Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.091281

ARTICLE

Effects of Different Cultivation Substrates on Rooting and Phloem Secondary Metabolite Profiles of *Maclura tricuspidata* Cuttings

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Received: 19 August 2026; Accepted: 28 September 2026; Published: 19 October 2026

ABSTRACT: *Maclura tricuspidata* (Carrière) Bur. (Moraceae) is a traditional medicinal plant with roots and stem bark rich in bioactive secondary metabolites, particularly xanthones and flavonoids with anti-tumor, antioxidant, and anti-inflammatory activities. Although cutting propagation is the primary method of seedling production, how cultivation substrates affect rooting and secondary metabolite accumulation in cutting phloem remains unexplored. Four substrate treatments were evaluated for *M. tricuspidata* cuttings: peat soil + perlite (Group A), peat soil (Group B), sandy soil + vermiculite (Group C), and sandy soil (Group D) as the control. Rooting rates were recorded, and phloem tissues were analyzed using untargeted ultra-performance liquid chromatography-tandem mass spectrometry metabolomics. Differential metabolites were identified using multivariate statistical analyses and subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The rooting rate was the highest in Group C (77.78%), which significantly exceeded other treatments, and the lowest was observed in Group D (7.78%). A total of 849 metabolites were identified. Compared with Group D, 336 differential metabolites were identified in Group A (106 upregulated and 230 downregulated), 257 in Group B (79 upregulated and 178 downregulated), and 256 in Group C (86 upregulated and 170 downregulated). Group A contained significantly greater abundances of flavonoids and xanthones than Group D, and differential metabolites were enriched mainly in flavonoid and isoflavonoid biosynthesis pathways. Therefore, the cultivation substrate significantly influenced both rooting rate and phloem secondary metabolite accumulation in *M. tricuspidata* cuttings. Sandy soil + vermiculite was optimal for rooting, whereas peat soil + perlite was optimal for bioactive secondary metabolite accumulation, indicating a trade-off between rooting and secondary metabolite production in this species.

KEYWORDS: *Maclura tricuspidata*; cutting propagation; cultivation substrate; rooting rate; phloem; secondary metabolites; metabolomics

1 Introduction

Maclura tricuspidata is a deciduous shrub or small tree widely distributed across East, Central, and Southwest China, as well as the Korean Peninsula and northern Vietnam (Fig. 1a) [1]. Its wood is valued for high-grade furniture, and the fruits are consumed fresh or processed into juices and wines (Fig. 1b). As a traditional Chinese medicinal plant, the roots of *M. tricuspidata* have been used in folk medicine to treat gonorrhea, rheumatism, jaundice, boils, scabies, bruising, and dysmenorrhea (Fig. 1c) [2]. Phytochemical investigations have identified diverse secondary metabolites in *M. tricuspidata* (e.g., xanthones, flavonoids, isoflavonoids, lignans, and triterpenoids), with xanthones being the most abundant, followed by flavonoids [3–5]. *In vitro* studies have shown that these compounds exhibit anti-tumor, anti-inflammatory,

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antioxidant, antimicrobial, neuroprotective, hepatoprotective, and anti-obesity activities [6,7]. Extracts from the roots and stems of *M. tricuspidata* have been developed into proprietary Chinese medicines, namely 'Zhe-Mu Syrup' and 'Zhe-Mu Injection'. Zhe-Mu Syrup is listed in the Chinese National Drug Standard (WS3-B-3275-98). Clinical studies have reported its use as an adjuvant therapy for gastric cancer and chronic gastritis.



(**a**) (**b**) (**c**)

Figure 1: Overview of *Maclura tricuspidata*: (**a**) complete view of *M. tricuspidata*; (**b**) mature fruit of *M. tricuspidata*; and (**c**) root slice of *M. tricuspidata* used as a medicinal material.

Root sucker propagation has traditionally been the principal method for producing *M. tricuspidata* seedlings. However, cutting propagation can provide more uniform plant propagation and support large-scale production, with particular advantages for root tissue development. Studies have shown that substrate selection plays a critical role in the rooting success and subsequent growth of hardwood cuttings [8]. With coarse soil used as the bed soil, reported rooting rates for *M. tricuspidata* were 56.9% for hardwood cuttings and 62.3% for softwood cuttings [9]. Cultivation substrates can influence plant growth and cutting rooting, as well as secondary metabolite synthesis and accumulation through changes in the root microenvironment, including nutrient availability, water status, and aeration [10]. Phloem is a critical tissue for secondary metabolite synthesis and transport in plants, and the stem bark of *M. tricuspidata* containing the phloem is an important source of pharmacologically active constituents [11]. Thus, determining how cultivation substrates affect phloem secondary metabolites in *M. tricuspidata* cuttings is crucial for high-quality seedling production and regulation of medicinal quality.

By enabling simultaneous qualitative and quantitative analysis of thousands of metabolites in a single sample, untargeted metabolomics provides a broad view of treatment-induced changes in plant metabolic profiles [12]. Previous profiling of *M. tricuspidata* fruits at different maturity stages using gas chromatography–time-of-flight-mass spectrometry and ultra-performance liquid chromatography–quadrupole/time-of-flight-mass spectrometry has shown that flavonoids, lysophosphatidylcholines, and anthocyanins are the principal metabolites that discriminate between stages [13]. In the present study, four cultivation substrates were compared, with sandy soil serving as a control. Untargeted metabolomics based on ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) was used to characterize the differences in phloem secondary metabolite profiles of *M. tricuspidata* cuttings. Rooting rates recorded under the same treatments were considered alongside the metabolomic profiles to evaluate substrate-dependent changes in rooting and phloem secondary metabolite accumulation and the potential relationship between these processes. These findings provide a scientific basis for high-quality seedling cultivation and medicinal quality regulation in this species.

2 Materials and Methods

2.1 Plant Materials

Cuttings of *M. tricuspidata* were obtained from the germplasm repository of Jiangsu Yanjiang Institute of Agricultural Sciences, Jiangsu, China. The donor plants were grown from seeds collected from a single wild population in Nantong, Jiangsu (32°01′ N, 120°51′ E). Before cutting collection, they were cultivated for two years under uniform open-field conditions with a natural photoperiod and supplemental irrigation during dry periods. All donor plants were morphologically uniform and selected for vigorous growth and the absence of visible pests or diseases. Current-year semi-hardwood shoots were collected from all donor plants during active vegetative growth, approximately 30 days after bud break. Shoots were collected from the middle to upper canopy, 1.2–1.5 m above the ground. The shoots from all donor plants were pooled and thoroughly mixed. The cuttings were then randomly assigned to the treatments to evenly distribute genetic variation across the experimental groups. Each shoot was cut into a 10–12 cm segment, 2–3 leaves were retained at the apical end, and all thorns were removed. To facilitate water and nutrient uptake, the basal end of each cutting was trimmed at a 45° oblique angle.

2.2 Experimental Design

Four substrate formulations commonly used for nursery cutting propagation were selected to represent contrasting physical and chemical properties. Peat soil (Group B) was selected because of its high organic matter content and water-holding capacity. Peat soil + perlite (Group A, 3:1 v/v) represented an organic substrate amended with perlite to improve aeration and drainage. Sandy soil + vermiculite (Group C, 3:1 v/v) represented a mineral-based substrate amended with vermiculite to enhance water retention while maintaining aeration. Sandy soil alone (Group D) served as the control and represented a well-drained mineral substrate with low nutrient content and minimal water-holding capacity. The design compared complete substrate formulations without independently testing the effects of peat, sand, perlite, and vermiculite. Therefore, the observed differences reflect the combined physical, chemical, and hydraulic properties of each formulation rather than the isolated effect of any component.

Each treatment was arranged in a completely randomized block design with three replicates of 30 cuttings. The basal 2–3 cm of the cuttings were immersed in 200 mg/L indole-3-butyric acid solution for 2 h before planting, after which the plants were maintained at 20–30°C and 60%–80% relative humidity in a controlled-environment greenhouse (12 h photoperiod, photosynthetic photon flux density of 150 ± 20 μmol m^{-2} s^{-1} provided by full-spectrum LED lamps). On day 100, the rooting rate was determined as the number of rooted cuttings/total cuttings × 100%. Phloem tissues were subsequently collected, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. Metabolomic analysis included 12 samples, comprising three biological replicates from each of the four treatments.

2.3 Metabolite Extraction and Detection

2.3.1 Metabolite Extraction

Each 50-mg phloem sample was combined with 1000 μL of pre-cooled methanol/water (4:1, v/v), to which 10 μL of L-2-chlorophenylalanine (0.06 mg/mL) was added as the internal standard. Following vortex mixing and the addition of steel beads, the samples were pre-cooled at −20°C for 2 min and ground for 2 min at 60 Hz. The extraction continued with 10 min of ultrasonication in an ice-water bath and subsequent 30-min incubation at −20°C. The samples were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were collected and passed through a 0.22 μm microporous membrane before UPLC-MS/MS analysis.

2.3.2 Chromatographic Conditions

A Waters ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) was used for chromatographic separation. The mobile phase system consisted of 0.1% formic acid in water as phase A and 0.1% formic acid in acetonitrile as phase B. The elution began with 5% B from 0 to 1 min and increased from 5% to 95% B between 1 and 9 min. Phase B was maintained at 95% from 9 to 12 min, decreased to 5% between 12 and 12.1 min, and then maintained at 5% until 15 min. Finally, the separation was conducted at a column temperature of 40°C and a flow rate of 0.35 mL/min, with an injection volume of 5 μL.

2.3.3 Mass Spectrometry

Positive- and negative-ion data were acquired on a Thermo Q-Exactive Orbitrap mass spectrometer fitted with an electrospray ionization (ESI) source. Spray voltages of 3.8 and 3.2 kV were applied in the positive and negative modes, respectively. The sheath gas flow was 35 arb, the auxiliary gas flow was 10 arb, and the capillary temperature was maintained at 320°C. For full-scan acquisition, the resolution was set to 70,000 across m/z 70–1050. A resolution of 17,500 was used for MS/MS.

2.4 Data Processing and Analysis

The conversion of the raw mass spectrometry data to mzXML format was carried out with MS Convert in the Proteo Wizard software package [14]. The resulting data were processed using XCMS (v3.12.0) for peak detection, filtering, and alignment [15]. Systematic errors were corrected using support vector regression based on quality control (QC) samples. Metabolites showing a coefficient of variation >30% in QC samples were excluded from further analysis.

The identification of metabolites relied on concordance of accurate molecular weight, retention time, and MS/MS spectra with records in the Human Metabolome Database (HMDB), MassBank, the Kyoto Encyclopedia of Genes and Genomes (KEGG), and an in-house plant spectral library [16–18].

A multivariate analysis was conducted in the R package ropls (v1.22.0). It included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) [19]. A variable importance in projection (VIP) value > 1, together with $p < 0.05$, defined differential metabolites. Enriched KEGG pathways were identified using the hypergeometric distribution [20]. The correlations between rooting rate and the relative abundances of differential metabolites were examined by Pearson correlation analysis. Rooting rate data were analyzed separately using one-way analysis of variance, followed by Duncan's multiple range test.

3 Results

3.1 Effects of Different Substrates on Rooting Rate

The rooting rate differed highly significantly among cultivation substrates (Table 1). Group C (sandy soil + vermiculite) had the highest rooting rate at 77.78%, significantly exceeding other treatments ($p < 0.01$). The rooting rates were 42.22% in Group A (peat soil + perlite) and 35.56% in Group B (peat soil), without significant difference between them ($p > 0.05$). As the sandy soil control, Group D produced the lowest rooting rate (7.78%), significantly below all other treatments.

Table 1: Rooting rates of *Maclura tricuspidata* cuttings under different cultivation substrate treatments.

Treatment	Substrate Composition	Rooting Rate (Mean ± SD, %)
A	peat soil + perlite	42.22 ± 12.62Bb
B	peat soil	35.56 ± 13.47BCb
C	sandy soil + vermiculite	77.78 ± 6.94Aa
D	sandy soil control	7.78 ± 6.94Cc

Note: Different lowercase letters indicate significant differences ($p < 0.05$), and different uppercase letters indicate highly significant differences ($p < 0.01$).

3.2 Quality Control of the Metabolomic Data

Base peak chromatograms were first used to evaluate the metabolomic data quality and showed normal peak shapes with effective separation. After metabolic features with a relative standard deviation (RSD) >30% in QC samples were removed, 88.7% of the remaining features had RSD values below 30%. This confirmed the high reproducibility and stability of the dataset for downstream analyses.

3.3 Multivariate Statistical Analysis

The overall metabolic variation among the four substrate treatments was visualized using PCA. In both positive and negative ion modes, the PCA score plots (Fig. 2a,d) clearly separated the four groups, indicating that different cultivation substrates induced systematic alterations in the phloem metabolic profiles of *M. tricuspidata*. The cumulative R2X(cum) values were 0.501 and 0.547 for the positive and negative ion modes, respectively. Because an R2X value >0.5 can generally indicate good explanatory power for the data matrix, these values suggested that the PCA models effectively represented the metabolic differences among samples.

PLS-DA was used to maximize the discrimination among groups (Fig. 2b,e). The models exhibited excellent predictive ability and group discrimination, while the cumulative Q^2(cum) values approaching 1 across all pairwise comparisons indicated high stability and reliability. In the positive ion mode, the Q^2(cum) values were 0.967, 0.894, and 0.970 for A vs. D, B vs. D, and C vs. D, respectively, while the corresponding values in the negative ion mode were 0.938, 0.989, and 0.986. A Q^2 value closer to 1 indicates a stronger predictive ability for new samples. For every comparison, permuted models had consistently lower R^2 and Q^2 values than the original models, confirming that the PLS-DA models were not overfitted and that the results were reliable.

Metabolites contributing most strongly to group discrimination were resolved by OPLS-DA (Fig. 2c,f). The first predictive component captured most inter-group differences, whereas the orthogonal components separated the intra-group variation. Tight clustering within each treatment group supported high reproducibility and reliable group separation. The OPLS-DA S-plot was used to select metabolites meeting both a high-contribution threshold (VIP > 1) and a high-reliability threshold ($|p(\text{corr})| > 0.5$) for subsequent differential metabolite screening.

3.4 Screening and Identification of Differential Metabolites

Screening at VIP > 1 and $p < 0.05$ identified 849 differential metabolites across comparisons of the three substrate treatments with the sandy soil control (Table 2). Notably, the comparison with Group D yielded 336 differential metabolites for Group A (106 upregulated and 230 downregulated), 257 for Group B (79 upregulated and 178 downregulated), and 256 for Group C (86 upregulated and 170 downregulated). In each comparison, downregulated metabolites outnumbered those that were upregulated (Fig. 3).

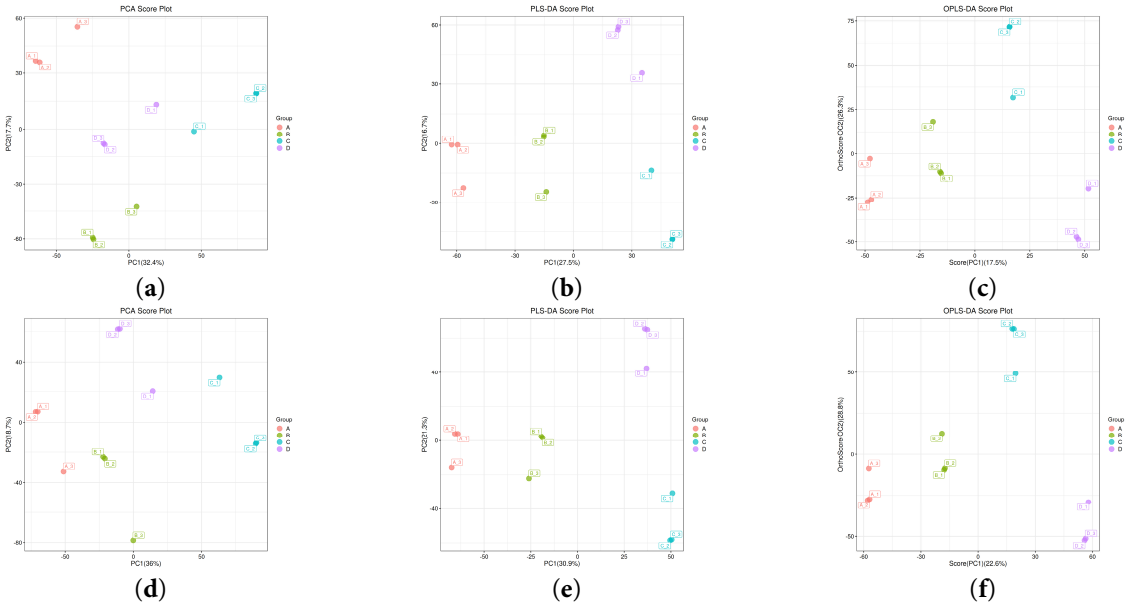


Figure 2: Score plots of multivariate statistical analyses for phloem metabolites of *Maclura tricuspidata* cuttings under different substrate treatments in the positive and negative ion modes. (**a**) OPLS-DA score plot (positive ion mode); (**b**) PCA score plot (positive ion mode); (**c**) PLS-DA score plot (positive ion mode); (**d**) OPLS-DA score plot (negative ion mode); (**e**) PCA score plot (negative ion mode); and (**f**) PLS-DA score plot (negative ion mode).

Table 2: Statistics of differential metabolites between substrate treatments and sandy soil control.

Comparison	Total Differential Metabolites	Upregulated	Downregulated
A vs. D	336	106	230
B vs. D	257	79	178
C vs. D	256	86	170

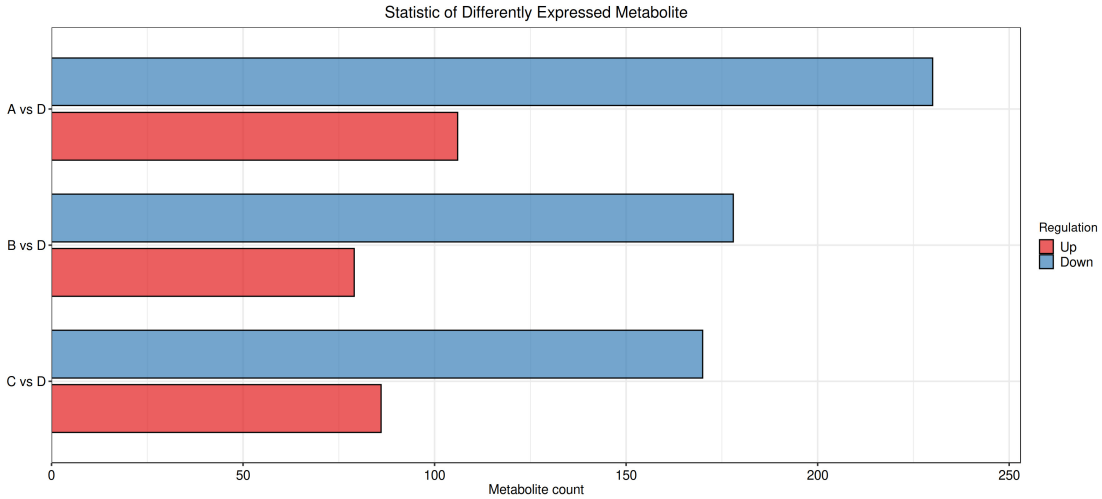


Figure 3: Statistical bar chart of differential metabolites. The *x*-axis shows the number of differential metabolites, and the *y*-axis shows the comparison groups. Red bars indicate upregulated metabolites, and blue bars indicate downregulated metabolites.

KEGG database classification assigned the identified metabolites to a broad range of chemical categories. The major classes included flavonoids (197 compounds), organooxygen compounds (68), prenol lipids (58), fatty acyls (50), carboxylic acids and their derivatives (47), and benzene and substituted derivatives (32). Other classes included amino acids, organic acids, carbohydrates, and their derivatives (Fig. 4). Flavonoids were the most abundant class, accounting for approximately 19.3% of all identified metabolites.

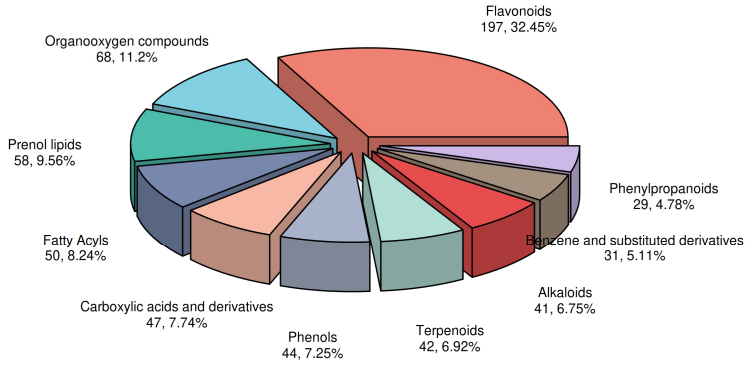


Figure 4: Pie chart of identified metabolite classes in the phloem of *M. tricuspidata*.

3.5 Analysis of Differential Metabolites

3.5.1 Differential Metabolites in Group A vs. Group D

Between Group A (peat soil + perlite) and Group D (sandy soil), 336 differential metabolites were detected, of which 106 were upregulated and 230 were downregulated in Group A compared with the control (Fig. 5a). The largest increases among the upregulated metabolites occurred in pimara-7,15-diene, a diterpene hydrocarbon; chorismate, a key shikimate pathway intermediate; xylopentaose, an oligosaccharide; and papaverine, an isoquinoline alkaloid. Substantial increases were also observed in the levels of citbismine C, 2-oxoadipic acid, and 2-methylcitric acid.

Significant upregulation occurred among several flavonoids and xanthones. Group A contained significantly higher abundances of isorhamnetin, a methylated flavonol, and secoisolariciresinol, a lignan. The prenylated flavonoid, cudraflavanone B, and the xanthone derivative, 1,6-dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone, also increased markedly.

The most strongly down-regulated metabolites included dihydrophaseic acid, a plant hormone metabolite; leucocyanidin; ciceritol; and fumarate. Thus, the peat soil + perlite substrate promoted the bioactive flavonoid and xanthone accumulation while suppressing certain stress-related metabolites.

3.5.2 Differential Metabolites in Group B vs. Group D

Compared with Group D, Group B (peat soil alone) contained 257 differential metabolites, including 79 upregulated and 178 downregulated compounds. Because this total was lower than that in Group A, peat soil alone appeared to induce milder metabolic perturbation than the peat soil + perlite composite (Fig. 5b).

Among the metabolites upregulated in Group B, phenylethylamine as a biogenic amine exhibited a significant increase, and pimara-7,15-diene and D-sedoheptulose 7-phosphate were significantly upregulated. Genipic acid, secoisolariciresinol, and colforsin accumulated substantially.

Numerous metabolites downregulated in Group B were affected in Group A, including aureusidin, bracteatin, glyceollin I, and quercetin 7-glucoside. Their predominance among the differential metabolites suggests that peat soil alleviated the stress responses present in the sandy soil control.

3.5.3 Differential Metabolites in Group C vs. Group D

Compared with Group D, Group C (sandy soil + vermiculite) contained 256 differential metabolites, including 86 upregulated and 170 downregulated compounds. This total closely matched that of Group B (256 vs. 257), suggesting that vermiculite addition to sandy soil induced metabolic changes similar in magnitude to those caused by peat soil alone (Fig. 5c).

The strongest upregulation in Group C was observed for the xanthone derivative 1,6-dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone, which increased 25-fold. In addition, the phytoalexin phaseollin and the alkaloid strychnine were significantly increased. Significant upregulation was detected for casticin, a polymethoxyflavonoid, and cudraflavanone B, and dihydrochelirubine, oripavine, tetracycline, and diphyllin presented additional large increases.

Among the downregulated metabolites, the greatest decrease was observed in vitexin, a flavonoid C-glycoside, with dihydrophaseic acid and ciceritol decreasing markedly. These results indicate that despite the most favorable rooting response, sandy soil + vermiculite effectively promoted the accumulation of diverse defense-related secondary metabolites.

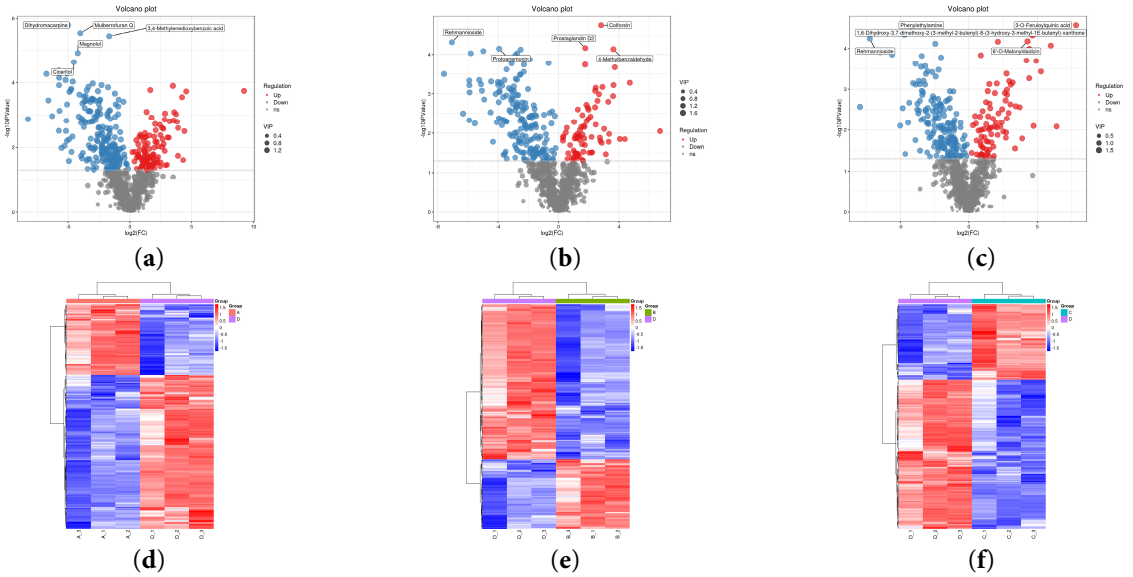


Figure 5: Differential metabolite analysis across three treatment comparisons. Volcano plots show (**a**) A vs. D, (**b**) B vs. D, and (**c**) C vs. D. Cluster heatmaps show (**d**) A vs. D, (**e**) B vs. D, and (**f**) C vs. D. In volcano plots, red and blue dots denote significantly upregulated and downregulated metabolites, respectively. Gray dots denote non-significant metabolite changes. In heatmaps, red and blue indicate high and low relative abundances, respectively.

3.5.4 Comparative Overview

The number of downregulated metabolites exceeded that of upregulated metabolites across all three treatments, with ratios of 2.17, 2.25, and 1.98 for Groups A, B, and C, respectively. Therefore, the stress-related metabolite accumulation induced by sandy soil control may be alleviated by substrate improvement. The accumulation of the xanthone derivative 1,6-dihydroxy-3,7-dimethoxy-2-(3-methyl-2-butenyl)-8-(3-hydroxy-3-methyl-1E-butenyl)-xanthone was the highest in Group C, intermediate in Group A, and the lowest in Group D. Moreover, both Group A and Group C contained higher abundances of cudraflavanone B and isorhamnetin than Group D, indicating that peat soil + perlite and sandy soil + vermiculite promoted

the flavonoid and xanthone accumulation to different extents. These expression patterns are visualized in the cluster heatmaps (Fig. 5d–f).

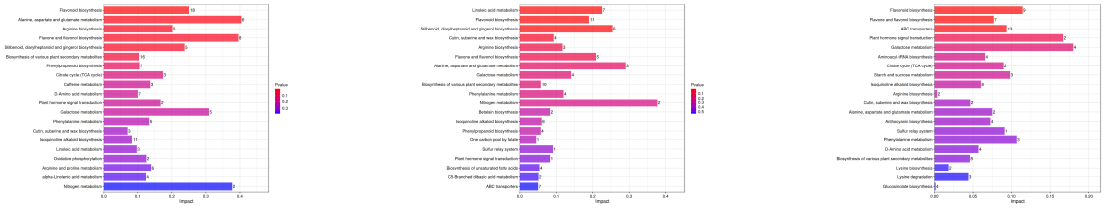
3.6 KEGG Pathway Enrichment Analysis

KEGG pathway enrichment analysis was applied to the differential metabolites from each comparison to further characterize the biological functions and metabolic pathways affected by cultivation substrate.

In the Group A vs. Group D comparison, 20 KEGG pathways were significantly enriched (Fig. 6a). Flavonoid biosynthesis was the most significantly enriched pathway, containing 18 differential metabolites. It was followed by alanine, aspartate, and glutamate metabolism (6 metabolites), arginine biosynthesis (5 metabolites), flavone and flavonol biosynthesis (8 metabolites), and stilbenoid, diarylheptanoid, and gingerol biosynthesis (5 metabolites). Furthermore, phenylpropanoid biosynthesis, which can serve as an upstream precursor for flavonoid and lignin biosynthesis, was notably enriched with 11 differential metabolites. Together, these results indicate that the peat soil + perlite primarily affected the secondary metabolic pathways associated with phenylpropanoid and flavonoid metabolism, consistent with the accumulation of flavonoids and xanthones observed in Group A.

Significant enrichment of 20 KEGG pathways was detected in Group B vs. Group D comparison (Fig. 6b). Galactose metabolism contained 10 differential metabolites, the biosynthesis of various plant secondary metabolites contained 8, flavonoid biosynthesis and linoleic acid metabolism each contained 7, and phenylpropanoid biosynthesis and isoquinoline alkaloid biosynthesis each contained 4. Although fewer differential metabolites were detected in flavonoid-related pathways in Group B than in Group A, the enrichment extended to fatty acid and carbohydrate metabolism. This suggests that peat soil alone may affect a broader range of primary metabolic processes.

Another 20 KEGG pathways were significantly enriched when Group C was compared with Group D (Fig. 6c). Among them, plant hormone signal transduction contained 8 differential metabolites, whereas flavonoid biosynthesis, flavone and flavonol biosynthesis, galactose metabolism, and ATP-binding cassette (ABC) transporters each contained 4. Anthocyanin biosynthesis was enriched only in Group C and was absent in the other two comparison groups. The enrichment of plant hormone signal transduction and ABC transporter pathways suggests a possible influence of sandy soil + vermiculite on hormone signaling and transport processes, which could be associated with the superior rooting performance of Group C.



(**a**) (**b**) (**c**)

Figure 6: KEGG pathway enrichment bar plots for differential metabolites. (**a**) A vs. D, (**b**) B vs. D, and (**c**) C vs. D. The *x*-axis represents the pathway impact values, and the *y*-axis represents the pathway names. The bar colors indicate the *p*-values, and the numbers at the end of the bars represent the differential metabolites (Hits) annotated to each pathway.

A shared response across the three comparisons was the enrichment of both flavonoid biosynthesis and flavone and flavonol biosynthesis, confirming that cultivation substrates primarily modulated flavonoid metabolism in the *M. tricuspidata* phloem. Within the flavonoid biosynthesis pathway, the number of

differential metabolites decreased from 18 in Group A to 7 in Group B and 4 in Group C. This indicates the strongest regulatory effect of peat soil + perlite on this pathway. Additionally, the same three comparisons showed the enrichment of phenylpropanoid biosynthesis, involving 11, 4, and 2 metabolites in Groups A, B, and C, respectively. This pattern supports the central role of the upstream pathway in substrate-induced metabolic changes. Plant hormone signal transduction was enriched in Groups B and C, whereas isoquinoline alkaloid biosynthesis was enriched in Groups A and B. Group A was additionally characterized by the specific enrichment of arginine biosynthesis and alanine, aspartate, and glutamate metabolism, suggesting that peat soil + perlite may also affect amino acid metabolism.

4 Discussion

4.1 Effects of Different Cultivation Substrates on Rooting

The cultivation substrate had a highly significant effect on the rooting rate of *M. tricuspidata* cuttings ($p < 0.01$). In particular, Group C (sandy soil + vermiculite) produced the highest rate (77.78%), whereas Group D (sandy soil alone) produced the lowest (7.78%). The differences in substrate physical properties were closely related to this response. Sandy soil provides good aeration but retains water and nutrients poorly, and thus fails to maintain the constant supply required for root initiation and development. Vermiculite combined the strong water absorption and retention with good aeration. When added to sandy soil, it significantly improves both the water-holding capacity and aeration, creating a more favorable microenvironment for adventitious root formation [21].

The rooting rates in the peat-based substrates, Groups A and B (42.22% and 35.56%, respectively), were significantly below that in the sandy soil + vermiculite treatment. Although peat soil is rich in organic matter and retains water and nutrients effectively, its aeration is relatively poor. Insufficient aeration in pure peat soil (Group B) may restrict root respiration. Perlite addition (Group A) improved aeration to some extent but did not significantly increase rooting compared with Group B. For *M. tricuspidata* cuttings, substrate aeration may be more critical than nutrient supply, with sandy soil + vermiculite providing the oxygen environment most conducive to adventitious root formation. This observation is consistent with the findings in other woody species, where substrate physical properties, particularly aeration and water-holding capacity, have been identified as key determinants of rooting success [22].

Few studies have examined cutting propagation of *M. tricuspidata*. In coarse sand, Kim et al. reported rooting rates of 56.9% and 62.3% for hardwood and softwood cuttings of *M. tricuspidata*, respectively [8]. The higher rate obtained with sandy soil + vermiculite in the present study (77.78%) may have resulted from the greater water and nutrient retention provided by vermiculite. A pending Chinese patent application (CN110832997A) reported an 85.66% rooting rate for hardwood *M. tricuspidata* cuttings grown in peat and coir substrate blocks. Coir in that formulation may improve aeration and water retention compared with our peat-based substrates. Collectively, these findings indicate that an optimal substrate for *M. tricuspidata* cutting propagation should balance aeration, water retention, and nutrient supply.

4.2 Effects of Different Cultivation Substrates on Phloem Secondary Metabolites

Among the tested substrates, peat soil + perlite (Group A) most strongly favored flavonoid and xanthone accumulation in *M. tricuspidata* phloem, whereas the sandy soil control (Group D) showed the lowest accumulation of these compounds. The differences in nutrient supply among substrates were closely associated with this pattern.

Peat soil supplies abundant organic matter and mineral nutrients, including nitrogen, phosphorus, and other essential elements. Mineral nutrition plays an important regulatory role in the secondary metabolism

of medicinal plants [23]. Moderate nutrient supply facilitates the synthesis and accumulation of secondary metabolite precursors, whereas excessive nutrient stress may suppress overall metabolic activity. In the present study, the peat soil + perlite combined balanced nutrient availability with suitable water and aeration conditions, which supplied sufficient substrates and energy for flavonoid and xanthone biosynthesis.

Aromatic compounds, particularly xanthones and flavonoids, are abundant in *M. tricuspidata*, with 158 flavonoids and 99 xanthones isolated from this species [2]. The substantial accumulation of these compounds under Group A treatment further confirms the advantage of using peat soil + perlite in promoting the biosynthesis of bioactive components in *M. tricuspidata*.

Although sandy soil + vermiculite (Group C) produced the highest rooting rate, it did not produce the greatest phloem accumulation of flavonoids and xanthones. The substrate optimum for rooting differed from that for secondary metabolite accumulation, with sandy soil + vermiculite and peat soil + perlite favoring the former and latter, respectively. For *M. tricuspidata* seedling production, this distinction could provide a basis for selecting substrate formulations according to the cultivation objective, whether rapid propagation or improvement of medicinal quality.

KEGG pathway enrichment analysis supported these observations. Flavonoid biosynthesis was enriched in all three comparisons, whereas Group A vs. Group D contained the highest number of differential metabolites in this pathway. Phenylpropanoid biosynthesis, which serves as the upstream precursor for flavonoid and lignin biosynthesis, was also notably enriched across all comparisons. Consistent with the accumulation of bioactive flavonoids and xanthones in Group A, these results confirmed that peat soil + perlite exerted the strongest regulatory effect on flavonoid metabolism.

4.3 Trade-Off between Rooting and Secondary Metabolite Accumulation

The negative correlation between rooting rate and phloem secondary metabolite accumulation in *M. tricuspidata* cuttings was an important finding of this study. Group C (sandy soil + vermiculite) achieved the highest rooting rate (77.78%) but not the greatest accumulation of flavonoids and xanthones. In contrast, Group A (peat soil + perlite) had a lower rooting rate (42.22%) and exhibited the highest accumulation of these compounds.

This negative correlation reflects a resource allocation trade-off in plants. Secondary metabolite synthesis consumes substantial photosynthates and energy [24,25]. As a traditional anti-cancer medicinal herb, *M. tricuspidata* exerts pharmacological effects that are primarily attributed to its bioactive secondary metabolites. Under rooting-favorable conditions, such as good aeration supplied by sandy soil + vermiculite, cuttings can allocate more resources to adventitious root formation and development and relatively less to secondary metabolite synthesis. In contrast, in peat-based substrates with abundant nutrients but suboptimal aeration, cuttings may experience stress, such as hypoxia. Such stresses can induce the synthesis of defensive secondary metabolites while simultaneously inhibiting root growth.

The observed pattern was consistent with the "growth–defense trade-off" theory in plant physiology [26,27]. As the major bioactive components of *M. tricuspidata*, xanthones and flavonoids contribute significantly to plant defense. When resources are limited, growth represented by rooting competes with defense represented by secondary metabolite accumulation. Hence, the medicinal seedling production of *M. tricuspidata* involves a choice or a balance between "rapid rooting and seedling establishment" and "high accumulation of bioactive components".

Additional indirect support for this trade-off emerged from the contrasting KEGG enrichment profiles of Groups C and A. In Group C, plant hormone signal transduction and ABC transporter pathways were enriched, suggesting that sandy soil + vermiculite may enhance hormone signaling and transport

processes associated with root development. In contrast, Group A exhibited specific enrichment of arginine biosynthesis and alanine, aspartate, and glutamate metabolism. This pattern suggests that peat soil + perlite may promote amino acid metabolism, potentially providing precursors for secondary metabolite biosynthesis. The divergence between these pathway responses could support a physiological trade-off between rooting capacity and secondary metabolite accumulation.

4.4 Medicinal Value and Implications of Phloem Secondary Metabolites

Phloem in the stem bark of *M. tricuspidata* is an important source of medicinally active constituents. Previous studies have demonstrated anti-tumor, anti-inflammatory, and antioxidant activities of stem bark extracts [6,7]. However, most pharmacological studies have used *in vitro* models, and clinical evidence for the efficacy of *M. tricuspidata*-derived compounds remains limited. The abundant flavonoids and xanthones detected in the cutting phloem in the present study support its potential as a medicinal tissue. Xanthones are the most characteristic and abundant components of *M. tricuspidata*. Accordingly, the significant substrate-dependent differences in xanthone accumulation observed provide direct evidence that cultivation practices can regulate the medicinal quality of *M. tricuspidata*.

Flavonoids are the major anti-tumor active components of *M. tricuspidate*, with potential anti-tumor activity. For instance, a comprehensive review reported 158 flavonoids and 99 xanthones isolated from this species, of which 78 were considered potential antineoplastic agents [2]. Furthermore, specific compounds such as macluraxanthone B have shown cytotoxic activity against human digestive tract tumor cell lines, with IC_{50} values ranging from 2.70 to 12.66 Mm [28]. The highest content in Group A (peat soil + perlite) suggested that this substrate may be most conducive to enhancing phloem medicinal value. KEGG enrichment analysis supported the conclusion that flavonoid biosynthesis was the most significantly enriched pathway in Group A vs. Group D, with 18 differential metabolites and the highest impact value.

These findings have practical implications for the medicinal cultivation of *M. tricuspidata*. Peat soil + perlite is the recommended substrate when production targets high-quality medicinal materials with elevated bioactive compound content, whereas sandy soil + vermiculite is more appropriate for nursery operations prioritizing rapid seedling propagation. Manipulating secondary metabolite accumulation through substrate selection can provide a practical, non-genetic approach to improving the medicinal quality of *M. tricuspidata*.

4.5 Limitations and Future Perspectives

Despite providing valuable insights into substrate-dependent variations in the rooting and secondary metabolism of *M. tricuspidata*, the present study has several limitations. The application of untargeted metabolomics restricts differential metabolite measurements to relative quantification. Moreover, absolute quantification and validation would require targeted metabolomics with authentic standards. The molecular responses underlying the effects of substrates on secondary metabolism remain unresolved because changes in the expression of key biosynthetic enzymes have not been examined. Finally, all experiments were performed under controlled conditions, leaving the long-term responses of field-grown plants and their medicinal quality to different substrates to be evaluated.

Addressing these limitations will require complementary molecular and cultivation-based approaches. Integration of transcriptomics with targeted metabolomics could clarify the molecular mechanisms through which cultivation substrates regulate secondary metabolism in *M. tricuspidata*. Particular attention should be given to genes involved in flavonoid and xanthone biosynthesis, including chalcone synthase (CHS), chalcone isomerase (CHI), and xanthone synthase. A broader evaluation of transplant survival, field

performance, and consistency of medicinal quality under different substrate treatments would provide more comprehensive technical support for commercial cultivation. Extending the cultivation period and monitoring temporal changes in bioactive compound accumulation could additionally help establish the optimal harvest time for different cultivation objectives.

Future research should examine how substrate management can be incorporated into Good Agricultural and Collection Practices and standardized production protocols. Quantitative models linking cultivation conditions to reproducible concentrations of pharmaceutically relevant metabolites would provide a scientific basis for quality control and standardization of *M. tricuspidata* medicinal materials. The contrasting substrate responses observed here provide a rationale for investigating a two-stage production system. This system uses sandy soil + vermiculite to maximize rooting during nursery propagation, followed by transfer to conditions that favor flavonoid and xanthone accumulation. The effects of post-harvest drying, storage, and extraction methods on medicinal quality also warrant systematic investigation to connect cultivation practices with the quality of pharmaceutical raw materials. Comprehensive cost-benefit analyses should be conducted to assess the commercial scalability of different substrate treatments, accounting for substrate costs, seedling survival rates, biomass yield, active compound productivity per unit area, and overall farm profitability. Linking cultivation practices, metabolite profiles, raw material grading, and downstream pharmaceutical processing could support traceable medicinal plant value chains and reduce variability in herbal materials used in the industry.

5 Conclusions

The responses of *M. tricuspidata* cuttings to four cultivation substrates were evaluated through rooting measurements and UPLC-MS/MS-based untargeted profiling of phloem secondary metabolites, with sandy soil used as the control. The two principal cultivation responses differed by substrate, where the sandy soil + vermiculite produced the highest rooting rate, and peat soil + perlite most strongly promoted the phloem accumulation of bioactive flavonoids and xanthones. Under the latter treatment, 336 differential metabolites were identified, and flavonoid biosynthesis was the most significantly enriched pathway. Rooting rate and secondary metabolite accumulation exhibited a negative correlation, consistent with a resource trade-off between vegetative propagation and defense metabolism. Together, these results provide the first systematic evidence that substrate selection can differentially regulate rooting success and medicinal quality in *M. tricuspidata* cuttings. Nursery production aimed at rapid propagation would favor sandy soil + vermiculite, whereas peat soil + perlite would be preferable when the objective is to enhance the medicinal value of phloem tissues. The selection of a cultivation substrate can provide a practical, non-genetic approach for optimizing both seedling production and quality regulation in this economically important medicinal plant.

Acknowledgement: Not applicable.

Funding Statement: This study was funded by (1) Jiangsu Provincial Forestry Development Special Project (Project: Conservation and Utilization of *Maclura tricuspidata* Germplasm Resources; No. 02651007202501); (2) Doctoral Fund of Jiangsu Yanjiang Institute of Agricultural Sciences (Project: Association Mechanism Between Growth Rate and Lignin Synthesis in *Maclura tricuspidata* and Mining of Key Genes; No. YJBS(2026)001); and (3) Nantong Natural Science Foundation Livelihood Project (Project: Resource and Timber Species Identification, Rapid Propagation of Superior Varieties, and Demonstration Application of the Rare Native Tree Species *Maclura tricuspidata* in Nantong; No. MSZ2024112).

Author Contributions: The authors confirm contribution to the paper as follows: conceptualization, Fan Cao and Yujuan Li; methodology, Fan Cao, Yan Chen and Ling Wu; software, Yihang Ning, Xin Huang and Yuqing Jin; validation, Fan Cao, Cong Guo and Qingqing Gu; formal analysis, Fan Cao; investigation, Fan Cao; resources, Fan

Cao; data curation, Fan Cao; writing—original draft preparation, Fan Cao; writing—review and editing, Fan Cao; visualization, Fan Cao; supervision, Yujuan Li and Peibei Ke; project administration, Fan Cao. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author, Yujuan Li, upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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