LC-MS/MS-Based Analysis of Metabolite Changes in Rhododendron delavayi Flowers (Postprint)

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Abstract

To analyze the differences in metabolites and their associated pathways during the flowering to withering process of Rhododendron delavayi, this study employed LC-MS/MS technology to conduct untargeted metabolomics analysis of chemical components across six developmental stages: bud stage, cracking stage, pollination stage, full-bloom stage, senescence stage, and withering stage. The results demonstrated: (1) A total of 973 metabolites were identified, primarily comprising flavonoids, organic acids, phenolic acids, amino acids and their derivatives, lipids, and alkaloids. (2) Principal component analysis (PCA) revealed differences in metabolite profiles among samples. Differential metabolites were screened using orthogonal partial least squares discriminant analysis (OPLS-DA), P values from t-tests, and fold-change (Fc) from univariate analysis (VIP > 1, P < 0.05, Fc > 2 or Fc < 0.5), involving 591 metabolites. The number and expression levels of differential metabolites increased significantly when R. delavayi flowers entered the senescence and withering stages. Specifically, differential metabolite expression was predominantly down-regulated from bud stage to cracking stage, while up-regulation was predominant after entering senescence and withering stages. (3) KEGG annotation identified 68 metabolic pathways, among which three pathways showed extremely significant enrichment (P < 0.01) of differential metabolites, including phenylpropanoid biosynthesis, plant hormone biosynthesis, and flavonoid biosynthesis. (4) Combined analysis of biosynthetic pathways for active components such as phenylpropanoids and flavonoids screened 10 metabolites, including L-phenylalanine, trans-cinnamic acid, chalcone, naringenin, p-coumaroyl shikimic acid, ferulic acid, coniferyl alcohol, sinapic acid, syringin, and quercetin. Furthermore, differential metabolites of active components revealed that phenylpropanoid biosynthetic metabolic activity gradually enhanced with the development of R. delavayi flowers, whereas flavonoid biosynthesis gradually weakened. These key differential metabolites

may play important regulatory roles in the development of R. delavayi flowers. This study provides a metabolomics foundation for investigating active substances in metabolic pathways of effective components during the flowering to withering process of R. delavayi, and provides a reference for further research on the molecular mechanisms underlying floral stage regulation.

Full Text

Analysis of Metabolite Changes in Rhododendron delavayi Flowers from Flowering to Withering Based on LC-MS/MS

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Abstract

To analyze the differences in metabolites and their metabolic pathways during the flowering-to-withering process of *Rhododendron delavayi*, we performed non-targeted metabolomic analysis of chemical components at six developmental stages—bud stage, dehiscence stage, pollination stage, blooming stage, senescence stage, and withering stage—using LC-MS/MS technology. The results revealed: (1) A total of 973 metabolites were identified, primarily including flavonoids, organic acids, phenolic acids, amino acids and their derivatives, lipids, and alkaloids. (2) Principal component analysis (PCA) demonstrated significant differences in metabolites among samples. By combining orthogonal partial least squares discriminant analysis (OPLS-DA), t-test P-values, and fold-change (Fc) values from univariate analysis, we screened 591 differential metabolites (VIP $>$ 1, P $<$ 0.05, Fc $>$ 2 or Fc $<$ 0.5). Both the number and expression level of differential metabolites increased significantly after the flowers entered senescence and withering stages. Notably, differential metabolites were predominantly down-regulated from bud to dehiscence stage, but mainly up-regulated during senescence and withering. (3) KEGG annotation identified 68 metabolic pathways, with three pathways showing extremely significant enrichment (P $<$ 0.01): phenylpropanoid biosynthesis, plant hormone biosynthesis, and flavonoid biosynthesis. (4) By integrating the biosynthetic pathways of phenylpropanoids, flavonoids, and other active components, we identified 10 key metabolites: L-phenylalanine, trans-cinnamic acid, chalcone, naringenin, p-coumaroyl shikimic acid, ferulic acid, coniferyl alcohol, sinapic acid, syringin, and quercetin.

Furthermore, analysis of differential metabolites of active components indicated that phenylpropanoid biosynthetic activity gradually intensified with flower development, while flavonoid biosynthesis gradually weakened. These key differential metabolites may play important regulatory roles in *R. delavayi* flower development. This study provides a metabolomic foundation for investigating active substances in metabolic pathways during the flowering-to-withering process and offers a reference for further research on the molecular mechanisms underlying flowering regulation in *R. delavayi*.

Keywords: *Rhododendron delavayi*, flowering period, metabolome, LC-MS/MS, metabolic pathway

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Introduction

Rhododendron delavayi is an evergreen shrub or small tree named for its bright red flowers that resemble horse tassels. It is primarily distributed in southwestern China, including southwestern Sichuan, throughout Yunnan, and northwestern Guizhou (Zhang et al., 2015). In northwestern Guizhou, it is most extensively distributed in the Pudi and Jinpo scenic areas of Baili Rhododendron Forest, serving as a dominant and constructive species (Li & Chen, 2005). The flowering cycle typically spans approximately 25–30 days: initial flowering (from petal opening to 10% inflorescence bloom) occurs before mid-March; full bloom (10% to 10% withering inflorescences) lasts from late March to early April; and the withering stage (10% to 70% withered inflorescences) occurs in mid-April. Investigating metabolite changes during this process is crucial for extending and regulating the flowering period, thereby maximizing the ornamental and economic value of *R. delavayi* for local economic development and rural revitalization in Guizhou Province.

Current research on *R. delavayi* flowers has primarily focused on floral characteristics and pollen traits. Previous studies have examined floral trait differentiation and resource distribution in the Baili Rhododendron Nature Reserve (Hu et al., 2021) and investigated pollen morphology, size, exine ornamentation, and aperture characteristics using microscopy (Mao, 2000). Flowering regulation research has concentrated on nutrient elements, plant hormones, and temperature treatments, including effects of different N, P, K, and Ca concentrations on bud development and flowering (Hong et al., 2010), impacts of exogenous hormone spraying on flowering stages (Song et al., 2010), and exploration of flowering regulation methods using growth regulators and mineral nutrients (Yue, 2015). While a few studies have reported on metabolites from *R. delavayi* stems and roots—identifying 10 compounds using chromatographic techniques (Xu et al., 2012)—no research has yet investigated metabolites and metabolic pathways during flower development.

In recent years, plant metabolomics has been widely applied to analyze metabolite changes during fruit tree growth, development, and maturation (Aaron et

al., 2008; Zhang et al., 2011; Li et al., 2020) and has begun to illuminate the metabolic mechanisms underlying flower formation and development (Jia et al., 2017; Cheng et al., 2020). For example, GC-MS analysis of metabolomes at different developmental stages of coltsfoot flowers revealed significant changes in characteristic metabolites during bud development (Xue et al., 2012). Transcriptomic and metabolomic studies of *Dendrobium officinale* flower development found that secondary metabolism and carbohydrate changes were associated with flower development (He et al., 2020). Similarly, GC-MS detection of metabolites during loquat flower development revealed physiological and metabolic mechanisms affecting the process (Xu et al., 2020). These studies demonstrate that metabolomics provides valuable methodology for investigating plant flower development.

This research focuses on *R. delavayi* flowers from the Baili Rhododendron Scenic Area in Dafang County, Bijie City, Guizhou Province. Using non-targeted metabolomics (LC-MS/MS) analysis of six developmental stages (bud, dehiscence, pollination, blooming, senescence, and withering), we address three key questions: (1) How do metabolites change across different stages from flowering to withering? (2) Which metabolites and metabolic pathways show significant differences? (3) Which metabolites are crucial for flower development? The results provide a theoretical foundation and scientific basis for flowering regulation in *R. delavayi*.

1.1 Experimental Materials

Experimental materials were *R. delavayi* Franch. flowers collected from the Baili Rhododendron Scenic Area in Dafang County, Bijie City, Guizhou Province. In 2020, we selected *R. delavayi* plants with consistent growth status and collected flower samples from six individual trees at six time points: bud stage (March 20), dehiscence stage (March 24), pollination stage (March 30), blooming stage (April 3), senescence stage (April 10), and withering stage (April 15). Samples were frozen in liquid nitrogen immediately after collection, transported to the laboratory for metabolomic analysis, with six biological replicates per stage.

1.2 Metabolite Extraction from *R. delavayi* Flowers

Non-targeted metabolomics was performed using LC-MS/MS technology (Warwick et al., 2011; Want et al., 2010). For each sample, 100 mg of liquid nitrogen-ground *R. delavayi* flower tissue was placed in an EP tube, mixed with 500 L of 80% methanol aqueous solution containing 0.1% formic acid, vortexed, and incubated on ice for 5 minutes. After centrifugation at 15,000 rpm for 10 minutes at 4°C, 100 L of supernatant was diluted with ultrapure deionized water to achieve 53% methanol content, then centrifuged again at 15,000 g for 10 minutes at 4°C. The final supernatant was collected for LC-MS/MS analysis.

Quality control (QC) samples were prepared by mixing equal volumes from each experimental sample. Blank samples were prepared using 53% methanol

aqueous solution with 0.1% formic acid instead of plant material, following the same pretreatment procedure.

1.3 Non-Targeted Metabolomics (LC-MS/MS) Detection

Chromatographic separation was performed on a Thermo Hyperil Gold C18 column at 40°C with a flow rate of 0.2 mL/min. For positive ion mode, mobile phase A was 0.1% formic acid and mobile phase B was methanol. For negative ion mode, mobile phase A was 5 mM ammonium acetate (pH 9.0) and mobile phase B was methanol. The gradient elution program was: 0–1.5 min, 98% A, 2% B; 1.5–12 min, 100% B; 12–14 min, 100% B; 14–14.1 min, 98% A, 2% B; 14.1–17.0 min, 98% A, 2% B. The injection volume was 10 L.

Mass spectrometry was conducted using a Thermo Vanquish UHPLC system coupled with a Thermo QE series mass spectrometer in both positive and negative ion modes. The scan range was m/z 70–1,050. ESI source parameters were: spray voltage 3.2 kV, sheath gas flow rate 35 arb, auxiliary gas flow rate 10 arb, capillary temperature 320°C. MS/MS scans were performed in data-dependent mode. QC samples were analyzed every 10 samples to evaluate instrument stability and data accuracy throughout the experiment.

1.4 Data Analysis

Raw data were imported into Compound Discoverer 3.1 (CD) software for processing. Peaks were filtered based on retention time and mass-to-charge ratio, aligned using a retention time tolerance of 0.2 min and mass tolerance of 5 ppm, and extracted based on mass deviation (5 ppm) and signal intensity deviation (30%) (Dai et al., 2017). Peak areas were quantified, target ions integrated, and molecular formulas predicted using molecular and fragment ions. Compounds were identified by matching against mzCloud, mzVault, and MassList databases. Background ions were removed using blank samples, and quantitative results were normalized to obtain final identification and quantification data.

MetaX software (Wen et al., 2017) was used for log transformation of the data. All experimental and QC samples were imported into SIMCA 14.1 for multivariate statistical analysis (A JiYe, 2010). Principal component analysis (PCA) was performed to assess overall sample stability, followed by supervised orthogonal partial least squares discriminant analysis (OPLS-DA) to differentiate metabolite profiles between groups. Differential metabolites were screened using variable importance in projection (VIP $>$ 1) from the OPLS-DA model, combined with t-test P-values ($P < 0.05$) and fold-change values (Fc $>$ 2 or Fc $<$ 0.5). Venn diagrams were generated using TBtools 0.6 software, and metabolic pathway enrichment analysis was performed based on the KEGG database.

2.1 Morphological Changes in *R. delavayi* from Flowering to Withering

The flowering-to-withering process was divided into six stages, as shown in [Figure 1: see original paper]. **Bud stage (A):** Flowers remained closed as buds, with immature anthers and undehisced stamens. **Dehiscence stage (B):** Buds began to open, stamens had just dehisced, and no pollen was present on the stigma. **Pollination stage (C):** Buds were more open, stamens were fully dehisced, and pollen had covered the stigma. **Blooming stage (D):** Flowers were fully open with vibrant colors; stamens were completely dehisced with all pollen released, and little pollen remained on the stigma, indicating completed pollination. **Senescence stage (E):** Flowers began to wither and show wilting. **Withering stage (F):** Flowers were completely wilted, withered, and desiccated.

2.2 Principal Component Analysis of *R. delavayi* Flower Samples

To analyze relationships among metabolomes at different stages, PCA was performed to simplify and reduce dimensionality through orthogonal transformation, revealing internal structural relationships among metabolic profiles. The PCA results ([Figure 2: see original paper], where A represents positive ion mode and B represents negative ion mode) showed clear separation trends among different stages, indicating reliable data processing and significant differences between samples. Good overlap among biological replicates within each group demonstrated high reproducibility, validating subsequent differential metabolite analysis. Metabolite changes were relatively large from bud to dehiscence, pollination, and blooming stages, with the greatest differences observed between senescence and withering stages.

To identify differential metabolites during the flowering-to-withering process, OPLS-DA was used to distinguish overall metabolic differences among the six stages ([Figure 3: see original paper]). The six stages—bud, dehiscence, pollination, blooming, senescence, and withering—showed clear metabolic separation, indicating significant differential changes. Model quality parameters R^2 and Q^2 were both >0.9, confirming the stability and predictive capability of the OPLS-DA model for identifying potential differential metabolites.

2.3 Metabolite Profiling of *R. delavayi* Flowers

LC-MS/MS multi-peak chromatograms were generated for metabolites across six developmental stages, with the bud stage chromatogram shown in [Figure 4: see original paper] and other stages in Appendix 1. A total of 973 metabolites were detected: 587 in positive ion mode and 386 in negative ion mode. These included 114 flavonoids, 114 organic acids, 112 phenolic acids, 75 amino acids and derivatives, 61 lipids, 57 alkaloids, 45 terpenoids, 43 nucleotides and derivatives, 36 carbohydrates and derivatives, 33 phenylpropanoids, 33 alcohols and polyols, 11 tannins, 3 steroids, and 236 others.

2.4 Analysis of Differential Metabolites

Differential metabolites across the six stages were screened using OPLS-DA (VIP $>$ 1, P $<$ 0.05, Fc $>$ 2 or Fc $<$ 0.5). Pairwise comparisons between adjacent stages revealed 591 differential metabolites in total: 170 between bud and dehiscence (87 positive, 83 negative; 48 up-regulated, 122 down-regulated), 144 between dehiscence and pollination (89 positive, 55 negative; 68 up-regulated, 76 down-regulated), 111 between pollination and blooming (67 positive, 44 negative; 75 up-regulated, 36 down-regulated), 284 between blooming and senescence (184 positive, 100 negative; 177 up-regulated, 107 down-regulated), and 314 between senescence and withering (180 positive, 134 negative; 260 up-regulated, 54 down-regulated). Differential metabolite numbers increased markedly during senescence and withering compared to earlier stages, with most showing significantly elevated expression. Conversely, most differential metabolites were down-regulated from bud to dehiscence. Venn diagram analysis ([Figure 5: see original paper]) showed that the vast majority ($ $46.8%) of differential metabolites appeared only in one adjacent transition, with no metabolites present across all stages.

2.5 Metabolic Pathway Analysis

KEGG Pathway enrichment analysis of significantly differential metabolites (Kanehisa & Goto, 2000; Jun Rao et al., 2014) identified 68 metabolic pathways, with six showing significant enrichment (P $<$ 0.05): phenylpropanoid biosynthesis, plant hormone biosynthesis, flavonoid biosynthesis, arginine and proline metabolism, phenylalanine/tyrosine/tryptophan biosynthesis, and ornithine/lysine/nicotinate alkaloid biosynthesis. Three pathways were extremely significant (P $<$ 0.01): phenylpropanoid biosynthesis, plant hormone biosynthesis, and flavonoid biosynthesis ([Figure 6: see original paper]).

By integrating metabolic pathway analysis ([Figure 7: see original paper]), we identified 10 key differential metabolites: L-phenylalanine, trans-cinnamic acid, chalcone, naringenin, p-coumaroyl shikimic acid, ferulic acid, coniferyl alcohol, sinapic acid, syringin, and quercetin. Expression patterns across six stages revealed that phenylpropanoid-related metabolites (L-phenylalanine, trans-cinnamic acid, p-coumaroyl shikimic acid, coniferyl alcohol, sinapic acid, syringin) were up-regulated during senescence and withering, peaking at the withering stage. In contrast, flavonoid-related metabolites (naringenin, quercetin, chalcone) were down-regulated during flower development. Previous studies have shown that during critical winter bud differentiation in 'Summer Black' grape secondary fruit, CCC treatment induces up- or down-regulation of genes in phenylpropanoid and flavonoid pathways, promoting flowering by altering flower-related gene expression and secondary metabolite synthesis (Shi et al., 2021). Transcriptomic analysis of coltsfoot leaves at different developmental stages also revealed increasing expression of phenylpropanoid biosynthesis genes during leaf growth (Nie et al., 2018). The gradual increase in phenylpropanoid metabolites and decrease in flavonoid metabolites during

R. delavayi flowering may be closely related to flower development.

3.1 Differential Metabolites in *R. delavayi* Flowers

Metabolites form the basis of organismal phenotypes and provide direct insight into biological processes and mechanisms (Xiong et al., 2019). GC-MS analysis of loquat flower development identified the C/N ratio as a key determinant of developmental progression (Xu et al., 2020). Flowering regulation in *R. delavayi* has long been a research focus, as the flowering-to-withering process involves complex physiological changes closely associated with metabolite types and quantities. This non-targeted metabolomics study investigated metabolite changes across six developmental stages. We identified 591 differentially expressed metabolites, primarily flavonoids, organic acids, phenolic acids, amino acids and derivatives, lipids, and alkaloids. These differential metabolites showed stage-specific expression patterns, decreasing from bud to blooming stages but increasing significantly during senescence and withering. Up-regulated metabolites gradually increased throughout the process, peaking at withering, while down-regulated metabolites decreased from bud to blooming, increased during senescence, then decreased at withering. These patterns suggest that differential metabolite types and expression are intimately linked to the flowering-to-withering progression.

3.2 Differential Metabolic Pathways in *R. delavayi* Flowers

KEGG pathway enrichment analysis annotated 68 metabolic pathways, with three being extremely significant ($P < 0.01$): phenylpropanoid biosynthesis, plant hormone biosynthesis, and flavonoid biosynthesis. Phenylpropanoid biosynthesis was the most significant pathway, with L-phenylalanine providing precursors for downstream secondary metabolites. We identified 10 key differential metabolites related to active component biosynthesis. Phenylpropanoid-related metabolites showed gradually increasing expression during senescence and withering, while flavonoid-related metabolites decreased during development, suggesting important regulatory roles in flower withering.

Phenylpropanoid biosynthesis is a crucial plant secondary metabolic pathway (Dong & Lin, 2021) that produces numerous secondary metabolites including pigments, phenolic acids, flavonoids, and lignin (Wen et al., 2017). Studies on coltsfoot flowers revealed distinct secondary metabolite compositions at different developmental stages, with high phenylpropanoid expression from early to middle stages and fluctuating flavonoid levels that overall promoted flower development (Jia et al., 2017). Transcriptomic analysis of *Citrus medica* var. *sarcodactylis* showed seven phenylpropanoid biosynthesis genes were differentially expressed during fruit development and color change, with increased expression promoting fruit development (Pan et al., 2020). The phenylpropanoid pathway converts L-phenylalanine to trans-cinnamic acid, which is hydroxylated by cinnamate-4-hydroxylase (C4H) to p-coumaric acid, then catalyzed by 4-coumarate-CoA ligase (4CL) to 4-coumaroyl-CoA, which is further con-

verted to various phenylpropanoid products including coumarins, flavonoids, terpenoids, and lignin (Wang et al., 2019). In *R. delavayi*, phenylpropanoid-related metabolites were up-regulated during development, peaking at withering, while flavonoid-related metabolites were down-regulated. Studies on Chinese cabbage flowers revealed that phenylpropanoids are major floral scent components, while flavonoids are primary pigments in flowers, fruits, and seeds that also affect petal elongation and pollen germination (Li et al., 2017). Intermediates such as L-phenylalanine (Hou et al., 2015), naringenin (Tu et al., 2016), and ferulic acid (Louie et al., 2010) directly limit downstream pathways and influence flowering. As important secondary metabolites, phenylpropanoids and flavonoids likely play crucial regulatory roles in *R. delavayi* flowering. Further molecular-level studies are needed to fully elucidate these relationships.

This study employed non-targeted metabolomics to analyze metabolic components at different developmental stages, annotated differential metabolites using the KEGG database, and constructed biosynthetic pathways for phenylpropanoids and flavonoids. These results provide a reference for investigating gene regulatory mechanisms and comprehensively analyzing metabolic pathways, offering a theoretical foundation for mechanisms to extend the flowering period of *R. delavayi*.

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Note: Figure translations are in progress. See original paper for figures.