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Study on dynamic alterations of volatile organic compounds reveals aroma development over enzymatic-catalyzed process of Tieguanyin oolong tea production

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ABSTRACT

To elucidate the formation of characteristic aroma over enzymatic-catalyzed processes (ECP), GC–MS-based volatile-metabolomic combined with desorption-electrospray-ionization coupled mass-spectrometry-imaging (DESI-MSI) were employed to analyze the changes of volatile organic compounds (VOCs) in Tieguanyin tea. A total of 579 VOCs were obtained, from which 24 components involved in five pathways were identified as biomarkers. Among these, four VOCs including 2-furancarboxylic acid, 4-methylbenzaldehyde, N-benzylformamide, cuminaldehyde, were detected in both DESI-MSI and GC–MS analysis, exhibiting dynamic changes along processing steps. RNA-sequencing analysis indicated the genes referring to stress response were activated during tea processing, facilitating the accumulation of flora-fruity aroma in tea leaf. Metabolic pathways analysis revealed that the increase in floral-fruity related components such as volatile terpenoids, phenylpropanoids/benzenoids, indole, alongside a decrease in green leaf volatiles including (*E*)-2-Hexenal, (*Z*)-3-Hexenol, played a crucial role in development of characteristic aroma, which could be a feasible index for evaluating processing techniques or quality of oolong tea.

1. Introduction

Tea is a worldwide consumed beverage for its special flavor and healthy benefits. As the most attractive property, aroma is considered to be the symbol of premium quality tea (Zhai, et al., 2022; X. Zheng, et al., 2016). Oolong tea is a unique type of tea, which is famous for its distinct floral and fruity aroma. There have been more than 300 volatiles identified in oolong tea, some of which could be vital indicators for assessing the quality (Wan, et al., 2015). The characteristic aroma of oolong tea is formed by two major stages: enzymatic and the followed non-enzymatic process (Zeng, et al., 2024). The gradual dehydration and moderate bruising bring in and intensify the volatile organic compounds (VOCs) over enzymatic-catalyzed process (ECP, including plucking, withering and turning-over steps), supplying the substance foundation for the subsequent non-enzymatic-catalyzed process (NCP, including fixing, rolling and drying), facilitating the final quality of oolong tea (Zeng, Watanabe, et al., 2019). Therefore, the investigation on the biosynthesis and transformation of VOCs over ECP stage provides a theoretical basis for understanding the development of characteristic fragrance of oolong tea.

The continuous wounding increased binding ability of transcription factor Cs*MYC*2a to the promoter of tryptophan synthase β-subunit 2 by 3 times comparing to the CK sample, regulating the accumulation of indole over oolong tea manufacturing (Yang, et al., 2021). Cs*RLIS*, the gene encoding (*R*)-linalool synthase can be induced by shaking treatment over ECP stage of oolong tea manufacturing, accounting for the significant accumulation of lavender fragrance associated (*R*)-linalool (~10 nmol/g) in comparison to that in CK group (~5 nmol/g) (Y. Zhou, Deng, et al., 2020). The persistent wounding evoked the enzyme activities of *Cs*CYP79D73 and *Cs*CYP71AT96, promoting the formation of benzyl nitrile (~1500 ng/g) via the conversion of L-phenylalanine and phenylacetaldoxime (Liao, et al., 2020). The dual stresses of wounding

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and low temperature collectively contributed to accreting of jasmonic acid (JA), indirectly enhancing indole content to approximate 12 nmol/g in contrast to that in control group (~2 nmol/g) (Y. Zhou, Zeng, et al., 2020). Similarly, the combination of low temperature and mechanical wounding synergistically stimulated (*E*)-nerolidol synthase activity, increasing the expression level of Cs*NES* by 4 folds compared to the control, resulting in the acceleration of the catalysis of farnesyl diphosphate into (*E*)-nerolidol during the ECP steps of oolong tea production (Y. Zhou, et al., 2017). Moreover, the carotenoids were the key aroma precursors due to their ability to generate α-ionone, β-ionone, dammasone accounting for woody, floral or fruity scents, collectively determining the aroma quality of teas (Fang, et al., 2024; Shi, et al., 2023).

Although several pathways involved in the key aroma components have been revealed in the previous studies, the whole roadmap for the alteration of VOCs and formation mechanism of characteristic fragrance over ECP stage of oolong tea production is still unclear. Tieguanyin is one category of oolong tea that is widely appreciated for its elegant and generous fragrance (Deng, et al., 2020; Xu, et al., 2018; Zeng, et al., 2022). Tieguanyin flavor is derived from the reactions of stress-responsive in the postharvest tea shoots at ECP stage, as the final quality was largely determined by handlings or operations during sequential withering and turning-over steps (Chenxue Li, et al., 2023; Lin, et al., 2024). The typical aroma of Tieguanyin is close to the fragrance of orchid, which is reputed as “Guanyin Yun” (the characteristic charm of Tieguanyin). The typical aroma makes Tieguanyin a model to explore ordo development via plant defense responses, and figure out the flavor formation over the ECP stage.

The non-destructive desorption-electrospray-ionization coupled mass-spectrometry-imaging (DESI-MSI) has been emerged and developed as a feasible measurement to interpret the significance of biological pathways in specific tissues by detection and semi-quantification of a variety of small molecules. As for tea plants, a Teflon-imprint method was established to profile the spatial distribution of catechins and gallic acid within tea leaf samples (Liao, et al., 2019b). Moreover, the DESI-MSI analysis on tea leaf tissues showed that a distinct distribution pattern of B-ring trihydroxylated flavonoids, primarily concentrated in the outer layer of tea buds (Ruan, et al., 2024). Besides, strawberry cryosections maintained original structures prepared using adhesive film were subjected to DESI-MSI analysis, and the amino acids, sugars, organic acids, and flavonoids, were identified and visualized in strawberry tissues (Enomoto, 2021). In this study, we comprehensively investigated the dynamic changes of VOCs in oolong tea leaves during ECP stage. The relative contents of VOCs were monitored through gas chromatography mass spectrometry (GC–MS), and the non-destructive desorption-electrospray-ionization coupled mass-spectrometry-imaging (DESI-MSI) was employed visualize spatial distribution of volatile compounds. The transcriptomic dataset was used to reveal molecular mechanism underlying biochemical processes in response to environmental stimulus. The results in this study will shed light on the development of characteristic aroma of Tieguanyin oolong tea.

2. Material and methods

2.1. Tea sample preparation

Tea shoots with three leaves and a banjhi bud were freshly picked from tea plants (*Camellia sinensis* cv. *Tieguanyin*) cultivated on the tea farm of Fujian Agriculture and Forestry University, Fuzhou (26.04 °N, 119.25°E) in April 2023. The harvested tea shoots were withered under sunlight for 30 min, followed by an indoor-withering at 25 ℃ for 180 min. Then the tea shoots were subjected to the turning-over procedure, composed of alternant tossing and spreading operations. The first tossing was performed in a rotary machine (75D, equipped with 1.5 m × 3 m bucket, Nanda tea machinery factory, Quanzhou City) within 65 s, followed by a spreading-out on the bamboo mats for 1 h; the second tossing was performed within 5 min, followed by a spreading-out for 2 h; the third tossing was carried out within 4 min, with another 5 h spreading-out. Afterwards, the tea shoots were subjected to fixing operation in an oven at 270–350 ℃ for 5 min. Afterwards, the rolling operation was conducted at room temperature, and the tea shoots were dried at 95–100 ℃ until the moisture content dropped to approximately 5 %. The second leaf basipetal from the apical bud in one randomly selected shoot was cut off with scissors, then immediately immersed in liquid nitrogen for 2 min, subsequently stored at −75 ℃ prior to analysis. Each sample was composed of three biological replications.

2.2. Volatile components extraction and quantification

The sample pretreatment and volatiles metabolomic determination were performed by a commercial service company (MetWare Technology, Wuhan, China) based on the protocol reported previously (Wu, et al., 2022). Briefly, the tea sample were subjected to freeze-drying handling in a lyophilizer (FD-551P, Tokyo chemical equipment co., LTD, Japan). The moisture content of grounded sample was measured using MB120 moisture-meter (OHAUS Company, NJ, USA). The moisture contents of the samples were used for the calibration of the following determination of volatile components. The dried samples were further grounded into powder in a grinder (MM 350, Retsch). An aliquot of tea powder (0.5 g) was transferred to a 20 mL head-space vial containing NaCl saturated solution. Then the vials were covered by crimp-top caps with TFE-silicone headspace septa. For solid-phase microextraction (SPME) analysis, each vial was placed in 60 °C for 300 s, then a 120 μm DVB/CWR/PDMS fiber was exposed to the headspace of the sample at 60 °C for 15 min. Afterwards, desorption of volatiles from the fiber was performed in the injection port of the GC apparatus at 251 °C for 6 min. The identification of volatiles was conducted on 7000D mass spectrometer (Agilent), equipped with a 30 m × 0.25 mm × 0.25 μm DB-5MScapillary column. Helium was adopted as the carrying gas with a linear velocity of 1.20 mL/min. The injector temperature was kept at 251 °C and the detector at 281 °C. The programmed temperature was initiated from 40 °C, increasing to 100 °C with 10 °C/min, then to 180 °C with 6 °C/min, finally to 281 °C with 24 °C/min, lasting for 5 min. The MS using ion monitoring mode was employed to identify and quantify volatile components.

2.3. Transcriptomic analysis

The total RNA of sampled leaf was isolated by using RNAprep pure Plant Kit (TIANGEN Biotech Co., Ltd., Shanghai, China) based on manual instruction, and mRNA was isolated by using Oligo (dT) magnetic beads. The cDNAs were synthesized using random hexamer primer, reverse transcriptase, Polymerase I and RNase H. The obtained cDNA was further purified using AMPure XP system (Beckman Coulter, Beverly, USA). The PCR was carried out using Phusion High-Fidelity DNA polymerase, Index (X) Primer, Universal PCR primers. The raw reads filtration was conducted to acquire clean reads by removing ambiguous sequences and adaptor. The clear reads were blasted against reference genome of *Camellia sinensis* cv. *Tieguanyin* using HISAT v2.1.0. The quantification of the gene expression level was calculated by using Fragments Per Kilobase of exon model per Million mapped fragments (FPKM). The different expressed genes (DEGs) were predicted with the criteria of $|\log_2 \text{foldchange}| \geq 1$ and false discovery rate < 0.05. The annotation gene functions and metabolic pathways were performed according to Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A quality control (QC) sample by mixing 12 g of each sample powder was prepared and subjected to determination, to assess reliability and repeatability of analytical results.

2.4. The DESI-MSI analysis

The performance of DESI-MSI detection was conducted by MetWare

Technology (Wuhan, China) according to the protocol outlined in previous study (Liao, et al., 2019a). Each tea leaf was set on a sheet of porous polytetrafluoroethylene (PTFE) and covered with several layers of tissue paper to minimize metabolite delocalization. Two aluminum plates were then used to clamp the tissue paper-covered PTFE sheet, applying a pressure of approximately 4.5 MPa to create an imprint. Afterwards, the imprint of the tea sample on the PTFE was cleaned by removing leaf tissue residues and tissue paper with tweezers. The PTFE sheets were subsequently analyzed using G3 DESI XS (Waters Corporation, Wilmslow, UK). The DESI system settings adhered to the parameters established in prior study (Lin, et al., 2024). In brief, ionization mode, negative; mass range, 50–1200; solvent, acetonitrile with 0.1 % formic acid; flow rate, 2 μL/min; collection angle, 10°; incident spray angle, 50°; sprayer-to-inlet distance, 4 mm; sprayer-to-surface distance, 1 mm; pixel resolution, 100 × 100 μm. For the identification of peaks from the mass spectrometry data, the peaks with high intensity were selected as target peaks and subjected to in situ secondary fragmentation within the tissue. This process generated secondary spectra (MS/MS secondary fragment peak information) specific to each target peak. Subsequently, compound identification was carried out by aligning the collected secondary spectra with entries in both our in-house database and public repositories. For target peaks where secondary spectra could not be obtained due to low intensity, compounds were identified by searching our in-house database and public repositories using their primary molecular weights within an error range of 10 ppm, compounds whose molecular weights closely matched those detection by the instrument were retrieved. The mass spectra collected as raw data were processed with Masslynx software v4.1, and images were viewed using

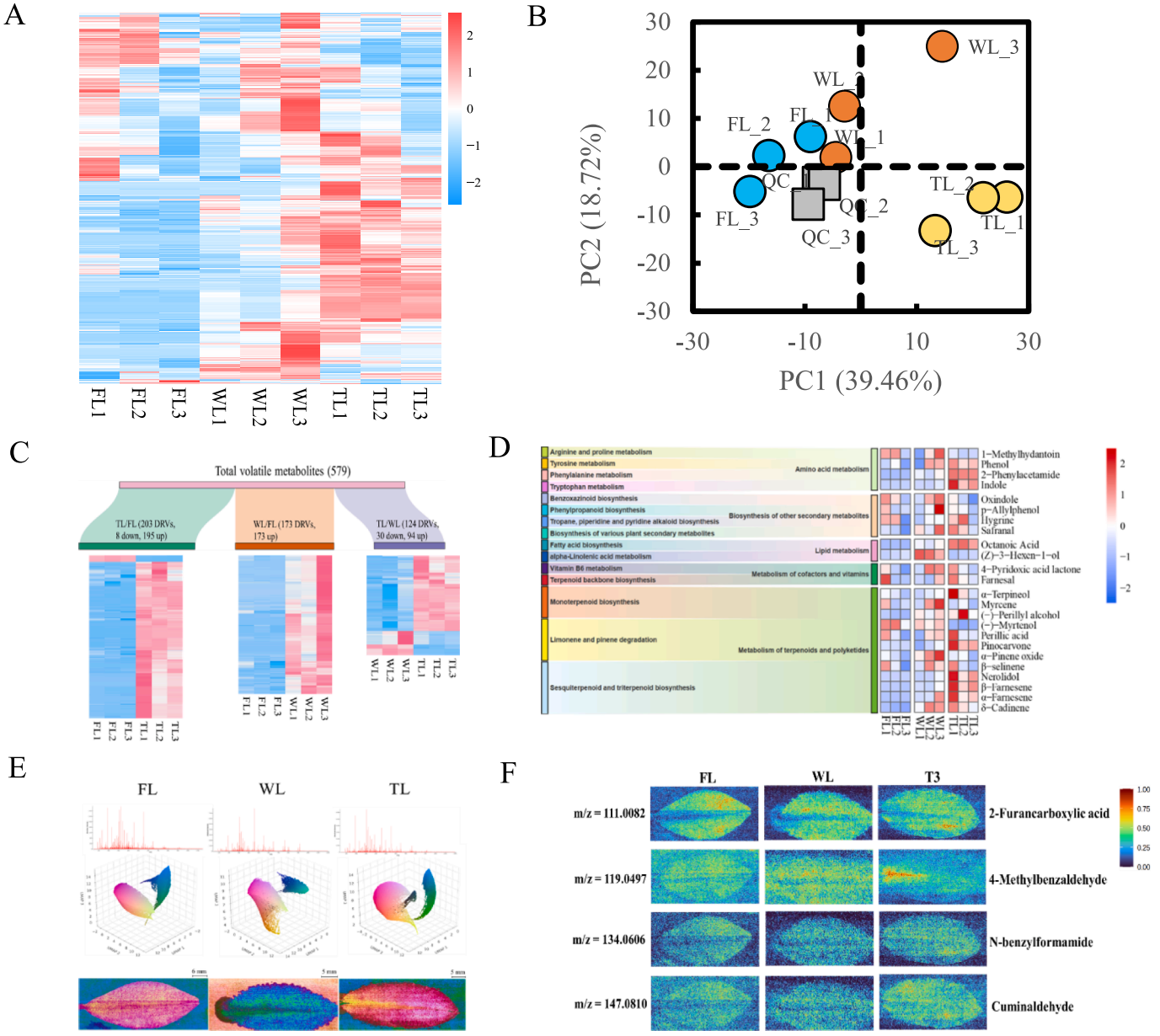

Fig. 1. The volatile-metabolite profiles of tea sample under processing. (A) The heatmap of volatile profiles of tea samples. (B) The score plot of PCA of tea samples. (C) The number of significantly different regulated metabolites in each paired-comparison. (D) The pathway clustering analysis on the biomarkers of tea samples. The left to right represented the Class II pathways, Class I pathways and the biomarkers. (E) The results of DESI-MSI detection on tea samples. Upper: the mean spectrum of each sample; middle: The hyperspectral visualization of mass spectrometry imaging data by using uniform manifold approximation and projection (UMAP); lower: the UMAP image of each sample in this study. (F) The spatial distribution of four volatile compounds in tea samples under processing steps. The different colors in the image represent the varying strength of the components in the area as indicated by the legend ranging from 0 to 1 of increasing relative contents.

HDImaging v1.4.

2.5. Data analysis

The analysis on volatile metabolomic dataset were conducted by using principal component analysis (PCA), and orthogonal projection to latent structures discriminant analysis (OPLS-DA). The significant regulated volatiles (DRVs) in paired-comparisons were predicted using the criteria of $\log_2|(\text{Fold change})| > 1$, and variable importance projection (VIP) values higher than 1.0 in this study.

3. Results and discussion

3.1. The variations of the volatiles during ECP stage

A total of 579 VOCs were identified and quantified from FL, WL, and TL samples in this study, which could be classified into 16 categories, including 118 terpenoids, 106 esters, 51 alcohols, *etc.* (Table S1). As presented in Fig. 1A, the abundance of the identified volatiles demonstrated dynamic changes along the processing steps, suggesting these volatile components were induced or regulated under the stimulus of adverse environment over the oolong tea production. Further, the results of PCA showed that the first two principal components accounted for 58.18 % (PC1 = 39.46 %, PC2 = 18.72 %) of the total variance (Fig. 1B). The replicates of each sample were gathered, while each group was dramatically separated from the others, QC group was located around the origin of orthogonal coordinate; moreover, the points of each sample showed a distribution along the negative to positive direction of PC1 (Fig. 1B), indicating the determining result is reliable, and the metabolomic dataset of each sample altered with the production process. The significant DRVs were predicted by using OPLS-DA algorithm. Consequently, a distinct division amongst FL, WL and TL samples was observed as illustrated in Fig. S1, underscoring that the dramatical influence of processing operation on the VOCs of tea samples. Moreover, the cross-validation of permutation test showed a good model quality with $R^2X > 0.65$, $R^2Y > 0.99$, $Q^2 > 0.88$ (Fig S1), attesting to the strong accuracy and reliability of OPLS-DA model. As presented in Fig. 1C, the most abundant DRVs were in the comparison of TL/FL (203 DRVs), followed by the comparisons of WL/FL (173 DRVs), and TL/WL (124 DRVs). This observation is congruous with the result of previous study, in which the DRVs enriched in turning-over was obviously more than those in withering (Lin, et al., 2024; Wu, et al., 2020), implying the increasing amounts of DRVs were elicited by the progressively enhanced adversity along the chronological producing steps.

Furthermore, the DRVs referring to the three pair-comparisons were subjected to the KEGG pathways analysis, to predict biomarkers during ECP stage of Tieguanyin tea processing. As a result, 24 volatile components involving in five pathways were clustered as the biomarkers as present in Fig. 1D. The overall trend showed the abundance of most biomarkers in this study were elevated along the producing process, moreover the characteristic VOCs of oolong tea such as indole, nerolidol, α-farnesene, *etc* (Fig. 1D), were accumulated at turning-over stage, which is congruous with the observation of previous study (Y. Zheng, Hu, Wu, et al., 2022). The Class I pathways enriched in this study included “Amico acid metabolism”, “Biosynthesis of other secondary metabolites”, “Metabolism of terpenoids and polyketides”, “Lipid metabolism”, as well as “Metabolism of cofactors and vitamins”, suggesting that the alterations of volatiles were impacted by the dynamic changes of primary metabolites, including amino acids and lipids. This result suggests that stress-responsive reactions in plants are activated in the face of adversity, then most secondary metabolites are synthesized from a limited number of primary metabolites, leading to competition for carbon allocation among the production of various VOCs (Paul, et al., 2022). Notably, a few of Class II pathways enriched in this study were associated with terpenoids metabolism, such as “Terpenoid backbone biosynthesis”, “Monoterpenoid biosynthesis”, as well as “Sesquiterpenoid and triterpenoid biosynthesis” (Fig. 1D). Volatile terpenoids (VTs) are the most diversity secondary metabolites in plants with aromatic scents, and the contents of most volatile terpenoids would be dramatically induced by abiotic stress. Ultraviolet B (UV-B) exposure over solar withering step increased the terpenes such as α-farnesene, β-ocimene and 4-hexanolide, promoting fruity and floral odorants during oolong tea production (Wang, et al., 2024). (*E*)-nerolidol would accumulate during the turning-over stage under dual-stress of mechanical wounding and relatively low temperature, in contrast to intact tea leaf (Y. Zhou, et al., 2017). Besides, the mechanical wounding also induced the accumulation of linalool and geraniol in oolong tea leaves via promoting gene expression of biosynthesis cascades (Y. Zheng, Hu, Wu, et al., 2022). The results of biomarkers and pathway enrichments indicated the accumulation of several floral-associated volatiles over producing process may contribute to the characteristic aroma of oolong tea, which were due to the synergistic biochemical reactions responding to multiple stresses.

In this study, the raw data of DESI-MSI was converted and input into Cardinal software package to calculate the mean spectrum of each sample (Fig. 1E). Then the peaks acquired in the mass spectrometry were blasted to the self-built or public database for substance identification. The abundance of all components obtained from mass spectrometry imaging were embedded into three-dimension space using uniform manifold approximation and projection (UMAP) manifold learning technique for dimension reduction (McInnes, et al., 2018), then the locations of pixels on three UMAP axes were subjected to red–green–blue (RGB) color-coding, where RGB intensity were adjusted linearly between 0 and 1 for the minimum and maximum values on the UMAP axis. As shown in the middle row of Fig. 1E, the image after color-coding the pixels with RGB values showed visualization of the different tissue structures, exhibiting biochemical distinctions of different part of leaf. This results of UMAP indicated the heterogeneous distribution of small molecules among the vein, edge or central part of a given leaf, and this nonuniformity were intensified along the producing steps of oolong tea manufacturing (Fig. 1E). Subsequently, a cross-comparison between the identified compounds from DESI-MSI and GC–MS detections was conducted. As a result, four compounds were filtered out as the common metabolites, namely 2-furancarboxylic acid, 4-methylbenzaldehyde, N-benzylformamide, cuminaldehyde, whose abundance showed dynamic changes along the chronological processing steps (Fig. 1E). The level of N-benzylformamide was obviously accumulated along the ECP stage (Fig. 1E), which is agreed with the previous study that the content of N-benzylformamide would be elevated as a response to heat stress (Qin, et al., 2022). As a type of phytoalexin, cuminaldehyde have been reported to exhibit strong antioxidative and antibacterial activities when facing biotic or abiotic stress (Sowbhagya, 2013), and the accumulation of cuminaldehyde in this study (Fig. 1E) was probably ascribed to the enhancing biosynthesis during ECP stage of oolong tea processing. As a derivative of p-xylene degradation, 4-methylbenzaldehyde contributed to sweet or floral aroma of plant (Lu, et al., 2023). The stepwise enhanced content of 4-methylbenzaldehyde in oolong tea leaf in this study (Fig. 1E) suggested that the characteristic floral or fruity fragrance of oolong tea would be accumulated after the stimulating of environmental stress.

DESI-MSI appears to be a practical choice for the detection of plant tissue owing to its simple operation and low-cost. When DESI-MS was first applied to tea plant, an inhomogeneous distribution of catechin isomers were observed on different tissues of tea plant (Liao, Fu, Zhou, Rao, Zeng, & Yang, 2019a); Further, the redistributions of catechins, flavonols and amino acids were reported during oolong tea production (Lin, et al., 2024). These results suggest DESI-MSI can provide deeper insights into the synthesis and regulatory mechanisms underlying growth, development, and response to stresses. In this study, only four volatile components detected in DESI-MSI were covered by GC–MS, as most of metabolites identified by using mass spectrum imaging technique are non-volatiles. A lack of feasible method allowing high-

throughput volatile metabolites to be detected limits the application of DESI-MSI (Liao, et al., 2021). Nevertheless, the potential of DESI-MSI detection on spatial distribution of volatile metabolites in living tea tissue has been revealed in the present work, the scientific problems regarding precise measurement of volatiles in tea tissue remain to be solved by the development of advanced MSI methodology.

Coupled to GC–MS instruments represent high-throughput approaches for detecting and elucidating extremely low-abundance metabolites occurring in any type of biomass, although MS does not provide any information concerning the spatial and temporal distribution of metabolites in a biological sample (Parrot, et al., 2018). Imaging mass spectrometry (IMS) complements traditional qualitative and quantitative determination tools with spatiotemporal information, providing the capability to map specific molecules to multi-dimension coordinates of the original sample. In this study, 290 components were identified by DESI-MSI (Table S2), of which only four were detected by GC–MS. The differences in the number of detected components between DESI-MSI and GC–MS were due to the low volatility of the metabolites detected by DESI-MSI in tea leaf sample, making the determination of these metabolites by GC–MS difficult. The similar results were also reported in the previous studies, in which pre-treatments/ extracting method of samples potentially accounted for the discriminations among the DESI-MS and GC–MS (Cheng, et al., 2020; Jeng, et al., 2021). These results indicated that DESI-MSI can identify components on the surfaces of agricultural products, while GC–MS is inclination to the quantification of volatiles in the entire tissue in spite of the spatial distribution. Our work focused on heterogeneous damage can manifest across specific part of tea shoot under external stress, providing a deeper understanding on plant stress response mechanisms to environmental stimuli. Moreover, it offers a new perspective that when studying the damage caused by environmental stimuli to tea plants, it is essential to monitor the characteristic component accumulation in the sensitive or fragile parts due to the earlier signals of adversity-induced dynamic alterations in metabolism.

3.2. The transcriptomic analysis

The RNA-sequencing dataset was employed to quantify gene expression in tea samples. A total of 34,497 transcripts (Table S3) were identified and quantified as presented in Fig. 2A. This result showed a similar trend with the alterations of VOCs, implying that dynamic changes on mRNA level evoked the conversions of VOCs in the tea leaf under process, acting as a response to abiotic stress over ECP stage. The score plot of PCA indicated the transcriptomic profiles of different tea samples were obviously separated from each other, while the replicates in the same sample were closely clustered, with the first two PCs were made up to 54.7 % variance (Fig. 2B), exhibiting a fine representativeness in this study. The most DEGs were obtained in TL/FL comparison (6037 down-regulated, 5064 up-regulated), followed by TL/WL comparison (4645 down-regulated, 3255 up-regulated), as well as WL/FL (2391 down-regulated, 2400 up-regulated) as showed in Fig. 2C. This result is in line with the observation of previous study, in which multi-stress such as solar irradiation, heat, or dehydration, initiated the expression of genes associated with biosynthesis pathway of VOCs in tea leaf at withering step, then this process was accelerated by the continuous mechanical damage during turning-over, which was conducive to the final quality of oolong tea (Wu, et al., 2022; Zeng, et al., 2020).

Fig. 2D exhibited KEGG pathway enrichment analysis on DEGs in different comparisons (adjusted $P < 0.05$). For the pair of WL/FL, "flavonoid biosynthesis" was the most significant pathway in this comparison, which was also clustered in TL/FL (Fig. 2D). It has been demonstrated that flavonoid biosynthesis would be boosted by oxidative stress, especially the induction of reactive oxygen species (ROS) during metabolic process in plants. The B ring of flavonoids was able to absorb UV wavelengths to directly alleviate the solar withering triggered ROS overproduction, moreover the phenolic hydroxyl groups exerted scavenging free radicals by donating the hydrogen atom to neutralize the long-term dehydration and mechanical wounding evoked ROS generation over turning-over step (Nabavi, et al., 2020). A total of 16 pathways referring to primary and secondary metabolic process were enriched in TL/FL, which was more abundant than other pair-comparisons (Fig. 2D), suggesting a wider range of enzymatic-catalyzed reactions took place at turning-over than in plucking or bruising. The "Plant hormone signal transduction" was the most significantly pathway enriched in TL/FL. Plant hormones are signaling molecules that regulate responses to environmental stress by mediating gene expression. It has been found that drought and mechanical damage linked α-linolenic acid derived jasmonic acid (JA) increases promoted the genes expression of benzyl nitrile, jasmine lactone and methyl jasmonate (MeJA), resulting in the enhance of floral and fruity scents in oolong tea (Liao, Zeng, Tan, Cheng, Dong, & Yang, 2020; Wu, et al., 2022; Zeng, Wang, et al., 2019).

The joint analysis based on DEGs and DRVs were conducted by using MetaboAnalyst (https://www.metaboanalyst.ca/, accessed on 18 July 2024), to unravel the biological process in response to adverse environment during ECP stage of Tieguanyin tea processing. Totally, 25 pathways were clustered with P value < 0.05, of which 12 pathways, mainly including "Photosynthesis", "Starch and sucrose metabolism", "Carbon fixation in photosynthetic organisms", "α-Linolenic acid metabolism", "Phenylpropanoid biosynthesis", "Flavonoid biosynthesis" as well as "Isoflavonoid biosynthesis", were highlighted with colors indicating adjusted P value < 0.05 (Fig. 2E). The result of integrated analysis showed the overall defense system of tea leaf under manufacturing process. As photosynthesis is frequently impaired by environmental stress, the reallocation of carbon fixation is an early stress response that protect or restore the plant by providing energy and producing new molecules. Starch degradation and glycolysis is usually observed in response to multi-stress, allowing carbon to be reallocated to act osmoprotectants, scavenger of free radicals, or signals that refine stress response (Ribeiro, et al., 2022). Upon stress, the leaf release α-linolenic acid from membrane lipids, as a precursor participating the synthesis of JA and MeJA, facilitating the development of tea aroma (Wu, et al., 2022). As a pathway activated under-abiotic stress conditions, phenylpropanoid biosynthesis is elicited upon sensing the surge of L-phenylalanine, to generate the defense compounds derived from flavonoid or isoflavonoid metabolism, mediating stress tolerance in plants (Nabavi, et al., 2020).

3.3. The metabolic network of VOCs over ECP stage

Tea flavor develops by biochemical and chemical transformations over its processing. In this study, a simplified network of metabolic pathways related to fragrance formation over ECP stage was proposed based on the results of integrate analysis on volatile metabolomic and transcriptomic profiles. As shown in Fig. 3, the characteristic odors are mainly derived from several pathways derived from pyruvate and shikimic acid pathways. The significant generation and emission of VTs can be triggered by wounding, light, extreme temperatures, and other adverse conditions, serving as a defense response in plant-plant interactions (Changyan Li, et al., 2023). Terpenoids biosynthesis is mainly composed of two pathways: mevalonic acid (MVA) and methylerythritol phosphate (MEP) cascades. Geranyl pyrophosphate (GPP) is the precursor for monoterpenoids (C_{10} terpenoids) such as linalool and its oxidized derivatives on MEP pathway. In this study, the dramatical increasing of α-terpineol, geranial, citronellol, and linalool oxides during turning-over steps contributed to the fruity and floral aroma over ECP stage (Fig. 3), which is consistent with the previous study (He, et al., 2023). The terpineol synthase (*TES*) and alcohol dehydrogenase (*ALD*) are responsible for the generation of α-terpineol and geranial respectively. In this study, the expressions of TES and ALD were generally increasing over withering and turning-over process (Fig. S2 A, B, C), suggesting the adversity-enhanced transcription of structural genes gave

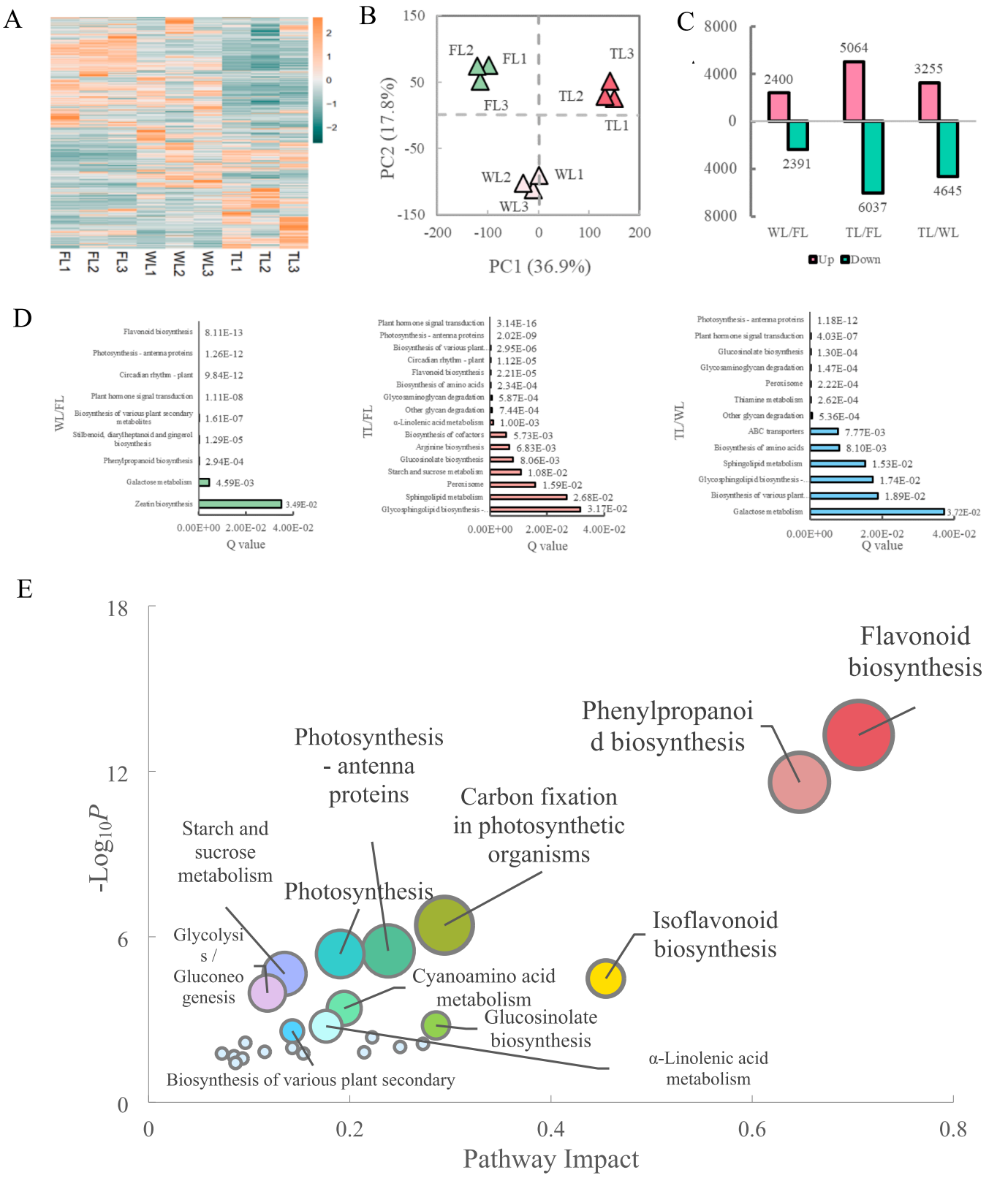

Fig. 2. The transcriptomic profiles of tea sample under processing. (A) The heatmap of expression dataset of transcriptomic detection. (B) The PCA score plot of tea samples. (C) The number of significantly different expressed genes (DEGs) in each paired-comparison. (D) The KEGG analysis of significantly enriched pathways (adjusted $P < 0.05$) on the DEGs in different paired-comparisons. The number represented the adjusted P value of each pathway. (E) The co-expressed metabolic pathways based on joint analysis on transcriptome-metabolite profiles.

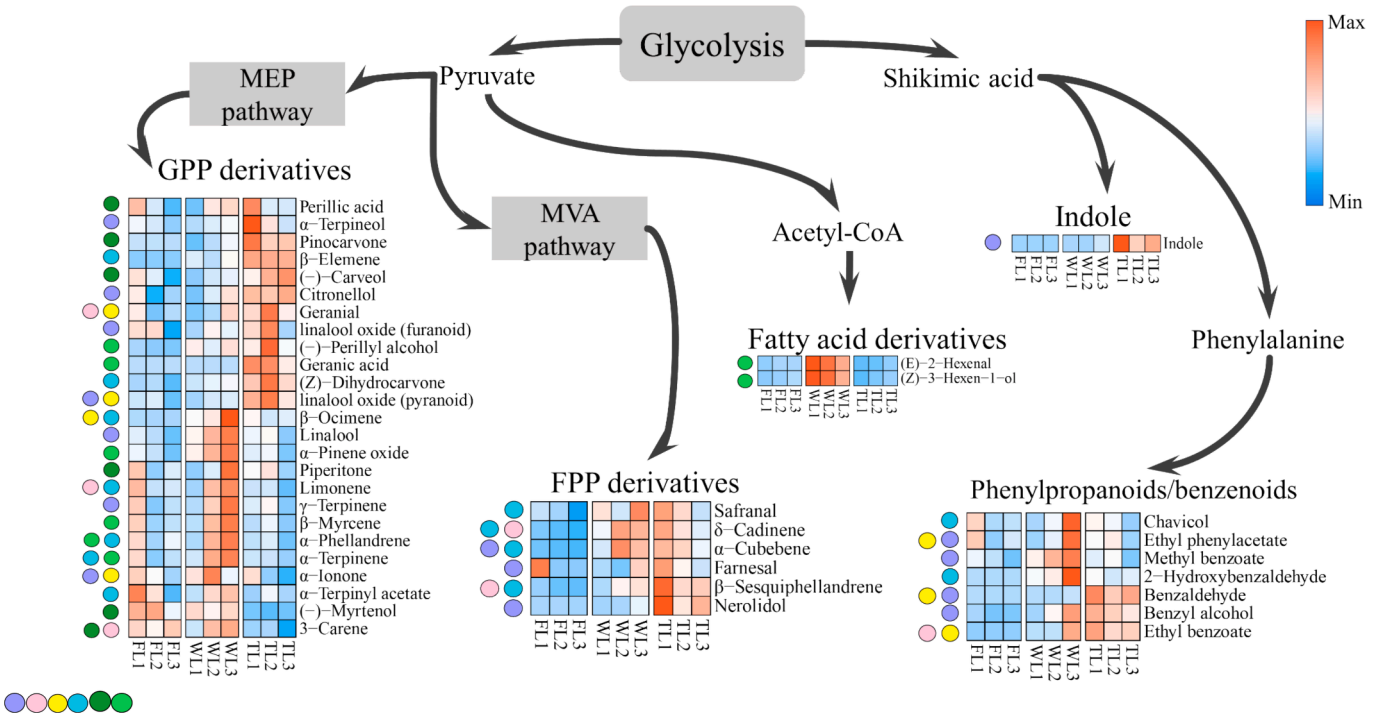

Fig. 3. The simplified proposed network of metabolic pathways associated with aroma formation during ECP stage of Tieguanyin tea production. MEP: methylerythritol phosphate; MVA: mevalonic acid; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate. The circle with different colors represented the odor of the components.: floral;: fruity;: sweet;: herbal;: minty;: grassy. The aroma description of the substance is , , , and accessed from http://www.thegoodscentscompany.comhttp://perflavory.comhttp://www.odour.org.uk/odour/index.htmlhttp://foodflavorlab.cn/#/home.

rise to the accumulation of α-terpineol and geranial in this study, agreed with the wounding-induced gene regulatory network observed during oolong tea production (Y. Zheng, Hu, Yang, et al., 2022). Apart from these floral-associated monoterpenoids, an increased content of linalool, limonene, β-ocimene, γ-terpinene, and α-ionone were also observed after withering, then sharply decreased at turning-over phase (Fig. 3). The VTs such as linalool, β-ocimene could be stimulated upon abiotic stresses such as dehydration and mechanical wounding in tea plant, while the decreasing of the VTs observed in this study might be ascribed to the accumulation and emission as a response to the adversity, which has also been observed in previous study that withering and bruising significantly increased the released terpenoid volatiles (F. Dong, et al., 2016; Hu, et al., 2018).

In contrast to monoterpenoids, sesquiterpenoids (C_{15} terpenoids) are a group of terpenoids consisting of three isoprene units that derived from MVA pathway. The representative sesquiterpenoids detected in this study, such as nerolidol, safranal, β-sesquiphellandrene, δ-cadinene, farnesal, and α-cubebene exhibited increasing trends along ECP stage (Fig. 3), leading to the intensified fruity and floral odors in Tieguanyin tea leaves. Nerolidol was one of the most quintessential aroma compounds prevalent in oolong teas, which would be produced under the catalyzation of nerolidol synthetase (*NES*). The expression of *NES* was enhanced over bruising process as presented in Fig. S2 D & E, in accordance with the study that continuous mechanical damage elevated the expression of NES, accounting for the accumulation of nerolidol during turning-over step.

Originated from shikimic acid pathways, phenylalanine acts as a precursor for a large array of multiple functional secondary metabolites, such as volatile phenylpropanoids and benzenoids (VPBs). The VPBs are the second most ubiquitous class of volatiles, exerting important ecological functions in plants. In this study, seven VPBs associated with typical scent of oolong tea, including methyl benzoate, chavicol, 2-hydroxybenzaldehyde, benzyl alcohol, benzaldehyde, ethyl phenylacetate, and ethyl benzoate, were quantified and exhibited dynamic change along ECP stage (Fig. 3). The abundance of benzyl alcohol, benzaldehyde, and ethyl benzoate showed overall increasing trend, peaked after turning-over step (Fig. 3), suggesting the combination of dehydration and mechanical wounding imposed on the tea leaf activated the gene expression on VPB biosynthesis pathway, such as benzoate *O*-methyltransferase (Fig. S2 F & G), enhancing the levels of VPBs. This result is in consistent with our previous stud that VPBs were significantly enhanced at the end of turning-over step, and the stresses such as mechanical wounding and water loss suffered during turning-over are indispensable for the augment of floral and fruity substances in oolong tea leaves (Wu, et al., 2022). In addition to VPBs, indole is a N-aromatic heterocyclic organic component that act as a precursor, core building block, and functional group of many important biochemical compound in plants. The synthesis of indole is to a large extent dependent on the interaction between α-subunit and β-subunit of tryptophan synthase (Zhang, et al., 2008). Continuous wounding on non-completed disrupted cell of oolong tea leaf elicited the expression of tryptophan synthase β-subunit (*TSB*), resulting in the aggregation of indole at bruising stage (Zeng, et al., 2016). The transcript levels of TSBs were significantly boosted during turning-over step in this study (Fig. S2 H & I), accounting for the accumulation of indole abundance in tea shoots after turning-over operation (Fig. 3).

Plants initiates the synthesis of fatty acid derivatives by using linolenic or linoleic acid as the starting point, then a series of C_6 or C_9 alcohols, aldehydes, and esters, such as (*E*)-2-hexenal and (*Z*)-3-hexenol, were produced under the catalysis of lipoxygenase (*LOX*), hydroperoxide lyase (*HPL*), alcohol dehydrogenase (*ADH*), *etc*. These compounds are termed as green leaf volatiles (GLVs) owing to their typical green leaf odor (Feussner, et al., 2002). In this study, the expressions of *LOX*, *HPL* and *ADH* peaked after withering, then sharply lowered over turning-over step (Fig. S2 J, K, L), potentially contributing to the observation that the contents of (*E*)-2-hexenal and (*Z*)-3-hexenol increased at withering, then reduced after turning-over process of Tieguanyin tea manufacturing (Fig. 3). The similar result has been reported in the study

on fatty acids dynamic change during postharvest process, in which *LOX*, *HPL*, as well as *ADH* were considered as the critical rate-limiting enzymes over long-chain unsaturated fatty acids biosynthesis, and the mechanical injury occurred during the postharvest process of oolong tea production greatly elevated the activities of these enzymes (Z. Zhou, et al., 2022). Furthermore, the enhancing level of VTs, VPBs, indole, in addition to the decreasing of GLVs over ECP stage led to an increasingly distinct floral-fruity aroma, promoting the characteristic fragrance of oolong tea.

Volatile components in plant tissues play crucial roles in reproduction and stress defense. The dynamic spatiotemporal location of various volatiles with tissue specific patterns would facilitate the exploration of the mechanism underlying the accumulation of key volatiles. Given the multiple detection on the tea leaf samples, this work revealed the spatial distribution and specific variations of volatile compounds, laying the groundwork for comprehensive understanding on the changes of metabolism process induced by oolong tea processing operations. Furthermore, the attempt of paralleled DESI-MSI and GC–MS detection for the same samples showed their difference in metabolites annotation, resolution, and data analysis. Although the lateral resolution of DESI-MSI is modest (50–200 μm) compared with that of other methods (Yoshimura, et al., 2020), its atmospheric operation allows for the analysis of various of samples without pre-treatments, resulting in minimal damage to the samples. However, diffraction limits remain a considerable obstacle, limiting further development of mass spectrum imaging with high spatial resolution and accurate quantification (Y. Dong, et al., 2020). Improvements in spatial resolution have increased the demand for detection sensitivity, and quantitative mass spectrum imaging remains a complex issue because the MS signal at each sampling pixel is strongly affected by modeling and visualization. It is expected that DESI may be combined with high resolution mass spectrometry in the future, to provide additional specific separation power for volatile detection.

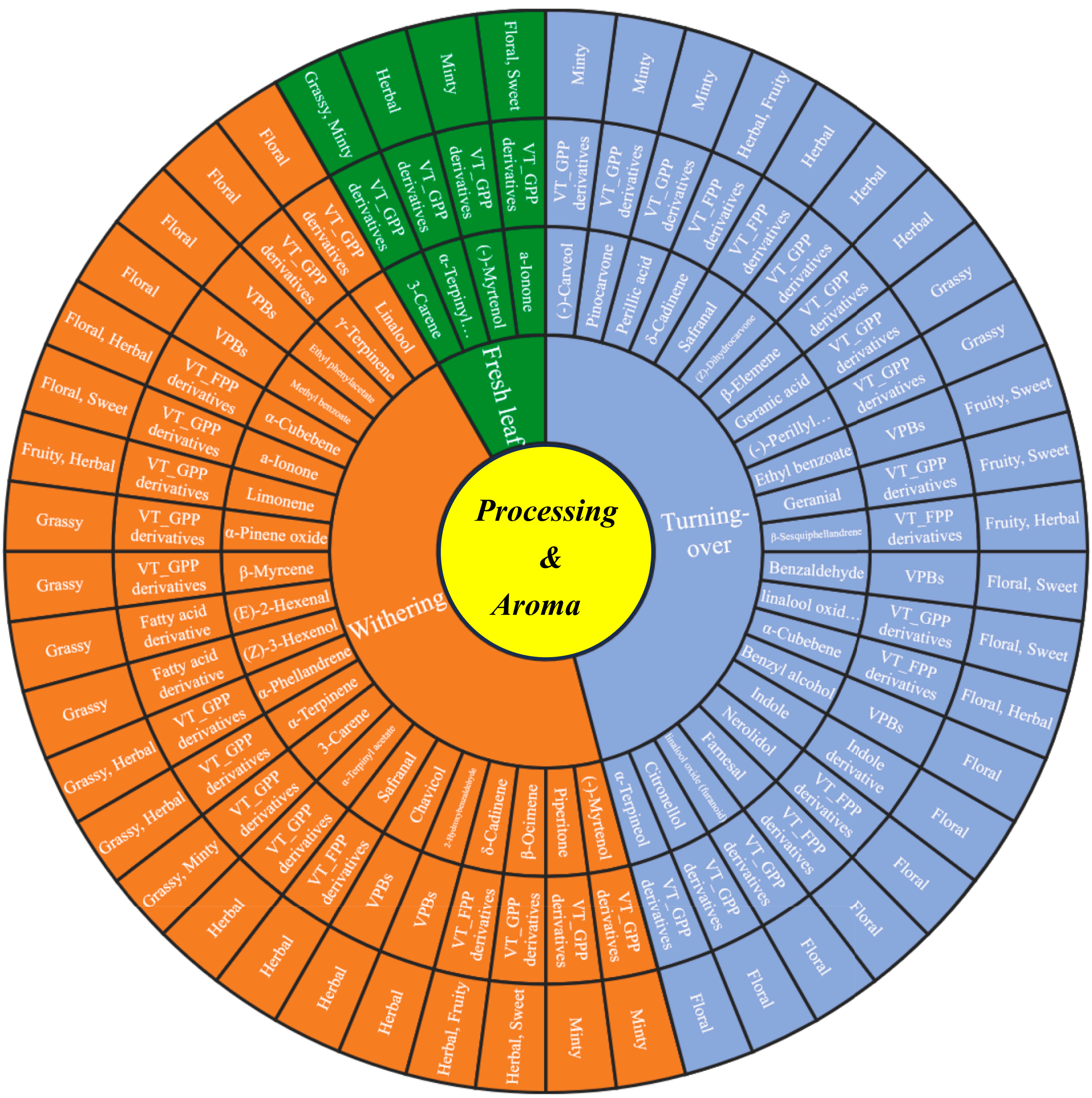

Fig. 4. The processing and aroma wheel of oolong tea sample over ECP stage. The first level is the processing steps including fresh leaf, withering and turning-over. The second level is the VOCs associated with the aroma. The third level is the categories of VOCs. The fourth level is the description of aroma. VT: Volatile terpenoid; GPP: Geranyl diphosphate; FPP: Farnesyl diphosphate; VPB: volatile phenylpropanoids & benzenoids.

3.4. The dynamic alterations of characteristic aroma

To elucidate the variations of characteristic aroma profiles during ECP stage, a processing and aroma wheel was proposed in Fig. 4 based on the metabolic network of aroma formation. The processing steps were divided into fresh leaf plucking (the green sector), withering (the red sector) as well as turning-over (the blue sector), as presented in Fig. 4. There were only four VOCs were clustered in fresh leaf (the green sector), mainly accounting for minty or grassy odors. This is primarily because most aroma compounds in oolong tea are generated along the manufacturing processes rather than being present in the original constituents, and the constituent odors predominantly refer to the volatiles originally present in cells and tissues, such as GLVs (Zeng, et al., 2024; Zeng, Zhou, Su, & Yang, 2020). The combination of representative VOCs, such as linalool, ethyl phenylacetate, methyl benzoate, β-ocimene, (*Z*)-3-hexenol, and (*E*)-2-hexenal, contributed to a mixed fragrance of floral, herbal as well as grassy in withered leaf (Fig. 4), which is agreed with the aroma profiles of previous study on VOCs in oolong tea (Y. Zheng, Hu, Yang, et al., 2022). During turning-over step, the overall fragrance has gradually converted into floral and fruity, attributing to the increasing proportion of indole, nerolidol, linalool oxides, benzyl alcohol, benzaldehyde, geranial, ethyl benzoate, *etc.* (Fig. 4), most of which were formed by *de novo* synthesis in response to environmental stresses (Zeng, Watanabe, & Yang, 2019). The processing and aroma wheel analysis revealed the chronological variations in the composition and proportion of VOCS accounted for the characteristic floral-fruity aroma of Tieguanyin oolong tea.

4. Conclusion

Withering and turning-over are critical steps for the flavor development over oolong tea production, ascribing to their impact on the aroma development. In this study, the analysis on transcriptome profile indicated the artificial environment stresses occurred over ECP stage induced the expression of structural genes on terpenoids and phenylpropanoids/benzenoids pathways, which was indispensable for the accumulation of oolong tea fragrance. The results of determination on VOCs showed that floral & fruity associated VTs, VPBs were increasing, while the green leaf related GLVs were reducing along the chronological producing steps. The enhancive proportion of floral & fruity associated volatiles to green leaf volatiles is the key chemical foundation for the formation of "Guanyin Yun" during the production of Tieguanyin tea, which could be a feasible index for the processing technique or quality assessment of oolong tea.

CRediT authorship contribution statement

Liangyu Wu: Writing – original draft, Investigation, Funding acquisition, Data curation. **Xiaolan Chen:** Resources, Methodology. **Jiaqi Lin:** Investigation, Data curation. **Hongzheng Lin:** Methodology, Investigation. **Ningkai Liao:** Methodology, Data curation. **Chenxue Li:** Software, Investigation, Data curation. **Yunfei Hu:** Resources, Funding acquisition. **Yun Sun:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2024.100227.

Data availability

Data will be made available on request.

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