



## Detection and classification of volatile compounds emitted by three fungi-infected citrus fruit using gas chromatography-mass spectrometry

Jue Wu <sup>a,b,c</sup>, Jinping Cao <sup>a,b,c</sup>, Jiebiao Chen <sup>a,b,c</sup>, Lingxia Huang <sup>d</sup>, Yue Wang <sup>a,b,c</sup>, Cui Sun <sup>a,b,c,\*</sup>, Chongde Sun <sup>a,b,c</sup>

<sup>a</sup> Laboratory of Fruit Quality Biology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China

<sup>b</sup> Horticultural Products Cold Chain Logistics Technology and Equipment National-Local Joint Engineering Laboratory, Hangzhou 310058, PR China

<sup>c</sup> Zhejiang Provincial Key Laboratory of Integrative Biology of Horticultural Plants, Zhejiang University, Hangzhou 310058, PR China

<sup>d</sup> College of Animal Sciences, Zhejiang University, Zijingang Campus, Hangzhou 310058, PR China

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### ABSTRACT

Citrus fruit produced some characteristic volatile compounds when infected by fungi compared with the healthy fruit. In the present study, volatile metabolites of postharvest citrus fruit with three different diseases including stem-end rot, blue mold and green mold were detected. Multivariate analysis such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were employed to classify the volatile compounds between the infected and non-infected citrus fruit. The results indicated that volatile compounds of unrotten, unrotten-rotten junction, and rotten tissues were successfully classified. Importantly, eight volatile compounds as biomarkers for stem-end rot and one biomarker for green mold of citrus were screened to discriminate the infected citrus fruit. This study offers the application potential of odor profiling of volatile compounds for detecting the fungi infection in postharvest citrus fruit.

### 1. Introduction

Citrus (*Citrus reticulata* Blanco) is one of the most productive fruits in the world and consumed preferentially by humans due to its special flavor and various nutrients including functional polysaccharides,  $\beta$ -carotene, vitamin C and flavonoids (Sharif, Khan, Iqbal, Azam, Lali, & Javed, 2018; Wang et al., 2022). However, citrus fruit after harvest was susceptible to be infected by pathogenic fungi such as *Penicillium italicum*, *Penicillium digitatum*, *Lasiodiplodia theobromae* and *Alternaria alternata*, leading to a great quantity of fruit decay during the postharvest transportation and storage (Liu et al., 2019; Wang et al., 2021). These fungi rots were very difficult to prevent and control by any registered fungicides once the fruits were infected by these pathogen (Liu et al., 2019; Soto-Munoz, Taberner, de la Fuente, Jerbi, & Palou, 2020). Therefore, early detection for infected citrus fruit with a rapid, sensitive and non-destructive approach and separation of these infected fruit from the healthy fruit is very important to reduce economic losses and enhance fruit quality.

In general, the identification of infected fruits was mainly dependent on the visual inspection based on the decayed symptoms on the fruit

surface by inspectors or based on touched-texture (Huang, Meng, Zhu, & Wu, 2017). The efficiency of this visual inspection or touch methods are not suitable for large-scale storage of citrus fruits, and the results are greatly affected by the subjective judgment of the inspector (Huang et al., 2017; Sanaeifar, ZakiDizaji, Jafari, & de la Guardia, 2017; Wen et al., 2019). Furthermore, in the early stage of pathogen infection, the infected fruit is difficult to be found because the disease spot has not been formed on the fruit surface, but the fungi had invaded the fruit issue at this time. On the other hand, the rotted tissues inside the fruit caused by pathogenic fungi could be discriminated through slicing the fruit (Wen et al., 2019). This destructive detection method has exhibited several shortcomings in practical application, such as enormous samples, considerable inspectors, seriously waste and time-exhausting. The identification techniques for infected fruit have been considerably advanced over past few years. For example, hyperspectral imaging, low-field nuclear magnetic resonance or their combination have been employed to detect the decayed blueberry and citrus fruit with high accuracy (Folch-Fortuny, Prats-Montalban, Cubero, Blasco, & Ferrer, 2016; Galed, Fernandez-Valle, Martinez, & Heras, 2004; Qiao, Tian, Wang, Song, & Song, 2021). The near-infrared spectroscopy technology

\* Corresponding author at: College of Agriculture and Biotechnology, Zhejiang University, PR China.

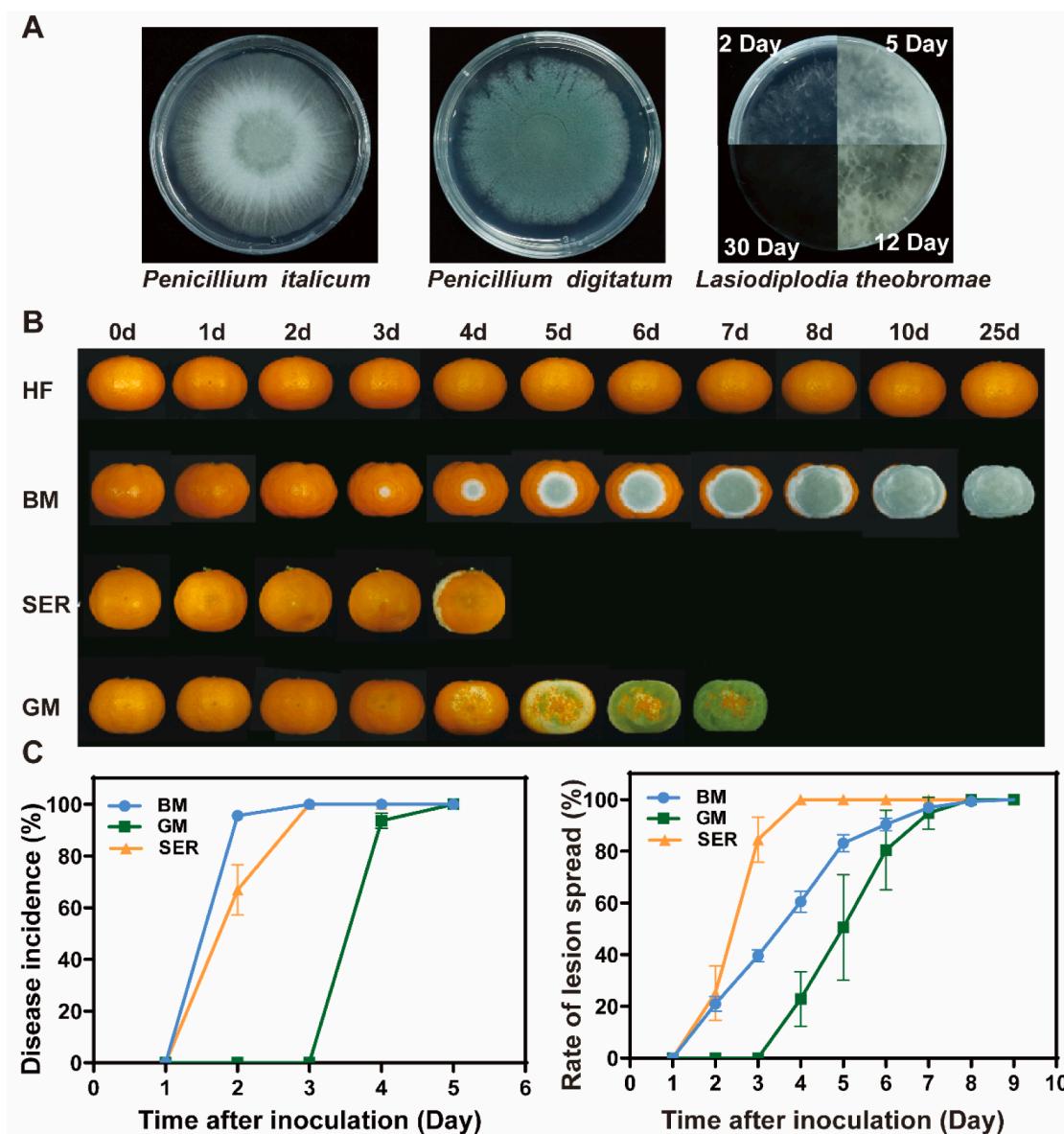
E-mail address: [suncui7@126.com](mailto:suncui7@126.com) (C. Sun).

was also applied to discriminate the decayed and healthy grape fruits (Ding, Dong, Jiao, & Zheng, 2017). These technologies have provided effective and precise methods in the detection of fungal infected post-harvest fruit, but the corresponding instruments were too expensive, and required skilled operating staff, which could not be popularized and applied in practical production.

Unlike the afore-mentioned methods, a fast, non-destructive and sensitive method was proposed for discriminating the fungal infected fruit through detecting the volatile organic compounds emitted by fruit using gas chromatography and mass spectrometry (GC-MS), electronic nose (E-nose) or gas chromatography ion-mobility spectrometry (GC-IMS) (Huang et al., 2017; Seong Mi, Sang Mi, Jeong-Ah, & Young-Suk, 2018). The volatile organic compounds in terms of the categories and levels changed when the pathogenic fungi infected the fruit (Ding et al., 2017; Seong Mi et al., 2018). An enormous amounts of studies have been providing the evidences that the volatile organic compounds emitted by the fungal infected fruit was different from that of non-infected fruit (Nouri, Mohtasebi, & Rafiee, 2020; Pallottino et al., 2012; Pan, Zhang,

Zhu, Mao, & Tu, 2014). These different volatile organic compounds might be the molecule markers to identify the rotted fruit by postharvest pathogenic fungi. Seong Mi et al. (2018) have detected some fungal volatile metabolites, including (E)-hex-2-enal, 1-methoxy-3-methylbenzene, methyl heptanoate, diethyl carbonate, ethyl 2-pheylacetate, propyl octanoate, as well as ethyl decanoate, which was considered as the “character marker” to identified the infected apples by *P. expansum*. Lopez et al. (2015) has determined two biomarkers of butanone and  $\alpha$ -pinene in the decayed pear fruit by *Rhizopus stolonifer* through comparing the volatile profile of *R. stolonifer* inoculated pear fruit with that of non-inoculated pear fruit. Although a lot of studies have identified the characteristic volatile organic compounds in the pathogenic fungal infected-fruit with different species of pathogenic fungi, there is still no report on the detection of volatile organic compounds from the citrus fruit infected with three different pathogens.

In the present study, citrus fruits were inoculated with three different postharvest pathogens that is *P. italicum*, *P. digitatum* and *L. theobromae*, and the corresponding peel and flesh tissues were collected at certain rot



**Fig. 1.** Pathogenicity analysis of three fungi to postharvest citrus fruit. (A) The colony morphology of *Penicillium italicum*, *Penicillium digitatum* and *Lasiodiplodia theobromae*. (B) Disease symptoms of citrus fruit during storage before inoculating with *P. italicum*, *P. digitatum* and *L. theobromae*. (C) Disease incidence and rate of lesion spread of citrus fruit with the stem-end rot (SER), blue mold (BM) and green mold (GM). Error bars represent standard deviation and three replicates for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stages. The volatile compounds emitted by fruit tissues and the whole fruit were detected and identified by GC-MS. Multivariate statistical analysis including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were applied to discriminate the infected and non-infected citrus fruit. Finally, the characteristic volatile compounds were screened as biomarkers to detect the infected-citrus fruit.

## 2. Materials and methods

### 2.1. Fruit

The citrus fruit (*Citrus unshiu* Marc. 'Miyagawa wase') at the maturity stage was picked from an orchard in Yongquan town, Linhai city, Zhejiang province. The fruits were transported to the lab of zhejiang university at the harvest day. The citrus fruits with uniform size, consistent maturity, no injury, and fungi infection were chosen for formal experiment, and stored at 10 °C in a cold storage before use.

### 2.2. Pathogen

The strains of *Penicillium italicum* was purchased from CGMCC and *Penicillium digitatum* was provided by the professor Zheng Xiaodong in Zhejiang university. *Lasiodiplodia theobromae* was isolated from the decayed citrus fruit with stem-end rot symptoms. 18S combined with ITS sequence were conducted to identified the strain. The colony morphology of these three strains were presented in Fig. 1A. The strains were incubated at 25 °C on PDA (Potato Dextrose Agar) medium for 7 days (*P. italicum* and *P. digitatum*) or 14 days (*L. theobromae*).

### 2.3. Validation of pathogenicity of the pathogenic fungi in citrus fruit

Citrus fruits were sterilized prior to use. Briefly, the fruit was soaked in 0.2 % (v/v) NaClO solution for 2 min to eliminate the bacteria and fungi on the fruit surface. Then the fruits were washed with water and dried by airing. The assay of pathogenicity of pathogenic fungi in citrus fruit was performed by injury inoculation method according to a previous report (An, Li, Li, Zhang, Qin, & Tian, 2016). A mechanical wound (2 mm diameter and 5 mm depth) was made at the equator part of the fruit with a sterile inoculation needle. Then 20 µL of spore suspension of pathogen (*P. italicum*, *P. digitatum* or *L. theobromae*) was injected to the wound. Equal sterile water was used as control. Each treatment included 3 replicates and each replicates contained 15 fruits. The inoculated citrus was placed in a sterilized plastic basket and covered with plastic wrap. The fruits were stored in a 25 °C constant temperature incubator, and the dynamic changes of fruit disease symptoms were observed and recorded.

### 2.4. Division of rot stage and sample preparation

The rot stage was divided based on the disease spot diameter. Rot stage 1 = 10 mm disease spot diameter, rot stage 2 = 30 mm disease spot diameter, rot stage 3 = 50 mm disease spot diameter, rot stage 4 = 70 mm disease spot diameter (just half fruit rots), and rot stage 5 = the whole fruit rots. The peel and flesh of the fruit with rot stage 3 (the diameter of the disease spot is 50 mm) were taken by a scalpel and then frozen with liquid nitrogen. The samples were stored at -80 °C prior to use.

### 2.5. Extraction and detection of volatile metabolites of citrus fruit tissues by GC-MS

The extraction of volatile metabolites of citrus tissue was conducted referring to the method reported by Cao et al. (2021). The frozen samples (0.1 g peel or 3 g flesh) of unrotten, unrotten-rotten junction, or rotten fruit tissues were ground into powder and then put into a 20 mL of

headspace extraction bottle containing 5 mL saturated sodium chloride solution and 50 µL 1-hexanol (0.1 %, v/v). The mixture was fully vortexed after sealing. The volatile metabolites were collected with the solid phase micro extraction (SPME). The samples were balanced at 42 °C for 30 min, and SPME head (model: 50/30 µM DVB/CAR/PDMS) was then inserted into the sample bottle for 30 min to enrich the volatile metabolites. Finally, the SPME head was transferred into the GC-MS sample inlet for desorption.

Volatile metabolites of peel and flesh tissues were measured by the Agilent 5975C-7890A gas chromatography-mass spectrometer (Agilent, USA). HP-5 capillary chromatographic column (30 m × 0.25 mm × 0.25 µm, Agilent, USA) was used to separate the volatiles with the carrier gas flow rate at 1.0 mL min<sup>-1</sup>. The separated volatiles were ionized at 70 eV electron energy with the ion source temperature at 230 °C. Temperature rise procedure was arranged from 40 to 70 °C (rise rate: 3 °C min<sup>-1</sup>), from 70 to 130 °C (rise rate: 1 °C min<sup>-1</sup>), and finally from 130 to 230 °C (rise rate: 15 °C min<sup>-1</sup>).

Qualitative analysis of unknown volatile metabolites was determined according to a previous study by Liu et al. (2017). (1) Retrieval and comparison are conducted through NIST/EPA/NIH Mass Spectral and Wiley Registry of Mass Spectral Data. (2) Comparing the calculated Retention Index (RI) with the results reported in the published literature. According to the retrieval on MS database and manual spectrogram analysis, volatiles with a similarity of more than 80 % are retained (siloxane impurity peaks are removed). The 1-hexanol (0.1 % v/v) was used as a reference to calculate the content of volatile compounds quantitatively. The formula is as follows:

$$C_t = \frac{C_i \times V_i \times A_t}{A_t \times M_t}$$

$M_t$  represents the weighing mass of the tested sample (mg Fw<sup>-1</sup>).  $C_t$  represents the concentration of the target (µg g<sup>-1</sup>).  $C_i$  represents the concentration of internal standard (mg mL<sup>-1</sup>).  $V_i$  represents the volume of internal standard (mL).  $A_i$  represents the peak area of the internal standard.  $A_t$  represents the peak area of the target.

### 2.6. Detection of volatile compounds produced by the whole fruit

The volatile compounds produced by the whole citrus fruit were evaluated by GC-MS. The citrus fruit inoculated with pathogen or sterilized water was sealed in a breaker covered with para-film and plastic film. The headspace gas was used for the measurement of volatile metabolites and the operation procedures were the same with that of citrus fruit tissues mentioned above.

### 2.7. Statistical analysis

Statistical analysis was conducted using SPSS 20.0 software (IBM Inc, USA) and the experimental data was expressed as mean ± standard deviation (SD). Graphpad Prism software (V7, Graphpad Software Inc, USA) and Adobe illustrator software (V2020, USA) were used for data visualization. PCA and PLS-DA analysis were performed by online analysis software MetaboAnalyst 5.0 (<https://www.Metaboanalyst.ca/>).

## 3. Results and discussion

### 3.1. Pathogenicity of three different pathogen to the postharvest citrus fruit

Great losses of citrus fruit were caused after harvest because of the fungi infection, which resulted in huge property loss to farmers (Folch-Fortuny et al., 2016; Liu et al., 2019; Pallottino et al., 2012). The fungi of *P. italicum*, *P. digitatum* and *L. theobromae* were the main pathogens for postharvest citrus fruit, causing blue mold rot, green mold rot and stem-end rot, respectively (Bhatta, 2022; Zheng et al., 2021). It is very important to identify the fungi and clarify its infected symptom for

controlling postharvest diseases of fruit. Previous researches had made great efforts to identify the fruit disease. The citrus pathogen of *P. italicum*, *P. digitatum* and *L. theobromae* has been isolated and identified from the decayed fruit. Zaheer et al. (2019) identified four pathogenic fungi in postharvest citrus fruit containing *P. digitatum* and *L. theobromae* based on ITS-rDNA sequencing. A lemon pathogen was identified as *P. italicum* according to its morphological criteria, ITS1, 5.8S and ITS2 region of the rDNA (Hernandez-Montiel & Ochoa, 2007). The pathogen of *P. italicum* and *P. digitatum* mainly infected citrus fruits, while *L. theobromae* could infect fruits of different species. Previous studies have reported that *L. theobromae* infected mango and banana and caused stem-end rot (Mortuza & Ilag, 1999; Yang, Dong, Wang, Xian, Wang, & Liang, 2021). In addition, jackfruit fruits were also decayed by *L. theobromae* infection (Ni, Chen, Chang, & Yang, 2008).

In the present study, to study the specific voltaic metabolites of different citrus disease, three fungi were selected and their colony morphology were shown in Fig. 1A. The pathogenicity of *P. italicum*, *P. digitatum* and *L. theobromae* were verified by a fruit-injury inoculation experiment. As shown in Fig. 1B, the citrus fruit in control group didn't show any disease spots or other signs of infection during the whole storage for 25 days. In contrast, citrus fruit inoculated with *P. italicum*, *L. theobromae* and *P. digitatum* had presented typical disease symptoms after inoculation at different times. At the early stage of the citrus fruit infected with *L. theobromae*, the peel showed light yellow water stains and the peel tissues were start to become soft and rotten. In the late stage, the fruit surface was covered with white mycelium and the juice flowed from the infected area, giving off the sour odor, and then the whole fruit rot soon. This disease symptoms and smells were typical for the stem-end rot of citrus reported by Zheng et al. (2021). Meanwhile, citrus fruit inoculated with *P. italicum* or *P. digitatum* exhibited specific symptoms of blue mold or green mold, which covered with thick mycelium and blue or green spores in the fruit surface at the infected later stage. These decayed symptoms were consistent with previous studies that reported the citrus postharvest diseases, such as blue mold and green mold (Bhatta, 2022). In addition to different disease symptoms, the infection speed of different pathogens also varies greatly. *P. italicum* and *L. theobromae* could quickly infected the citrus tissue through the chonical wound after inoculation for 2 days with the disease incidence of 95.56 % and 66.82 %, respectively (Fig. 1C). *P. digitatum* showed the obvious disease spots in citrus wound until the 4 days after inoculation, accompanied with a low rate of lesion spread (22.84 %). The results from this experiment determined that *P. italicum*, *P. digitatum* and *L. theobromae* presented well pathogenicity for citrus fruit, and the corresponding disease also displayed their own unique disease course characteristics.

### 3.2. Classification of the volatile compounds from the flesh or peel tissues of citrus fruit inoculated with fungi

#### 3.2.1. Changes of the total content of volatile compounds from flesh or peel tissues

Volatile compounds are the important components of fruit flavor. Recently, there are many researches about the changes of volatile compounds in citrus fruit after infection by fungi and a lot of characteristic compounds were identified (Ding et al., 2017; Ning, Nan, Nan, Wei, Zexiong, & Zhengguo, 2018; Seong Mi et al., 2018). Especially, terpene volatiles play an important role in the interaction between specific pathogens and fruits (Rodriguez et al., 2018). In this study, a total of 77 volatile compounds were identified from the unrotten, unrotten-rotten junction and rotten flesh tissues of green mold rot, blue mold rot and stem-end rot fruits (Table S1-S3). Based on their origin of carbon valence structure, these compounds were classified into 8 categories, including 12 monoterpenes, 13 monoterpene derivatives, 20 sesquiterpenes, 1 sesquiterpene derivatives, 5 alcohols, 8 aldehydes, 5 esters and 13 other compounds including alkanes, olefins, phenols, etc. Among these compounds, the content of monoterpenes was the highest,

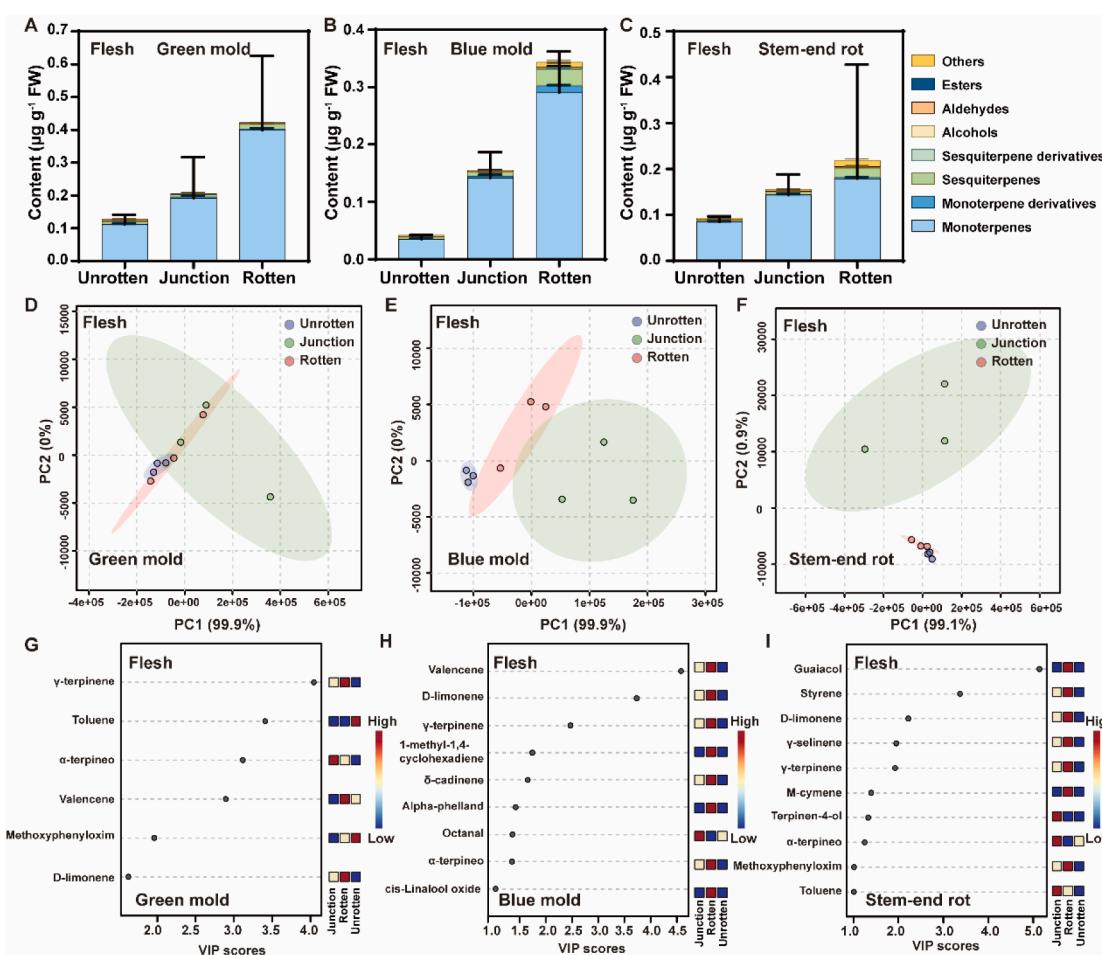
followed by sesquiterpenes (Fig. 2A-C). Moreover, the total content of volatile compounds was positively correlated with the decay degree of flesh tissues regardless of stem-end rot, blue mold or green mold. The increase of monoterpene content was the main reason for this correlation. This result was supported by a previous study that the content of monoterpene such as limonene and  $\beta$ -myrcene, was markedly released from the *P. digitatum*-infected citrus fruit (Chalupowicz, Veltman, Drobny, & Eltzov, 2020). Meanwhile, the content of sesquiterpenes from rotten flesh tissues was also higher than that in unrotten, unrotten-rotten junction flesh tissues in terms of the three fruit diseases. However, the content of alcohols, aldehydes and esters were relatively low, suggesting that they had the little impact on the odor of infected flesh tissues.

For peel tissues, 122 volatile compounds were identified, including 18 monoterpenes, 25 monoterpene derivatives, 38 sesquiterpenes, 4 sesquiterpene derivatives, 3 alcohols, 8 aldehydes, 10 esters and 18 other compounds (Table S4-S6). Terpenes compounds such as monoterpenes and sesquiterpenes, accounted for the main components and sesquiterpene derivatives, alcohols and esters were rare (Fig. 3A-C). This result was consistent with that of flesh tissues mentioned above. Interestingly, the content of monoterpenes was decreased with the peel rot developing heavier, which was contrary to the results in the flesh tissues. In particular, the monoterpenes from green mold tissue decreased the most. In addition, the content of sesquiterpenes and their derivatives increased in the stem-end rot tissues, while decreased significantly in the green mold tissues. Combined with the results of flesh tissues, it was speculated that monoterpenes were the main odor source of rotted citrus peel and flesh tissues.

#### 3.2.2. Classification of volatile compounds by principal components analysis

Principal components analysis (PCA) is one of the pattern recognition methods for analyzing the differences of a series of dataset, which could transform a set of relevant variables into the linearly unrelated variables through orthogonal transformation (Nouri et al., 2020; Rojas-Flores, Ventura-Aguilar, Bautista-Banos, Revah, & Saucedo-Lucero, 2019). Many previous studies have used PCA method to discriminate the database information in postharvest fruit field. The apple fruit inoculated with pathogenic fungi could be discriminated by applying PCA analysis to the volatile compounds (Seong Mi et al., 2018). In cold stored strawberry fruit, volatile compounds were analyzed by PCA to distinguish infected and non-infected fruit (Rojas-Flores et al., 2019). To further analyze the potential difference of volatile compounds between the infected and non-infected fruit tissues, the PCA analysis was carried out after the relevant data were normalized. As illustrated in Fig. 2D-F, the volatile compounds produced by the three parts were well separated in the blue mold and stem-end rot flesh tissues, while the three parts in green mold tissues had overlapping areas, indicating that the difference of volatile compounds from the three parts in stem-end rot and blue mold flesh tissues was greater than that in green mold flesh tissues.

For the peel tissues of citrus fruit samples, the principal component contribution rates of the PCA models of the three fruit diseases were 98.7 % for stem-end rot, 100 % for blue mold and 99.8 % for green mold peel tissues, respectively, indicating that the PCA results represent the whole information of citrus peel samples, and were highly representative (Fig. 3D-F). The volatile compounds from rotten, unrotten-rotten junction and rotten peel tissues with blue mold rot were well differentiated. Meanwhile, the volatile compounds from unrotten and junction peel tissues with stem-end rot were also separated, but there was overlap between the unrotten part and rotten part. The three parts of green mold rot fruit were not well separated. The results implied that there was a significant difference in the volatile compounds from the unrotten, unrotten-rotten junction and rotten peel tissues of the blue mold rot fruit. In the stem-end rot fruit, there was also a significant difference between the unrotten and rotten peel tissues. In contrast, volatile compounds from the three parts of peel tissues with green mold could not be discriminated by PCA.



**Fig. 2.** Content (A-C), principal component analysis (PCA) (D-F) and partial least squares discriminant analysis (PLS-DA) (G-I) of volatile compounds in flesh tissues of different parts of citrus fruits with different diseases. Error bars represent standard deviation and three replicates for each treatment.

### 3.2.3. Identification of characteristic volatile compounds based on VIP value

Partial least squares discriminant analysis (PLS-DA) is a multivariate statistical analysis method used for sample classification based on the feature information (Xin et al., 2018). It can establish the relationship model between the content of volatile compounds and the sample category to predict the sample category (Rojas-Flores et al., 2019; Xin et al., 2018). In particular, the variable importance for the projection (VIP) is used to weigh the influence intensity of volatile compounds and interpret the ability of each volatile compound on the classification and discrimination of each group of samples (Nouri et al., 2020; Seong Mi et al., 2018). Here, with the content of all volatile compounds as variables, the VIP score of each volatile compound was calculated and the volatile compounds with VIP above 1 were shown in Fig. 2G-I. It was found that volatile compounds in each sample with VIP above 1 were mainly focused on monoterpenes and their derivatives. There were 10, 6 and 9 of volatile compounds with VIP above 1 in the flesh tissues, respectively, corresponding to stem-end rot, green mold rot and blue mold rot fruit. These volatile compounds were considered to have important contributions to the change of odor in the flesh tissues of different diseased fruits. Among these volatiles, the compounds with the highest VIP values in the flesh tissues of stem-end rot, green mold rot and blue mold rot fruits were guaiacol,  $\gamma$ -terpinene, valencene, respectively, suggesting that they might be the most significant factors as biomarkers for the infected fruit. Ning et al. (2018) also found similar results that d-limonene,  $\beta$ -pinene, 3-carene,  $\alpha$ -terpinene, terpinene and other monoterpene compounds were markedly released after

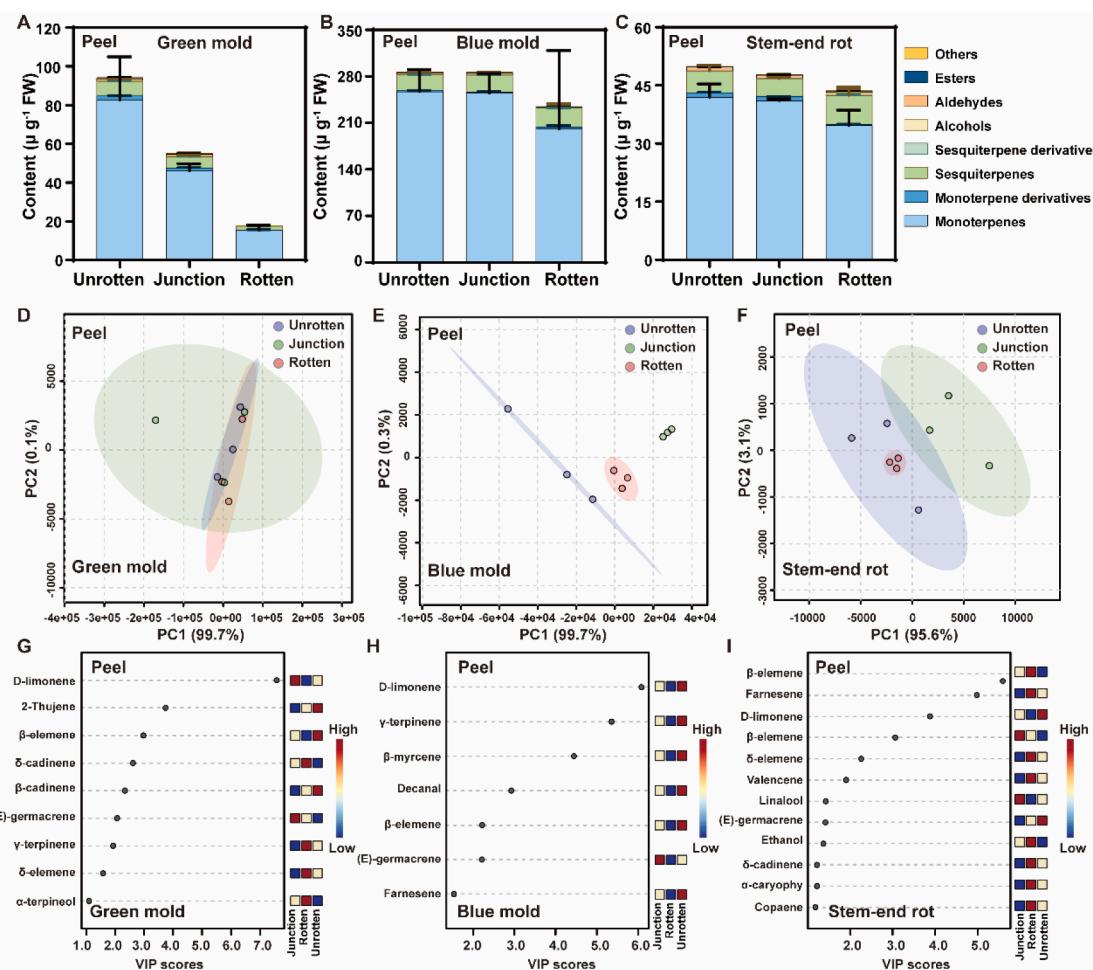
inoculation with *P. digitatum* on citrus for 60 h. It is reported that these compounds have strong odor and involved in the process of interaction between fruits and pathogens (Gonzalez-Mas, Rambla, Lopez-Gresa, Blazquez, & Granell, 2019; Seong Mi et al., 2018). Taken together, the identified compounds in this study with special smell might have important impact on the generation of peculiar smell of decayed citrus fruit.

In the peel tissues of decayed fruit, 12 of volatile compounds for stem-end rot fruit, 9 of volatile compounds for green mold rot fruit, and 7 of volatile compounds for blue mold rot fruit were identified with the VIP above 1 (Fig. 3G-I). Sesquiterpenes were the main compounds in stem-end rot fruits, and a small amount of monoterpene and their derivatives were included. Monoterpenes and sesquiterpenes were dominant in green mold rot and blue mold rot fruits. The top two compounds with the highest VIP value in the stem-end rot fruits were sesquiterpenes, while blue mold rot and green mold rot fruits were both monoterpenes. All in all, the volatile compounds emitted by citrus fruit were significantly affected due to the fungi infection and the identified volatile compounds partly explained the separation of citrus fruit with different postharvest diseases.

### 3.3. Changes of volatile compounds during the different rot stages of the whole citrus fruit

#### 3.3.1. Changes of volatile compounds during the rot stages in the whole citrus fruit

When plants are infected by pathogenic fungi or the tissues are



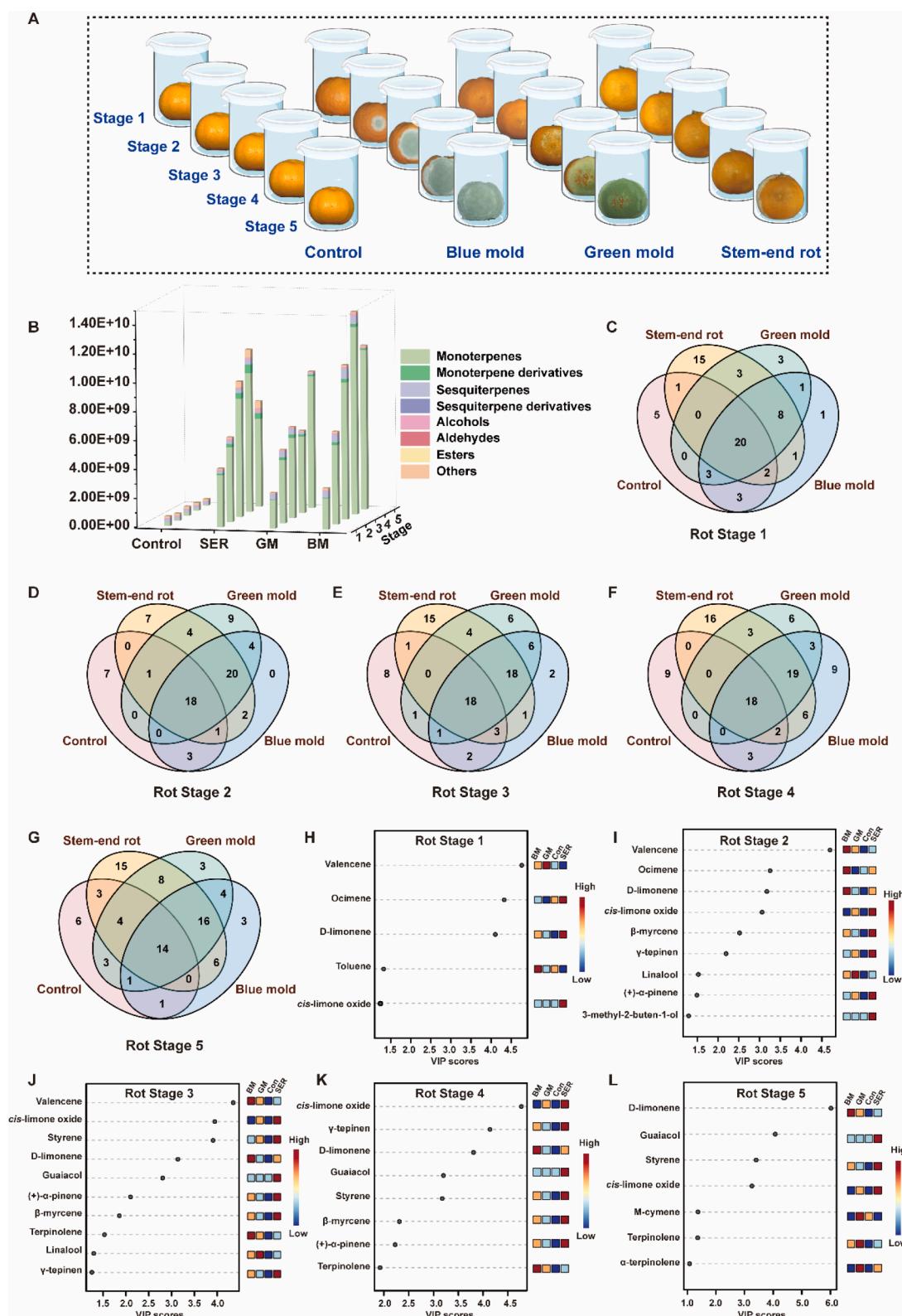
**Fig. 3.** Content (A-C), principal component analysis (PCA) (D-F) and partial least squares discriminant analysis (PLS-DA) (G-I) of volatile compounds in peel tissues of different parts of citrus fruits with different diseases. Error bars represent standard deviation and three replicates for each treatment.

damaged, a large amount of volatile compound would be released due to the combined effects of many factors such as their own tissue degradation and fungal metabolism (Lopez et al., 2015; Parthasarathy, Thiribhuvanamala, Subramanian, Paliyath, Jayasankar, & Prabakar, 2018). To further clarify the differential metabolites between the infected and non-infected citrus fruit, GC-MS was applied to detect the volatile compounds emitted by the whole fruit at different rot stages and the fruit treatment mode was shown in Fig. 4A. A total of 101 volatile compounds were detected, containing 20 monoterpenes, 23 monoterpene derivatives, 17 sesquiterpenes, 1 sesquiterpene derivative, 9 alcohols, 3 aldehydes, 5 esters and 23 other compounds including alkanes, olefins, ketones, phenols and etc. The change of volatile components caused by biology stress is also related to the specific pathogens (Seong Mi et al., 2018). In the present study, the content of volatile compounds varied greatly in the rotted fruit compared with the healthy fruit (Fig. 4B). In the stem-end rot and blue mold fruits, the total content of volatiles increased with the development of rot stage and reached the maximum at the rot stage 4. The result was similar with the previous report that the concentration of volatile compounds in oranges increased significantly after infected with *P. digitatum* (Chalupowicz et al., 2020). In the blue mold rot fruit, the content of volatile compounds kept rising mostly with the development of rot stage and reached the maximum at the stage 5. Monoterpene derivatives were not detected in the control fruits, but identified in most diseased fruits. Monoterpene derivatives were accumulated in stem-end rot fruits at the early rot stage, which significantly higher than that in blue mold rot and green mold rot fruits. More sesquiterpenes in all diseased fruit were emitted in the early rot stage, but

its volatilization level went down markedly when the fruit rotted completely. These results above indicated that citrus fruits might generate stress responses after being infected with fungi, which stimulated the changes of volatile compounds. Moreover, the stimulating effects varied with different fungi. On the other hand, many common and unique volatile compounds were observed (Fig. 4C-G). From the initial stage (stage 1) to the whole rotted fruit (stage 5), the amount of common volatile compounds from the three fruit diseases increased and went down afterwards. There were only 8 common VOCs in rot stage 1, and then increased to 20. When the whole fruit rotted (stage 5), 16 common volatile compounds were identified. Throughout the whole disease process, stem-end rot fruits had the most specific volatiles. Except for decayed stage 2, there were about 15 specific volatile compounds in other rot stages, which were more than green mold rot and blue mold rot fruits. Among these volatile compounds emitted by citrus fruit at different rot stage, 5, 9, 10, 8 and 7 of volatile compounds were identified based on the VIP above 1 corresponding to rot stage 1 to rot stage 5 (Fig. 4H-L). The results implied that these substances may play an important role in the odor contribution of fruits at different rot stage.

### 3.3.2. Screening of biomarkers of citrus fruit with different postharvest diseases

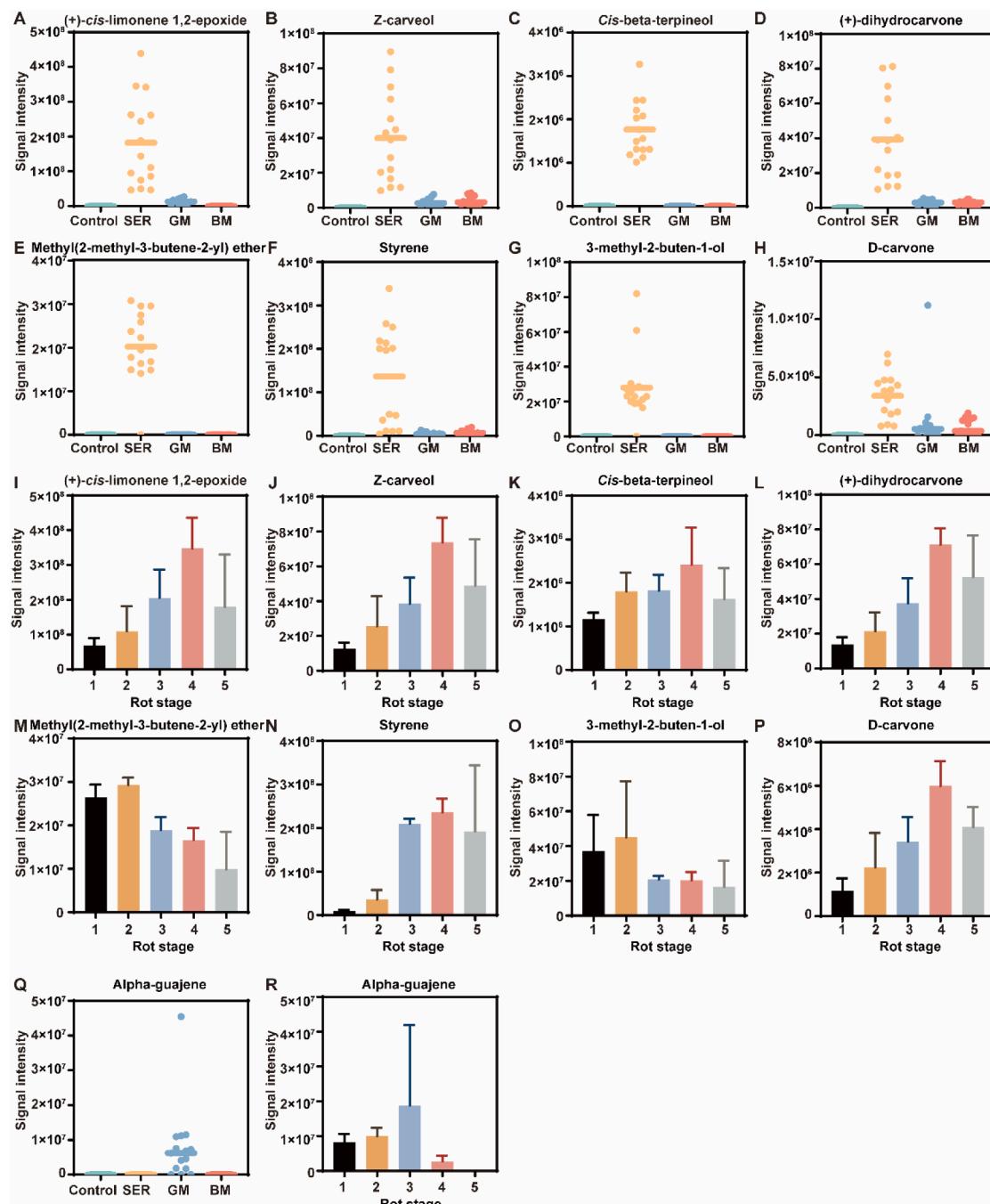
Many researchers were committed to identifying specific volatile compounds produced by the rotted fruit and these compounds were used as biomarkers to monitor the freshness of the fruit (Goldenberg, Yaniv, Choi, Doron-Faigenboim, Carmi, & Porat, 2016; Obenland, Collin, Sievert, & Arpaia, 2013). Through the changes of biomarker content, the



**Fig. 4.** Discrimination of volatile compounds from the citrus fruit with different diseases during stage 1 to stage 5. (A) Treatment model of citrus fruit with different diseases at different rot stages. (B) Signal intensity and quantity of volatiles in citrus fruits infected with different diseases from rot stage 1 to stage 5. (C-G) Venn diagrams of volatile compounds from citrus fruits infected with different fungi during the whole rot stage. (H-L) Volatiles with VIP greater than 1 in different rot stages of citrus fruits with different diseases.

fruit diseases could be detected at the early stage and then the control strategies could be formulated in time to prevent large-scale fungi infection and decay (Lopez et al., 2015; Tietel, Lewinsohn, Fallik, & Porat, 2011). Lopez et al. (2015) reported that Z-3-hexenyl 2-methylbutanoate could be as a potential biomarker to distinguish the infected and non-infected apple fruit. 13 volatile organic compounds were considered as biomarkers to track shelf-life of apple fruit during low-temperature postharvest storage (Waghmode, Masoodi, Kushwaha, Mir, & Sircar, 2021). In the present study, nine biomarkers were screened out and these compounds appeared specifically in each rot stage, which were

corresponding to the unique fruit disease. Specifically, 8 volatile compounds as biomarkers from stem-end rot fruits were screened, including five monoterpene derivatives ((+)-dihydrocarvone, Z-carveol, *cis*-beta-terpineol, (+)-*cis*-limonene 1,2-epoxide and D-carvone), one semiterpene derivative (3-methyl-2-butene-1-ol), and two other compounds (styrene and methyl(2-methyl-3-butene-2-yl) ether). These eight volatile compounds were specific in the whole stage of stem-end rot fruit (Fig. 5A-H). Moreover, (+)-dihydrocarvone, Z-carveol, *cis*-beta-terpineol, (+)-*cis*-limonene 1,2-epoxide, styrene and D-carvone all increased in the early decayed stages, and then decreased after the half



**Fig. 5.** The content of biomarkers of stem-end rot or green mold in citrus fruit. (A-H) The content distribution of eight biomarkers of stem-end rot in different fruit diseases throughout the whole rot stage. (I-P) Changes of eight biomarkers of stem-end rot with the development of rot stage 1 to stage 5. (Q) The content distribution of biomarkers of green mold in different fruit diseases throughout the whole rot stage. (R) Changes of biomarkers of green mold with the development of rot stage 1 to stage 5. Error bars represent standard deviation and three replicates for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the fruit rotted. 3-methoxy-3-methyl-1-butene and isopentenol were higher at the initial stages than the late decayed stages (Fig. 5I-P). The alpha-guajene was screened for green mold rot fruit as biomarker. Unfortunately, no suitable biomarkers have been screened from blue mold rot fruit. Through the detection of biomarkers, the operator can quickly and nondestructive judge whether citrus fruit is infected by pathogens and the types of pathogens. The method is simple in operation, reduces the cost of manual detection and decreases the loss of fruit storage after harvest. It can be used for the detection and monitoring of citrus fruit quality during the postharvest circulation, storage and marketing of citrus fruit.

#### 4. Conclusion

In summary, the present study compared and analyzed the volatile profiling of citrus fruit with or without the fungi infection. The unrotten, unrotten-rotten junction, and rotten citrus tissues could be discriminated successfully regardless of flesh or peel tissues. Eight volatile compounds were identified as biomarkers for citrus fruit to monitor the presence or absence of stem-end rot fruits, including (+)-dihydrocarvone, Z-carveol, cis-beta-terpineol, (+)-cis-limonene 1,2-epoxide, d-carvone, 3-methyl-2-buten-1-ol, styrene and methyl(2-methyl-3-butene-2-yl) ether, and alpha-guajene was screened to monitor the green mold fruit.

#### CRediT authorship contribution statement

**Jue Wu:** Conceptualization, Software, Data curation, Methodology, Formal analysis. **Jinping Cao:** Investigation, Methodology, Formal analysis, Data curation, Writing – review & editing, Project administration. **Jiebiao Chen:** Methodology, Software, Conceptualization. **Lingxia Huang:** Conceptualization, Investigation, Visualization, Formal analysis. **Yue Wang:** Conceptualization, Investigation, Methodology. **Cui Sun:** Writing – original draft, Conceptualization, Methodology, Software, Funding acquisition. **Chongde Sun:** Investigation, Writing – review & editing, Project administration, Funding acquisition, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

The data that has been used is confidential.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2023.135524>.

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