507.1715	−0.766	$[M - H]^-$	$C_{21}H_{32}O_{14}$	89.02, 101.02, 327.11, 357.12	secologanoside A	CFA
16	8.84_698.2270n	8.84	743.2260	1.109	$[M + HCOO^-]^-$	$C_{28}H_{42}O_{20}$	341.11, 697.22, 355.10, 179.06, 193.05	feruloyl-triglucoside	CFA
17	9.05_744.2334n	9.05	743.2262	1.364	$[M + HCOO^-]^-$	$C_{28}H_{42}O_{20}$	341.11, 697.22, 355.10, 179.06, 193.05	feruloyl-triglucoside	CFA
18*	9.86_436.1580n	9.86	435.1508	−0.091	$[M - H]^-$	$C_{17}H_{26}O_{10}$	227.09, 101.02, 127.04, 317.18, 389.15	loganin	IMI
19*	10.10_404.1319n	10.10	403.1247	0.162	$[M - H]^-$	$C_{17}H_{24}O_{11}$	125.02, 195.07, 179.06, 357.12	sweroside	CFA
20*	11.33_404.1317n	11.33	403.1244	−0.433	$[M - H]^-$	$C_{17}H_{24}O_{11}$	371.10, 223.06, 165.05, 179.06, 121.03	secoxyloganin	IMI, CFA
21	11.78_730.2322n	11.78	729.2249	0.217	$[M - H]^-$	$C_{32}H_{42}O_{19}$	453.14, 409.15, 281.12, 229.09, 177.06	unknown glucoside	IMI
22	12.00_452.1525n	12.00	433.1350	−0.414	$[M + HCOO^-]^-$	$C_{17}H_{24}O_{10}$	179.05, 155.03, 225.08, 101.02, 89.02	secologanin	IMI, CFA
23	12.03_537.2209n	12.03	536.2136	−0.269	$[M - H]^-$	$C_{26}H_{35}NO_{11}$	164.07, 147.04, 228.10, 272.09, 312.16, 356.15	*L*-phenylalaninosecologanin	CFA
24	12.10_730.2322n	12.10	729.2250	0.299	$[M - H]^-$	$C_{32}H_{42}O_{19}$	453.14, 399.13, 281.12, 229.09, 177.06	unknown glucoside	IMI, CFA
25	12.16_394.1840n	12.16	439.1818	−0.659	$[M + HCOO^-]^-$	$C_{17}H_{30}O_{10}$	393.18, 149.04, 89.02, 101.02, 191.06	hexenyl *O*−xylopyranosyl-glucopyranoside	IMI
26	13.46_744.2487n	13.46	743.2415	1.423	$[M - H]^-$	$C_{33}H_{44}O_{19}$	511.14, 467.15, 339.12, 287.09, 255.10, 203.03	strychoside A or isomer	IMI
27	13.79_744.2488n	13.79	743.2415	1.491	$[M - H]^-$	$C_{33}H_{44}O_{19}$	511.14, 467.15, 339.12, 287.09, 255.10, 203.03	strychoside A or isomer	IMI
28*	14.96_516.1266n	14.96	515.1194	−0.290	$[M - H]^-$	$C_{25}H_{24}O_{12}$	191.06, 179.03, 135.04, 353.09	isochlorogenic acid A	IMI
29*	15.87_516.1269n	15.87	515.1196	0.196	$[M - H]^-$	$C_{25}H_{24}O_{12}$	173.04, 179.03, 191.06, 135.04, 353.09	isochlorogenic acid C	IMI

*: Fully identified by comparing with reference standards

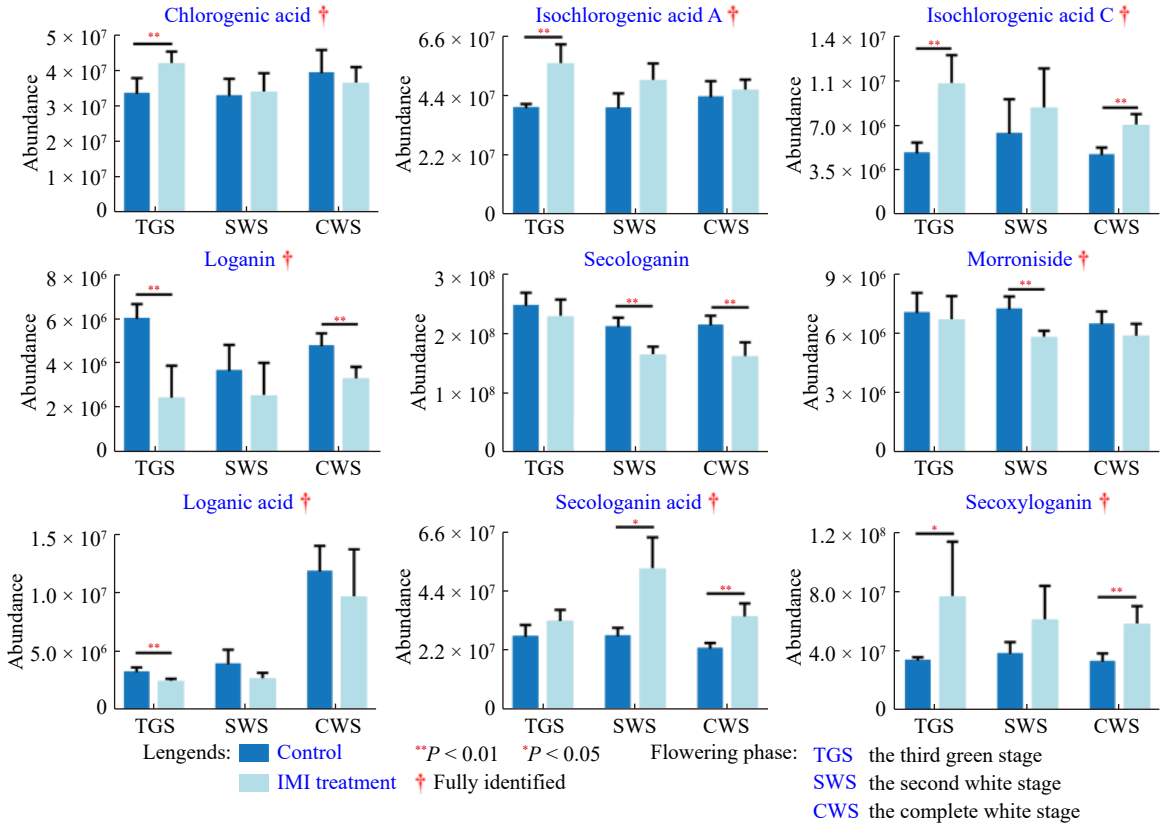

Fig. 3 IMI-induced metabolite variations across the flowering development through relative quantitative assay. (IMI-TGS, Ctrl-TGS and Ctrl-SWS, *n* = 6; IMI-SWS, *n* = 4; IMI-CWS, *n* = 7; Ctrl-CWS, *n* = 5)

flowering stages. Diglucose and one unknown glucoside also increased.

In comparison, the application of CFA did not substantially affect the metabolism of chlorogenic acids. The metabolic variations were mainly observed in iridoids, in which both positive and negative effects on the contents were detected through a relative quantitative analysis. These findings implied that the frequent utilization of CFA in the cultivation of *L. japonica* would also affect the related therapeutic effects contributed by iridoids. Therefore, proper utilization of CFA is important to ensure the quality, efficacy, and consistency of LJF.

Conclusively, using the untargeted plant metabolomics-based strategy, the present study revealed that the two insecticides, IMI and CFA, mainly caused a variation in the metabolism of iridoids and organic acid-glucosides. The detected negative effect on some iridoids, such as morroniside, secologanin, loganin, and loganic acid, indicated that these iridoids should be paid more attention in the holistic quality assessment of LJF. Additionally, a significant decrease in the contents of chlorogenic acids and flavonoids were not detected in this study. Our data demonstrated that the proposed strategy was powerful enough to assess systematically the metabolic variations induced by pesticides. Moreover, it is worthy to note that, for the purpose of guiding the scientific use of IMI and CFA, risk assessments of their terminal residues are needed.

Conclusion

To assess the holistic quality of LJF as influenced by pesticides, the present study systematically explored the effects of two frequently-used commercial insecticides in large-scale cultivation of *L. japonica*, IMI, and CFA, on the comprehensive constituents of LJF. For the first time, a plant metabolomics-based strategy, which integrated the design of field trials, untargeted chemical profiling, and multivariate statistical analysis, was proposed and applied to examine the unbiased metabolic variations induced by pesticides. In addition, this study took into account any inherent variations across the flowering development by analyzing the flower buds at TGS, SWS and CWS of *L. japonica*. Our findings revealed that both IMI and CFA influenced the global metabolic profiles of flower buds at three flowering stages of *L. japonica*. The results showed positive and negative regulations in the contents of iridoids, suggesting that the overuse of the two insecticides might impact the iridoid-related anti-inflammatory, anti-tumor, neuroprotective, and hepatoprotective activities. Accordingly, these decreased iridoids should be considered as important markers in the holistic quality assessment of LJF. With regard to the reasonable utilization of the two insecticides, further evaluation is needed in combination with their terminal residues in LJF. The study proved that the

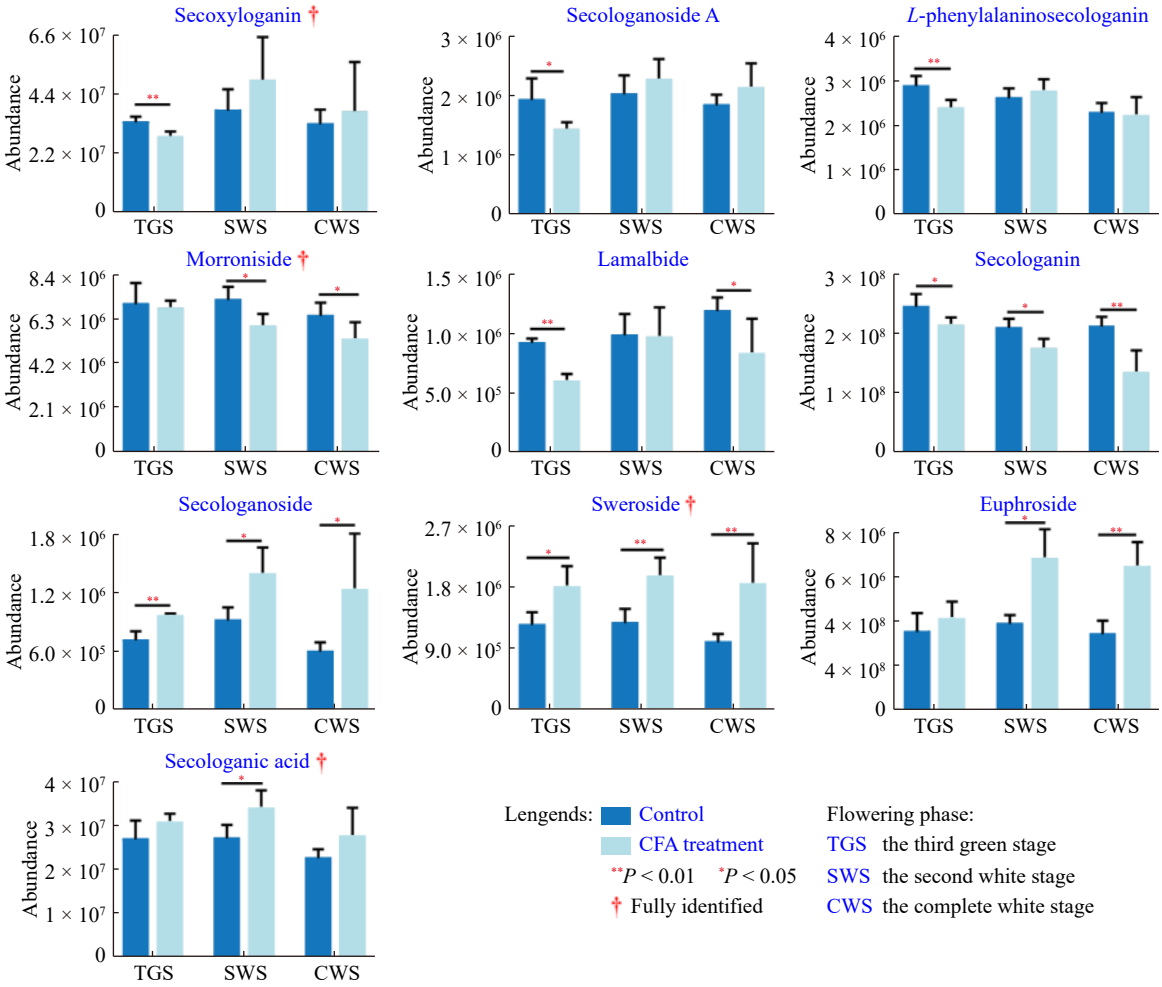

Fig. 4 Compound flonicamid and acetamiprid (CFA)-induced metabolite variations across the flowering development through relative quantitative assay. (CFA-TGS and Ctrl-CWS, *n* = 5; Ctrl-TGS and Ctrl-SWS, *n* = 6; CFA-SWS, *n* = 4; CFA-CWS, *n* = 7)

proposed strategy was powerful in a comprehensive evaluation of metabolic variations induced by pesticides.

Supplementary Materials

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

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