

Integrative Transcriptomic and Metabolomics Reveal Dynamic Differences in Pathogen Resistance Between Resistant and Susceptible Cultivars of *Myrica rubra*

Yingyao Liu

Zhejiang Institute of Subtropical Crops

Yang Song

Zhejiang Institute of Subtropical Crops

Peian Zhang

Zhejiang Institute of Subtropical Crops

Quan Zhao

Zhejiang Institute of Subtropical Crops

Hui Li

Yueqing City Bureau of Agriculture and Rural Affairs

Jun Chen

Zhejiang Institute of Subtropical Crops

Xiuzhu Guo

Zhejiang Institute of Subtropical Crops

Fayong Li

Zhejiang Institute of Subtropical Crops

Dongfeng Liu

liudongfeng001@126.com

Zhejiang Institute of Subtropical Crops

Research Article

Keywords: *M. rubra*, Pestalotiopsis, Immune response, Transcriptomics, Metabolomics

Posted Date: July 22nd, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7090173/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations:

No competing interests reported.

Supplemental figures S1-S4 are not available with this version.

Version of Record: A version of this preprint was published at BMC Plant Biology on December 5th, 2025.
See the published version at <https://doi.org/10.1186/s12870-025-07543-1>.

Integrative Transcriptomic and Metabolomics Reveal Dynamic Differences in Pathogen Resistance Between Resistant and Susceptible Cultivars of *Myrica rubra*

Yingyao Liu^{1,a}, Yang Song^{1,a}, Peian Zhang¹, Quan Zhao¹, Hui Li², Jun Chen¹, Xiuzhu Guo¹, Fayong Li¹, Dongfeng Liu^{1,*}

¹Zhejiang Institute of Subtropical Crops, Wenzhou, China

²Yueqing City Bureau of Agriculture and Rural Affairs, Wenzhou, China

^aThese authors correspond equally to the present work

Abstract

Background:

Twig blight (TB), caused by *Pestalotiopsis*-like species, induces twig dieback in *Myrica rubra*, leading to substantial economic losses in its production across China and posing a significant threat to *M. rubra* cultivation. To date, the immune response of *M. rubra* to *Pestalotiopsis*-like infections remains poorly understood.

Results:

In this study, full-length transcriptomics and untargeted metabolomics were employed to investigate the immune responses of resistant “Dingao” (DA) and susceptible “Dongkui” (DK) *M. rubra* cultivars to *Pestalotiopsis* pathogen infection at early (1DPI) and later (5DPI) stage. The main findings revealed that the resistant DA triggered a rapid and robust immune response at 1DPI, with 1,111 differentially expressed genes (DEGs), including 583 increased and 528 decreased. As the immune response progressed to 5DPI, the number of DEGs in DA was reduced to with 324 DEGs (114 upregulated and 210 downregulated). In contrast, the susceptible DK cultivar exhibited a weak and delayed defense response. At 1DPI only 99 DEGs (26 upregulated and 73 downregulated) in DK leaves; by 5DPI, a substantial shift occurred in DK samples, with 3,414 DEGs (2,141 upregulated and 1,273 downregulated), showing a late but massive defense response. In DA samples, WRKY,

AP2/ERF transcription factors (TFs) were the most up-regulated TFs at the early immune response stage; by 5DPI, NAC and WRKY families became the most prominently expressed TFs. In DK, notable TF upregulation (including WRKY, MYB and AP2/ERF) was only observed at 5DPI and there were not significantly expression changes at the early immune response stage. Untargeted metabolomics further revealed that DK exhibited 280 upregulated and 12 downregulated differentially accumulated metabolites (DMs) at 1DPI; 503 DMs upregulated and 166 downregulated DMs at 5DPI. In DA plants, 472 DMs were upregulated and 110 downregulated at 1DPI, whereas at 5DPI, 387 DMs were upregulated and 219 downregulated.

Conclusions: Through a combined analysis of full-length transcriptomics and untargeted metabolomics, we revealed distinct molecular and metabolic responses between resistant “Dingao” (DA) and susceptible “Dongkui” (DK) *M. rubra* cultivars under TB stress.

Keywords

M. rubra, *Pestalotiopsis*, Immune response, Transcriptomics, Metabolomics

Introduction

Chinese bayberry (*Myrica rubra*), also known as yangmei, is a subtropical fruit tree native to eastern Asia, with a cultivation history of over 2,000 years in China [1]. This fruit is celebrated for its sweet-tart flavor and is enjoyed fresh or used in various food products like juices, wines, jams, and traditional medicines. In addition to its culinary appeal, both the leaves and fruits of *M. rubra* are rich in phytochemicals including tannins, flavonoids, triterpenes, and diarylheptanoids. These components exhibit multiple biological activities, with particularly notable anti-inflammatory effects [2]. Chinese bayberry holds significant economic value, with an annual national production of approximately 1.5 million tons as of 2022. Zhejiang Province is a major

producer, yielding around 65 thousand tons with an estimated market value of ¥4.6 billion (approximately US\$740 million) [3]. Despite its commercial importance and high nutritional content, the yield and quality of Chinese bayberry are increasingly threatened by diseases caused by various pathogens. These diseases can severely impact both the quantity and quality of the fruit, leading to substantial economic losses.

Twig blight (TB), caused by *Pestalotiopsis*-like species, is one of the most severe diseases affecting Chinese bayberry cultivation in China [4]. The disease causes twig dieback and is widespread across all major Chinese bayberry producing areas.

Pestalotiopsis, a common plant pathogenic genus, is well-known for causing leaf blight in various plant species, including tea, apple, and guava, resulting in substantial economic losses worldwide [5, 6]. In *M. rubra*, TB manifests as brown spots on leaves and twigs, causing infected trees to shed leaves and eventually die within 1 to 4 years [7]. This disease spreads rapidly, posing a serious threat to the development of Chinese bayberry cultivation [8].

Some *M. rubra* cultivars, such as Dingao, exhibit significant resistance to TB, while others like Dongkui — the most widely cultivated and important cultivar, are highly susceptible [9]. To date, the molecular mechanisms underlying resistance and susceptibility between these cultivars remain limited understood. Therefore, extensive breeding research aimed at deepening understanding of the genetic basis of disease resistance is currently the most urgent priority for combating this disease and protect Chinese bayberry cultivation and production.

Plants have evolved intricate immune systems to resist pathogen attacks and minimize damage. These defenses are primarily divided into two layers: Pattern-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI). PTI is triggered when pattern recognition receptors (PRRs) on the plant cell membrane recognize pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) [10, 11]. This response triggers a signaling cascade that

strengthens cell walls, produces reactive oxygen species (ROS), and activates defense-related genes, providing broad-spectrum resistance [12]. On the contrary, ETI is a more precise and strong immune response triggered when plant intracellular receptors recognize specific pathogen effectors that attempt to suppress PTI. ETI typically leads to a stronger, more localized defense response, such as the hypersensitive response (HR), resulting in cell death at the site of infection to prevent disease spread [11], thereby offering targeted protection against specific pathogens. Both PTI and ETI activate various well-characterized downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) cascades, the transcription of defense-responsive genes, and hormone signaling transduction.

Plants respond to biotic and abiotic stresses through complex regulatory networks, involving transcription factors, signal transduction, plant hormones and secondary metabolites. Transcriptomics is a powerful tool for identifying gene-level regulatory mechanisms, while metabolomics provides insights into key metabolites such as amino acids, hormones, flavonoids, organic acids, and lipids.

To investigate the resistance mechanisms of *M. rubra* against *Pestalotiopsis*-induced twig blight, we conducted an integrative transcriptomic and metabolic analysis of two cultivars: the susceptible 'Dongkui' (DK) and the resistant 'Dingao' (DA). This comprehensive approach enabled us to find out vital defense signaling and metabolic pathways in the plant's time-course immune response to twig blight.

Our study provides an integrative view of Chinese bayberry's resistance to *Pestalotiopsis* and enhances our understanding of how *M. rubra* genetically and physiologically defends against *Pestalotiopsis* infection. These findings offer valuable insights for developing sustainable cultivation genetically and physiologically counters and breeding strategies for Chinese bayberry.

Material and Methods

Plant and Fungal material, growth conditions, and pathogenicity analysis

111 The Chinese bayberry (*M. rubra*) plants used in this study were grown in open fields
112 within the germplasm resources nursery of the Zhejiang Subtropical Crop Research
113 Institute, Wenzhou, Zhejiang, China. The cultivation area is located at approximately
114 28°00' N, 120°63' E, with an altitude of 100 m. These plants were cultivated using
115 standard agricultural practices, including regular watering, pest control, and
116 fertilization. Inside the nursery, the commercial cultivar "Dongkui" used in this study
117 was purchased from Wenzhou Ou Hai Chashan Xinpan Agriculture Co., Ltd. in 2024,
118 and the commercial cultivar "Dingao" was purchased from Yueqing Hongda Fruit
119 Cultivation Specialized Cooperative in 2024.

120 The pathogenic fungus, *Pestalotiopsis microspora*, were purchased from Biobw
121 (Beijing, China. For cultivation, the fungus was grown on prune agar medium (per L:
122 40 mL of prune juice, 1 g yeast extract, 5 g lactose, 5 g sucrose, and 20 g agar with
123 pH adjusted to 6.5) at 28 °C for 4-5 days [13].

124 For pathogenicity analysis, no field experiments were conducted in this study;
125 instead, these were performed indoors. we randomly selected six one-year-old
126 individual plants from both "Dongkui" and "Dingao" cultivars for pathogenicity,
127 transcriptomic, and metabolomic analyses. Following the method described in our
128 previous manuscript, two leaves were cut from each plant and combined into a single
129 group per cultivar. These were then inoculated with agar under laboratory conditions.
130 For inoculation, a 1mm² PA agar containing *P. microspora* was placed onto the
131 surface of plant leaves through the needle-pierced holes. For negative controls,
132 leaves were pierced and treated with plain PA agar (without the pathogen).

133 Inoculated leaves were placed in transparent flasks and covered with plastic wrap to
134 maintain continuous high humidity in a growth chamber (25 °C ± 2 °C). Photographs
135 of the disease phenotype, including both inoculated and systemic leaves, were taken
136 daily following pathogen inoculation. For each genotype, 10 leaves from three
137 individual plants were evaluated per experiment, with three independent replicates
138 performed [14].

RNA Isolation, Sequencing, and Bioinformatic Analyses

RNA extraction, reverse transcription, library preparation, and sequencing were conducted at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). RNA-seq libraries were constructed using the Illumina Stranded mRNA Prep Ligation Kit. Poly(A) mRNA was enriched by oligo(dT) beads, fragmented, and reverse transcribed into cDNA with random hexamer primers. After end repair, phosphorylation, and adapter ligation, ~300 bp fragments were selected and amplified with 15 PCR cycles. Final libraries were quantified with Qubit 4.0 and sequenced on the Illumina NovaSeq X Plus platform (PE150). Raw paired-end reads were trimmed and quality-checked using fastp [15]. Clean reads were aligned to the reference genome in orientation mode using HISAT2 [16], and assembled using StringTie [17] using a reference-based approach.

Gene expression levels were quantified as TPM method using RSEM [18]. Differential expression analysis was analysed by the DESeq2 [19] or DEGseq [20], with DEGs defined as $|\log_2FC| \geq 1$ and $FDR < 0.05$ (DESeq2) or $FDR < 0.001$ (DEGseq).

Functional enrichment analysis was conducted with Goatools for GO terms and Python scipy for KEGG pathways. All the alternative splice events were identified by rMATS [21], only isoforms matching the reference or containing new splice junctions were identified. Splicing differences were categorized into exon inclusion/exclusion, alternative 5'/3', and intron retention.

Metabolite Extraction, LC-MS/MS analysis, and Data Analysis

Solid Sample: Metabolites were extracted from 50 mg of leaves tissue with 400 μ l methanol: water (4:1, v/v) containing 0.02 mg/ml L-2-chlorophenylalanine (internal standard), followed by grinding, sonication, incubation, and centrifugation. The supernatant was collected for LC-MS/MS analysis. **Liquid Sample:** A 100 μ l liquid sample was mixed with 400 μ l of acetonitrile: methanol (1:1, v/v) containing 0.02 mg/ml internal standard. After vortexing, sonication, centrifugation, the supernatant was collected for LC-MS/MS analysis. **LC-MS/MS analysis:** Metabolite profiling was

conducted using a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, USA) at Majorbio BioPharm Technology Co., Ltd. MS conditions: data were collected in both positive and negative ESI modes. Full MS resolution was 60,000, and MS/MS resolution was 7,500. Data were collected in DDA mode with a mass range of 70–1050 *m/z*. Raw LC/MS data were processed using Progenesis QI (Waters Corporation, Milford, USA) to generate a CSV matrix containing sample IDs, metabolite names, and mass spectral intensities. Peaks from internal standards, known false positives and redundant features were removed. Metabolites were identified via HMDB (www.hmdb.ca/), Metlin (<https://metlin.scripps.edu/>), and the Majorbio Database. Data analysis was performed on the Majorbio Cloud Platform (cloud.majorbio.com) [22]. Features present in ≥ 80% of samples were retained. Missing values below the quantification limit were imputed with minimum value. Intensities were normalized by sum normalization to correct for technical variation. Variables with RSD >30% in QC samples were excluded. Finally, data were log₁₀ transformed for downstream analysis. Multivariate statistical analysis was performed by the R package “ropls” (V1.6.2) for PCA and OPLS-DA, with 7-fold cross-validation to evaluate model quality. Metabolites were considered significantly differentially accumulated if they met the criteria of VIP > 1 and *p* < 0.05. Differentially accumulated metabolites were mapped to pathways using KEGG (www.genome.jp/kegg/). Pathway enrichment was performed using Python’s “scipy.stats” package (<https://docs.scipy.org/doc/scipy/>), which identified significantly enriched pathways based on the functional or pathway classification of metabolites.

Results

Assessment of ‘Dingao’ and ‘Dongkui’ Response to *P. microspora* Infection

Two Chinese bayberry cultivars were analyzed in this study, including *Pestalotiopsis*-susceptible cultivar Dongkui which shows relatively high susceptible phenotype (S), and a *Pestalotiopsis* presumed-resistant line Dingao with a strong immune phenotype (R). First, we conducted plant pathogenicity analysis of the different symptoms induced by *P. microspora*. The young and tender Chinese bayberry leaves were cut and inoculated with agar containing *P. microspora*; we then recorded a series of macroscopic observations to track the pathogenic progress of TB (Fig. 1).

At 1 day post-inoculation (DPI), no symptoms were observed on the wounded leaves of either cultivar. From 5DPI, tiny and brown spots occurred around the *P. microspora* inoculated holes; and the size of these spots increased gradually and progressed to leaf necrosis after 10DPI in Dongkui plants (Fig. 1A). In contrast, few brown lesions were observed in the *P. microspora* inoculated Dingao leaves even after 10DPI (Fig. 1B). The control leaves did not present any symptoms throughout the experiments (Fig. S1 A&B).

General Transcriptome Analysis of Resistant and Susceptible *M. rubra* in Response to *P. microspora* Attack

To explore the molecular distinctions underlying plant resistance to *P. microspora* infection, we first constructed eight RNA-seq libraries for transcriptomic analysis. These libraries were prepared from young leaves of *M. rubra* cultivars: Dingao (R) and Dongkui (S). Samples were collected at 1DPI (early infection stage) and 5DPI (later infection stage) based on the macroscopic observations described above (Fig. 1). Additionally, mock-treated leaves at the indicated timepoints served as negative controls.

Transcriptome sequencing analysis yielded more than seven million clean reads per sample, with the Q20 base percentage exceeding 98.83%. A total of 6,818 genes were significantly differentially expressed (DEGs) (Fig. 2A and Table 1). To validate the quality of the RNA-seq results, 50 genes were randomly chosen for expression profiling across treatment groups. A heatmap generated using TBTool [23] presented

consistent gene expression patterns, demonstrating a strong correlation within the RNA-seq data (Fig. S2). The fewest DEGs were observed between DK infected with *P. microspora* (*P.m*) at 1DPI (DK *P.m* 1DPI) and its control (CK) samples (negative control DK leaves harvested at 1DPI), with only 99 DEGs (26 upregulated and 73 downregulated) (Fig. 2A). In contrast, the highest number of DEGs was observed between DK inoculated with *P.m* 5DPI (DK *P.m* 5DPI) and its control samples, with 3,414 DEGs (2,141 upregulated and 1,273 downregulated). Interestingly, DEGs in DA plants showed a rapid response at 1DPI, with 1,111 DEGs (583 upregulated and 528 downregulated). However, by 5DPI, the number of DEGs decreased to 324 DEGs (114 upregulated and 210 downregulated) (Fig. 2A and S1). Our findings indicate a marked difference in the dynamics of gene expression between the susceptible cultivar Dongkui and the resistant cultivar Dingao during *P. microspora* infection (Fig. 2B).

Functional Annotation and Enrichment Analysis of DEGs Provide Insight into Contrasting Host Responses

To analyze the functions of DEGs in response to TB infection, Gene Ontology (GO) classification and enrichment analysis were performed in this study. The GO annotation revealed similar results across samples, with the top five GO annotation terms were consistent, including cellular anatomical entity (GO:0110165), catalytic activity (GO:0003824), binding (GO:0005488), metabolic process (GO:0008152) and cellular process (GO:0009987) (Fig. S3 and Table S2).

GO enrichment analysis revealed significant differences between Dongkui and Dingao samples at different infection stages. In the early infection stage (1DPI) of Dongkui, the limited DEGs were predominantly associated with biological process (BP), including carboxylic acid metabolic process (GO:0019752), oxoacid metabolic process (GO:0043436) and organic acid metabolic process (GO:0006082). By 5DPI, the number of DEGs in Dongkui increased dramatically, with enrichment primarily in two categories: biological process (BP) and molecular function (MF), catalytic activity

(GO:0003824) and various binding-related terms (Fig. 3 and Table S3). In contrast, Dingao samples exhibited a broader range of enriched GO terms across all three categories: BP, MF and CC (cellular component). In Dingao samples at 1DPI, the prominent terms were oxidoreductase activity (GO:0016491), protein kinase activity (GO:0004672) and transmembrane transporter activity (GO:0022857). At 5DPI, the most enriched categories were extracellular region (GO:0005576), hormone-mediated signaling pathway (GO:0009755) and lipid catabolic process (GO:0016042) (Fig. 3 and Table S3).

To better understand the biological functions of these DEGs, we conducted pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KEGG analysis revealed distinguishable responses between Dongkui and Dingao under Pestalotiopsis infection. In resistant Dingao samples, the most upregulated pathways during early Pestalotiopsis infection (1DPI) included the Plant MAPK signaling pathway (map04016), Plant-pathogen interaction (map04626), Plant hormone signal transduction (map04075) and Phenylpropanoid biosynthesis (map00940). As the infection progressed to the later stages (5DPI), the number of DEGs in Dingao decreased, but the primary pathways remained Plant hormone signal transduction (map04075) and plant MAPK signaling pathway (map04016).

On the contrary, few DEGs were observed in Dongkui samples at 1DPI, with the main pathways being Phenylpropanoid biosynthesis (map00940) and Flavonoid biosynthesis (map00941). By 5DPI, as the number of DEGs in Dongkui increased significantly, the most prominently enriched pathways were Plant-pathogen interaction (map04626), Phenylpropanoid biosynthesis (map00940), Plant hormone signal transduction (map04075), and Amino sugar and nucleotide sugar metabolism (map00520) (Fig. 4 and Table S4).

Notably, the plant hormone signaling pathway — a vital regulator of plant immune responses — was enriched in Dingao (at both stages) and Dongkui at 5DPI, but not in Dongkui at 1DPI.

Transcription Factors Participate in Pestalotiopsis Stress Responses in *M. rubra*

Under *P. microspora* infection, a limited number of shared differentially expressed transcription factors (TFs) were identified between DK (susceptible cultivars) and DA (resistant cultivars) (Fig. 5B). In DA, the resistant cultivar, the most significantly changed TF families at 1DPI were MYB, WRKY, AP2/ERF, and HLH; most of WRKY and AP2/ERF TFs were upregulated. By 5DPI, most MYB and AP2/ERF TFs were downregulated; while the expression levels of NAC TFs were upregulated markedly. However, DK (susceptible cultivar) exhibited only three TFs at 1DPI: one from the MYB family was upregulated while the other from the AP2/ERF family was downregulated. By 5DPI, the number of expressed TFs increased significantly, with the most upregulated families being MYB, WRKY, AP2/ERF, HLH, and NAC (Fig. 5A).

While WRKY and AP2/ERF family TFs in DA samples at 1DPI, as well as WRKY family TFs in DK samples at 5DPI, were predominantly upregulated, the expression patterns of other TF families were more complex—with some TFs in the same family being upregulated and others downregulated (Fig. 5 and Table S6). Notably, NAC family TFs were upregulated in the later stage (5DPI) in both DK and DA samples, but were downregulated at 1DPI in the resistant DA samples (Fig. 5A). This suggests that NAC TFs play a distinct and significant role in the *Pestalotiopsis* stress response in *M. rubra*.

Overview of Untargeted Metabolomics

To further investigate the metabolite changes under the *Pestalotiopsis* attack in *M. rubra*, we performed untargeted metabolomic analyses for the control and infested Dongkui and Dingao leaves. A total of 2,512 (1,518 positive and 994 negative) and 2,611 (1,647 positive and 964 negative) annotated metabolites were detected from Dongkui and Dingao samples in comparisons between *P. microspora*-treated and control sample, respectively (Fig. 6). Principal component analysis (PCA) revealed that PC1, PC2, and PC3 contained 38.70%, 18.60%, and 10.80% of metabolites in the Dongkui samples, respectively; and for 42.00%, 16.70% and 9.97%

of the variance (Fig. S4 A&D), samples were clearly separated in the PCA models. Partial Least Squares Discriminant Analysis (PLS-DA) revealed clear separation of metabolites among all groups, except for the two Dingao mock treatments (Fig. S4 B&E), suggesting that *P. microspora* treatment significantly perturbed *M. rubra* metabolite profiles. The PLS-DA model validation results showed that R^2 values exceeded Q^2 in both models, with Q^2 regression line intercepts on the Y-axis were -0.9410 and -0.8925 , respectively (Fig. S4 C&F). These results indicate good model fit, high predictive ability, and suitability for subsequent data analysis.

Differential Metabolites Responses to *P. microspora* Stress

To identify the core metabolites involved in immune responses to *P. microspora* stress, we compared metabolites between the *P. microspora*-inoculated and control (CK) groups, and this analysis enabled the identification of specific differentially abundant metabolites (DMs).

At 1DPI, the DK *P. microspora*-inoculated group exhibited 280 upregulated and 12 downregulated DMs. Between DK *P. microspora* samples and their control samples at 5DPI, 503 DMs upregulated and 166 downregulated were observed. In DA plants, 472 DMs were upregulated and 110 downregulated at 1DPI, whereas by 5DPI, the number of DMs in DA samples shifted to 387 DMs were upregulated and 219 downregulated (Fig. 6 and Fig. S5).

A comparison of DMs between DK and DA samples showed that DA, the resistant cultivar, mounted a faster immune response to *P. microspora* attack. Interestingly, the dynamic DM patterns of DMs closely mirrored the gene expression patterns in both the susceptible cultivar Dongkui and the resistant cultivar Dingao during *P. microspora* infection.

Analysis of the Association between KEGG and Metabolic Data in Responses to the *P. microspora* Stress

To investigate the mechanisms underlying resistance to *P. microspora* infection, we analyzed the expression patterns of immune-related genes alongside their associated metabolites across key metabolic pathways.

Significant DMs in DK and DA were categorized according to the pathway classifications in the KEGG database (Fig. 7). In the resistant DA samples, by 1DPI, the top metabolic pathways were: linoleic acid metabolism, Glycerophospholipid metabolism, and Porphyrin metabolism. By 5DPI, these shifted to Biosynthesis of cofactors, Biosynthesis of various plant secondary metabolites and Phenylpropanoid biosynthesis. In the susceptible DK samples, the top three enriched pathways were: linoleic acid metabolism, Tryptophan metabolism and Biosynthesis of various plant secondary metabolites at 1DPI. By 5DPI, the most enriched metabolic categories had shifted to Biosynthesis of various plant secondary metabolites, Tyrosine metabolism and ABC transporters.

These results suggest that resistance to *P. microspora* in DA may be attributed to the early activation of lipid and porphyrin metabolism, followed by the induction of secondary metabolite and phenylpropanoid biosynthesis— processes that play critical roles in the plant immune response. In contrast, the delayed or altered metabolic responses in the susceptible DK, including the enrichment of tryptophan and tyrosine metabolism and ABC transporters, may reflect a less effective or misregulated defense strategy.

Discussion

DEGs Exhibit Distinct Expression Patterns Between Dongkui and Dingao in Comparative Transcriptomic Analysis Under *P. microspora* Attack

Over thousands of years of evolution, plants have developed complex and sophisticated immune responses to counter attacks from pathogenic microorganisms. In our study, we confirmed the distinct disease phenotypes between

a susceptible and a resistant *M. rubra* variety when challenged by *P. microspora*. Transcriptomic analysis revealed significant differences in immune responses between the resistant variety (Dingao) and the susceptible variety (Dongkui). The most striking difference was the number of DEGs at 1DPI (Fig. 2). Dingao exhibited a rapid and robust defense response, triggering thousands of DEGs at 1DPI, whereas Dongkui showed few DEGs at this early stage. Although Dongkui regulated more DEGs at a later stage (5DPI, this delayed response was unable to effectively contain pathogen progression. Based on our observations and analysis, we propose that the early immune response (1DPI) in *M. rubra* represents a critical window for repressing the development of Twig Blight in this species.

Signaling Pathways and Transcription Factors Involved in Immune Responses to *P. microspora* Stress

During pathogen invasion, plant immune responses including PTI and ETI trigger multiple signaling pathways for the resistance responses, including mitogen-activated protein kinases (MAPKs), defense hormones signalling pathways and calcium-dependent protein kinases (CDPKs), and so on [24, 25]. In our study, hundreds to thousands of DEGs in *M. rubra* were identified upon inoculation with *P. microspora*. Our research revealed that in the resistant Dingao samples, the predominant pathways throughout TB infection were plant MAPK signalling pathway and Plant hormone signal transduction, pathways that were absent in Dongkui at the early infection stage.

We further analyzed the transcription factors involved in the pathogenic process in *M. rubra*, as they play essential roles in regulating gene expression during plant immune responses.

Our investigation discovered that WRKY and AP2/ERF families were the most upregulated TFs in DA during early-stage immune responses; as time passed by, the most upregulated TFs belonged to NAC and WRKY families at 5DPI. In DK, the

susceptible cultivar by 5DPI, the most upregulated TFs families were WRKY, MYB, and AP2/ERF families, which were not detected at the early stage (1DPI). In addition, we noticed that the expression levels of NAC family TFs were increased in both DK and DA groups at 5DPI.

In plants, WRKY TFs regulate a wide range of biological functions, including receptor-mediated pathogen-triggered immunity, chromatin modulation for specific gene interactions, and signal transduction through complex gene networks [26]. AP2/ERF transcription factors initiate abiotic stress-responsive through both abscisic acid (ABA)-dependent and ABA-independent pathways, responding to hormones such as abscisic acid, auxin, ethylene, and gibberellin [27]. MYB TFs regulate the expression of resistance genes, hormone defense signaling, lignin/flavonoid/cuticular wax biosynthesis, and the hypersensitivity response [28]; NAC TFs play extensive roles in stress responses and disease resistance, acting through diverse and complex plant hormone signaling pathways involved in defense responses [29]. Combining our KEGG and TFs analyses, our results highlight the importance of plant hormones in *M. rubra* in response to *P. microspora*.

Plant Hormones and Secondary Metabolism Are Altered in Response to *P. microspora* Attack

Plant hormones play a crucial role in defense against pathogens, becoming activated upon the recognition of microbial or insect-derived molecules to initiate downstream defense signaling pathways through both PTI and ETI [30, 31]. Among these hormones, jasmonic acid (JA) and salicylic acid (SA) are the primary defense phytohormones in plant defense responses against pathogens [32, 33].

By 1DPI, both DA and DK samples exhibited linoleic acid metabolism as one of the dominant metabolic pathways. Linoleic acid a precursor in the oxylipin pathway, which leads to the production of jasmonic acid. Previous studies have found that JA can suppress the pathogenicity of *Pestalotiopsis* in *M. rubra*, although the underlying molecular mechanisms remain unclear [34].

In our study, trace amounts of JA were detected in the untargeted metabolomics of DA leaves under normal conditions, and the JA biosynthesis was further enhanced following the *P. microspora* attack. In contrast, in DK leaves, JA was synthesized only after *P. microspora* inoculation and was absent in the negative control groups (Table S3).

In addition, we observed complex expression patterns of gibberellins, a class of diterpenoid phytohormones, in both DA and DK samples. Overall, the baseline levels of gibberellins were higher in the susceptible DK leaves compared to the resistant DA samples. Following *P. microspora* infection, gibberellin synthesis increased further in DK leaves relative to their untreated controls. In contrast, gibberellin levels in DA samples remained relatively low and did not show significant changes under *P. microspora* attack. Gibberellins are known to regulate plant growth and development. Recently, their involvement in plant responses has attracted increasing attention [35]. Previous studies have reported complex expression patterns of gibberellins biosynthesis-related genes during the disease progression [9].

Taken together, we propose that the early conversion of linoleic acid to jasmonates in DA represents a critical defense stage, enabling the plant to promptly resist the invasion by the pathogenic fungus *P. microspora*. Taken together, our findings highlight the significant role that jasmonates play in the defense response of *M. rubra* against twig blight disease.

Conclusions

To summarize, by conducting comparative transcriptional profiling and metabolic analysis of two *M. rubra* cultivars: the resistant Dingao (DA) and susceptible Dongkui (DK) following the inoculation with *P. microspora*, we identified 8,399 and 2,313 shared DEGs at 1DPI and 5DPI, respectively. Additionally, we observed 2,512 and 2,611 DMs at 1DPI and 5DPI, respectively, between DA and DK under *P. microspora* stress.

441 The most notable difference between DA and DK was that the resistant Dingao
442 cultivar mounted a rapid and robust immune response to *P. microspora*'s invasion at
443 1DPI. Furthermore, DA exhibited jasmonate biosynthesis at this early stage (Table S3),
444 a phytohormone previously reported to suppress TB in *M. rubra*.
445 Our findings enhance the understanding of molecular defense mechanisms in *M.*
446 *rubra* and represent important progress toward developing disease-resistant cultivars
447 through molecular breeding.

448

449 **List of abbreviations**

450 BP: biological process
451 CDPK: calcium-dependent protein kinase
452 DA: Dingao
453 DEG: differentially expressed gene
454 DK: Dongkui
455 DM: differentially accumulated metabolite
456 DPI: day post-inoculation
457 ETI: effector-Triggered Immunity
458 GO: Gene Ontology
459 HR: hypersensitive response
460 JA: jasmonic acid
461 KEGG: Kyoto Encyclopanoid of Genes and Geneomes
462 MAMP: microbe-associated molecular pattern
463 MAPK: mitogen-activated protein kinase
464 PAMP: pathogen-associated molecular pattern
465 PCA: principal component analysis
466 PLS-DA: partial least squares discriminant analysis
467 PTI: pattern-triggered Immunity

ROS: reactive oxygen species

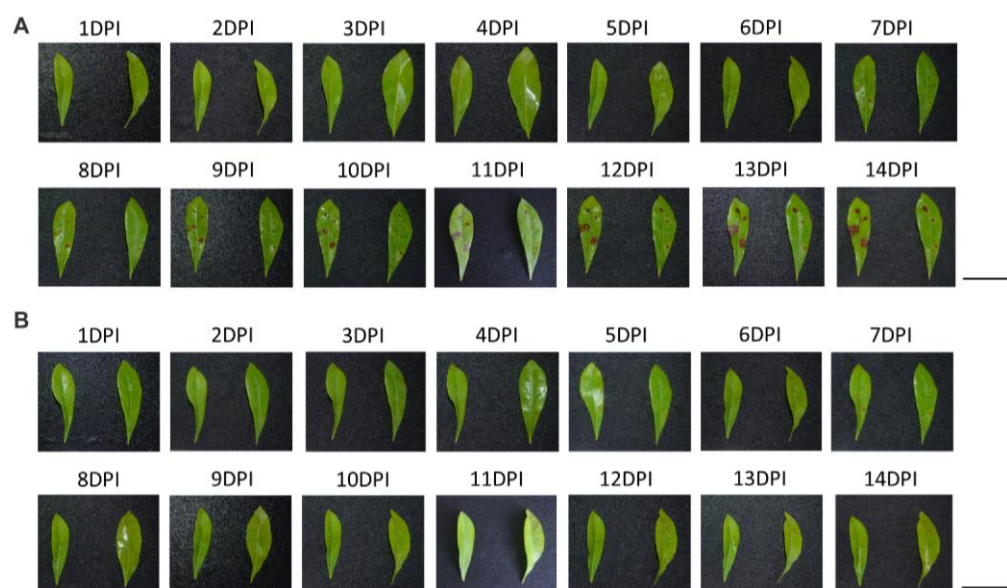
SA: salicylic acid

TB: Twig blight

TF: transcription factor

Figures

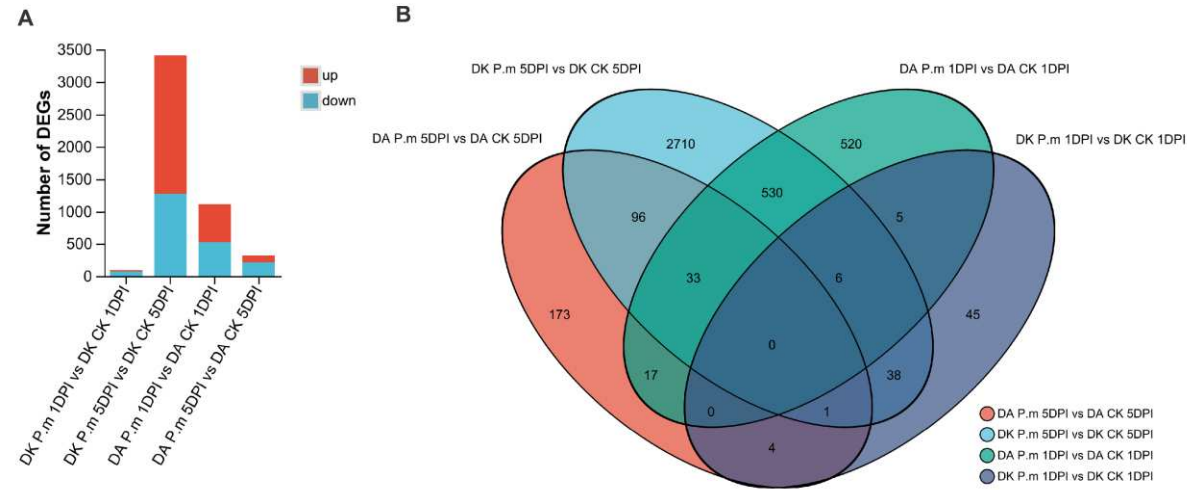
Fig. 1 Morphological characteristics and pathogenicity indices of *Myrica rubra* infected with *Pestalotiopsis microspora* at different times.



The pathogenic progression of *P. microspora* infection differed significantly between the susceptible cultivar Dongkui (A) and the resistant cultivar Dingao (B). Scale bar equals to 4 cm.

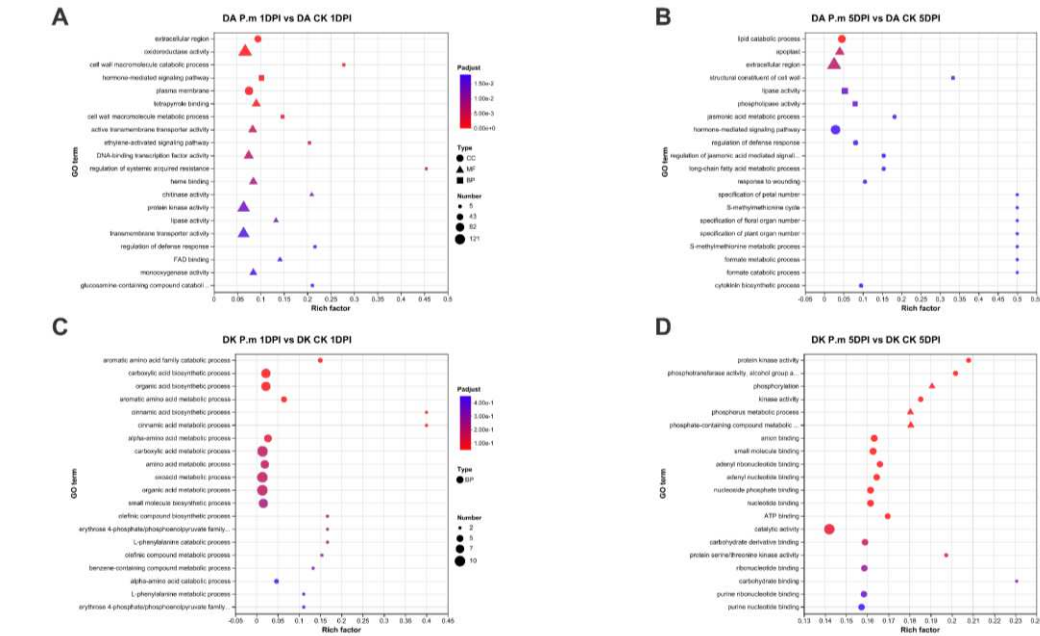
Fig. 2 Overview of differentially expressed genes (DEGs) in response to twig blight (TB) at different time points.

Fig. 2 Overview of differentially expressed genes (DEGs) in response to twig blight (TB) at different time points.



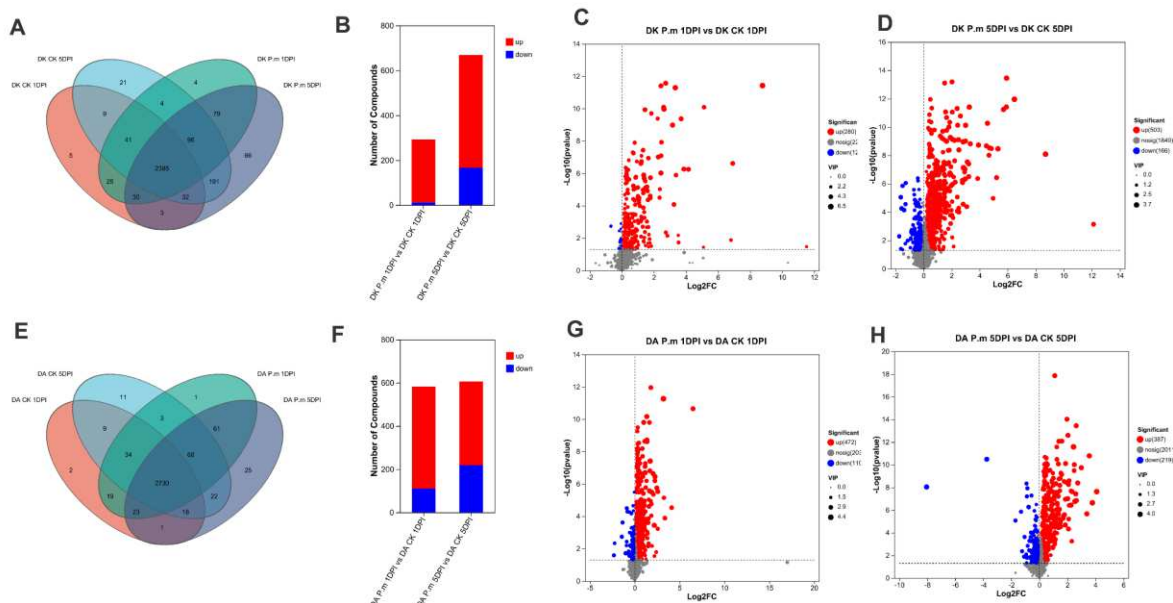
(A) Number of DEGs under *P. microspora* infection compared with control conditions at two time points; (B) Venn diagram showing common and unique DEGs in *M. rubra* leaves after inoculation with *P. microspora*.

Fig. 3 Gene Ontology enrichment of the identified DEGs.



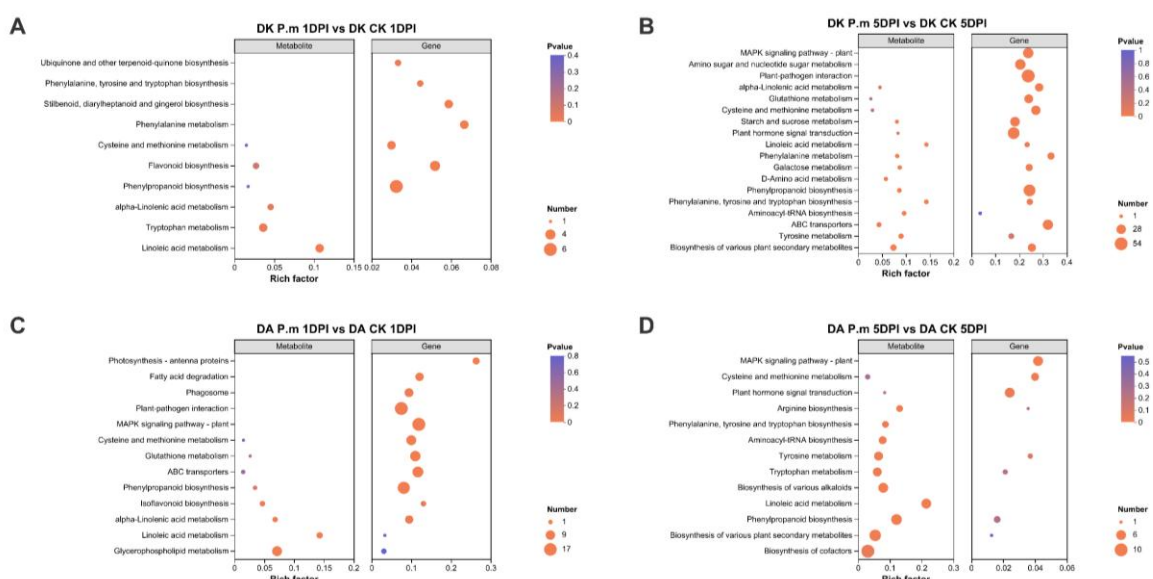
GO enrichment analysis identifies different gene expression for 1DPI and 5DPI between Dongkui (S, Susceptible) and Dingao (R, Resistant).

Fig. 4 Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways enriched in DEGs.



Differential metabolite abundances identified in comparisons between different treatments and stages (A, B, E, and F). Volcano plots of differential metabolites for *P. microspora*-treated groups vs. CK groups (C, D, G, and H). The x-axis shows $\log_2$ fold change in metabolite abundance between groups, and the y-axis shows $-\log_{10}$ P-value indicating significance. Each dot represents a metabolite. Higher values indicate greater significance in differential expression. Each dot indicates an individual metabolite.

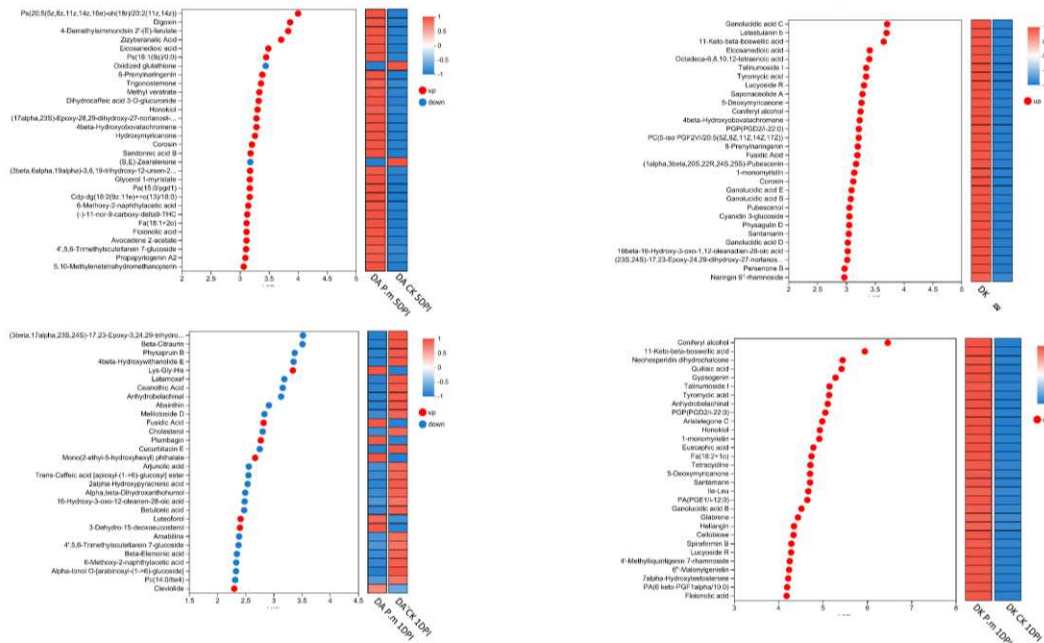
Fig. 7 KEGG enrichment of the differential metabolites.



(A) Metabolites identified in Dongkui 1DPI samples. (B) Metabolites identified in Dongkui 5DPI samples. Dot size reflects to the number of DMs in each pathway, while

color indicates the enrichment significance. Ratio refers to the number of metabolites in each pathway relative to the total number of enriched metabolites.

Fig. 8 Differentially abundant metabolites related to the plant defense responses.



VIP bar chart of differentially abundant metabolites. The default threshold for VIP values is ≥ 1 . The larger the VIP value, the greater the contribution of the metabolite to the differences between the two groups. Bar color indicates the significance of this difference: the smaller the P-value, the darker the color.

Fig. S1 Morphological characteristics and pathogenicity indices of *Myrica rubra* infected with plain agar (negative control) at different times.

The progression of plain agar inoculation in the susceptible cultivar Dongkui (A) and the resistant cultivar Dingao (B), serving as a negative control to assess baseline responses in the absence of *P. microspora* infection. Scale bar equals to 4cm.

Fig. S2 Heatmap of DEGs expression levels.

Heatmap showing the expression levels of 50 randomly selected DEGs based on the largest log fold change (logFC) values. Each tile indicates the expression level of a gene in a specific sample. Deeper red indicates higher expression, while deeper blue indicates lower expression. FC: fold change.

Fig. S3 Gene Ontology classification of identified DEGs.

GO classification distribution for 1DPI and 5DPI samples between Dingao (R, resistant) and Dongkui (S, susceptible).

Fig. S4 Quality analysis of metabolomics data.

PCA score plot of samples acquired in Dongkui (A) and Dingao (D). PLS-DA score plot for Dongkui (B) and Dingao (E) samples, respectively. Confidence ellipses indicate that the groups of actual samples are distributed within the marked region with 95% confidence. PLS-DA permutation tests for Dongkui (C) and Dingao (F) samples: The x-axis represents the permutation number from the permutation test, while the y-axis displays the R^2 values (red dots) and Q^2 values (blue triangles). The dashed lines indicate the regression lines for R^2 and Q^2 , respectively.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

1. Ethics approval and consent to participate

Not applicable.

2. Consent for publication

Not applicable.

3. Availability of data and materials

4. Competing interests

The authors declare no competing interests.

5. Funding

This research was supported by Sub-project of the Major Science and Technology Program for Agricultural (Fruit) Variety Breeding in Zhejiang Province, Grant No. 2021C02066-2-2; Wenzhou Agricultural New Variety Breeding Collaboration Project, Grant No. ZX2024002-4; Wenzhou Basic Public Welfare Research Project, Grant No. N2023015; Ou Hai Science and Innovation Center Project, Grant No. OHKC2024009.

6. Authors' contributions

All authors contributed to this study. Y. L & Y.S: Conceptualization, data curation, formal analysis, visualization, writing - original draft, writing, review and editing; P. Z, Q. Z, J. C, X. G & F. L: Data Correction; H. L: Data Collection; D. L: Conceptualization, funding acquisition, investigation, and writing - review and editing.

7. Corresponding author

Correspondence to Dongfeng Liu.

8. Acknowledgements

We extend our appreciation to the germplasm resources nursery of the Zhejiang Subtropical Crop Research Institute, Wenzhou, Zhejiang, China, for generously granting permission to utilize the plant material in this investigation.

9. Availability of data and materials

Sequence data that support the findings of this study have been deposited with the primary accession code PRJCA042809 at the China National Center for Bioinformation /Beijing Institute of Genomics, Chinese Academy of Sciences, and are publicly available at <https://ngdc.cncb.ac.cn/genbase>. All the data are public available and can be accessed at any time.

1. Sun C, Huang H, Xu C, Li X, Chen K: **Biological Activities of Extracts from Chinese Bayberry (*Myrica rubra* Sieb. et Zucc.): A Review**. *Plant Foods for Human Nutrition* 2013, **68**(2):97-106.
2. Kim HH, Kim DH, Kim MH, Oh MH, Kim SR, Park KJ, Lee MWJ: **Flavonoid constituents in the leaves of *Myrica rubra* sieb. et zucc. with anti-inflammatory activity**. 2013, **36**:1533-1540.
3. Saeed M, Elsadek MA, Chen Z, Zhao L, Wang G, Zhou C, Sun D, Gao Z, Jiao YJ: **Enhancing the terpenoid and flavonoid profiles and fruit quality in an elite Chinese bayberry line through hybridization**. 2025, **479**:143784.
4. 许渭根, 李国钧: **杨梅树枝叶凋萎症发生原因分析与防控对策** %J 中国植保导刊. 2015, **35**(10):38-42.
5. Wang S, Liu L, Mi X, Zhao S, An Y, Xia X, Guo R, Wei CJ: **Multi - omics analysis to visualize the dynamic roles of defense genes in the response of tea plants to gray blight**. 2021, **106**(3):862-875.
6. Maharachchikumbura SS, Guo L-D, Chukeatirote E, Bahkali AH, Hyde KD: **Pestalotiopsis—morphology, phylogeny, biochemistry and diversity**. 2011, **50**:167-187.

7. Chen F, Pu Z, Ni H, Wang Y, Yan B: **Multiple mycoviruses identified in *Pestalotiopsis* spp. from Chinese bayberry.** *Virology Journal* 2021, **18**(1).
8. Chen FY, Lu LM, Ni HZ, Wang Y, Wang YG, Li GQ: **First Report of *Pestalotiopsis mangiferae* and *P. vismiae* Causing Twig Dieback of *Myrica rubra* in China.** *Plant Disease* 2012, **96**(4):588-589.
9. Ren H, Wu Y, Ahmed T, Qi X, Li BJP: **Response of resistant and susceptible bayberry cultivars to infection of twig blight pathogen by histological observation and gibberellin related genes expression.** 2021, **10**(4):402.
10. Zhou F, Lin Q, Zhu L, Ren Y, Zhou K, Shabek N, Wu F, Mao H, Dong W, Gan LJN: **D14-SCFD3-dependent degradation of D53 regulates strigolactone signalling.** 2013, **504**(7480):406-410.
11. Cui H, Tsuda K, Parker JEJAropb: **Effector-triggered immunity: from pathogen perception to robust defense.** 2015, **66**(1):487-511.
12. Zhou J-M, Zhang YJC: **Plant immunity: danger perception and signaling.** 2020, **181**(5):978-989.
13. Liu Y, Pagac M, Yang F, Patkar RN, Naqvi NIJoF: **Fungal jasmonate as a novel morphogenetic signal for pathogenesis.** 2021, **7**(9):693.
14. Hu Y, Zhang M, Lu M, Wu Y, Jing T, Zhao M, Zhao Y, Feng Y, Wang J, Gao TJPP: **Salicylic acid carboxyl glucosyltransferase UGT87E7 regulates disease resistance in *Camellia sinensis*.** 2022, **188**(3):1507-1520.
15. Chen S, Zhou Y, Chen Y, Gu JJB: **fastp: an ultra-fast all-in-one FASTQ preprocessor.** 2018, **34**(17):i884-i890.
16. Kim D, Langmead B, Salzberg SLJNm: **HISAT: a fast spliced aligner with low memory requirements.** 2015, **12**(4):357-360.
17. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SLJNb: **StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.** 2015, **33**(3):290-295.
18. Li B, Dewey CNJBb: **RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome.** 2011, **12**:1-16.
19. Love MI, Huber W, Anders SJGb: **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.** 2014, **15**:1-21.
20. Wang L, Feng Z, Wang X, Wang X, Zhang XJB: **DEGseq: an R package for identifying differentially expressed genes from RNA-seq data.** 2010, **26**(1):136-138.
21. Shen S, Park JW, Lu Z-x, Lin L, Henry MD, Wu YN, Zhou Q, Xing YJPotnaos: **rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data.** 2014, **111**(51):E5593-E5601.
22. Ren Y, Yu G, Shi C, Liu L, Guo Q, Han C, Zhang D, Zhang L, Liu B, Gao H et al: **Majorbio Cloud: A one-stop, comprehensive bioinformatic platform for multiomics analyses.** *Imeta* 2022, **1**(2).

23. Chen C, Wu Y, Li J, Wang X, Zeng Z, Xu J, Liu Y, Feng J, Chen H, He YJMp: **TBtools-II: A “one for all, all for one” bioinformatics platform for biological big-data mining**. 2023, **16**(11):1733-1742.
24. Zhou J, Mu Q, Wang X, Zhang J, Yu H, Huang T, He Y, Dai S, Meng XJTPC: **Multilayered synergistic regulation of phytoalexin biosynthesis by ethylene, jasmonate, and MAPK signaling pathways in Arabidopsis**. 2022, **34**(8):3066-3087.
25. Zhang M, Zhang SJJoipb: **Mitogen - activated protein kinase cascades in plant signaling**. 2022, **64**(2):301-341.
26. Wani SH, Anand S, Singh B, Bohra A, Joshi RJPCR: **WRKY transcription factors and plant defense responses: latest discoveries and future prospects**. 2021, **40**:1071-1085.
27. Ma Z, Hu L, Jiang WJIJoMS: **Understanding AP2/ERF transcription factor responses and tolerance to various abiotic stresses in plants: A comprehensive review**. 2024, **25**(2):893.
28. Yu Y, Zhang S, Yu Y, Cui N, Yu G, Zhao H, Meng X, Fan HJP: **The pivotal role of MYB transcription factors in plant disease resistance**. 2023, **258**(1):16.
29. Han K, Zhao Y, Sun Y, Li YJPBJ: **NACs, generalist in plant life**. 2023, **21**(12):2433-2457.
30. Bari R, Jones JDJPmb: **Role of plant hormones in plant defence responses**. 2009, **69**:473-488.
31. Katagiri F, Tsuda KJMp-mi: **Understanding the plant immune system**. 2010, **23**(12):1531-1536.
32. Browse JJARoPB: **Jasmonate passes muster: a receptor and targets for the defense hormone**. 2009, **60**(1):183-205.
33. Vlot AC, Dempsey DMA, Klessig DFJArop: **Salicylic acid, a multifaceted hormone to combat disease**. 2009, **47**(1):177-206.
34. 俞浙萍, 张淑文, 梁森苗, 孙鹂, 任海英, 郑锡良, 戚行江: **3 种诱导剂对杨梅凋萎病抗性及其果实品质的影响** %J 浙江农业科学.1-5.
35. Castro-Camba R, Sánchez C, Vidal N, Vielba JMJP: **Plant development and crop yield: The role of gibberellins**. 2022, **11**(19):2650.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarytables.xlsx](#)