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Research Paper

PHYSIOLOGICAL AND METABOLOMIC APPROACH FROM DIFFERENT ACCESSIONS OF MULBERRY TREATED WITH SALT STRESS IN INDONESIA

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ARTICLE HIGHLIGHTS

- Information on salinity-tolerant mulberry accessions is currently unavailable. This research reveals the discovery of salinity-tolerant mulberry accessions from the exploration of various mulberry accessions in Indonesia.
- Salinity-tolerant mulberry accessions can be utilized for sustainable development and biodiversity conservation.
- Salinity stress treatment is known to increase secondary metabolites in mulberry plants.
- Profiling of secondary metabolites in mulberry plants in response to salinity stress is known.
- The integration of metabolomics and ecophysiology offers a new perspective on how plants adapt to stress.

ABSTRACT

Land salinity in Indonesia continues to increase, therefore research is needed to obtain saline-resistant plants such as mulberries. Mulberry plants are well recognized for their resilience to various abiotic stresses, including salinity. This study aimed to identify tolerant accessions to salinity stress, in accordance with their growth characteristics, and to examine the alterations in secondary metabolites as a reaction to salt stress. The experiment was designed using a factorial randomized block design, which included seven mulberry accessions, identified as MB1 through MB7, and varying NaCl concentrations of 0% (control), 0.2%, 0.3%, and 0.4%. The study's results showed that MB2-3 accession tolerates high salinity stress by accumulating proline, flavonoid compounds, phenolic and dissolved sugars, as well as secondary metabolites, with increasing NaCl concentration. Based on the results of UHPLC-Q-Orbitrap HRMS analysis of mulberry leaf extract, 100 selected metabolites were obtained. The heat map results showed metabolic substances such as phenylpropanoids, organic acids, lipids, and carbohydrates increased in response to salinity stress. The integration of metabolomics and ecophysiology called ecophysiolomics offers a new perspective on how plants adapt to stress. By analyzing the involvement of metabolites that facilitate tolerance to salinity stress, we can gain a valuable understanding of how mulberry plants endure this environmental challenge.

Keywords: flavonoid, metabolomics, proline, salinity stress, soluble sugar

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INTRODUCTION

Soil salinization is a major form of land degradation threatening agricultural sustainability, with an anticipated escalation attributed to human activities. It is estimated that approximately 20% of the world's irrigated land is currently affected by salinization processes. Furthermore, the risk of salinity-related challenges is projected to increase due to global warming. The stress caused by salinity adversely affects plant growth and development by disrupting various physiological and biochemical processes. These processes include antioxidant metabolism, nutrient uptake, photosynthesis, mineral homeostasis, hormone signaling, and osmolyte accumulation (Khan *et al.* 2012). According to Yu *et al.* (2013), mulberry plants undergo changes in chlorophyll content, antioxidant enzyme activity, and photosynthesis when exposed to salinity stress and elevated temperatures. Additionally, Vijayan *et al.* (2008) and Taiz *et al.* (2015) reported that morpho-biochemical changes and anatomical modifications in mulberry can decrease productivity by inhibiting photosynthesis and growth. Under stressful conditions, plants generate reactive oxygen species (ROS) as their initial response. Reactive oxygen species (ROS) and peroxides are neutralized by various protective proteins, including antioxidants and dehydrins, as well as secondary metabolites, such as flavonoids, anthocyanins, and phenolic compounds (Pardo 2010). Flavonoids, anthocyanins, and phenolic compounds were for plant defense. The activity of antioxidant enzymes can effectively identify mulberry genotypes that tolerate salinity (Harinasut *et al.* 2003). The extent of abiotic stress affecting plants can also influence the synthesis of secondary metabolites (Pacheco *et al.* 2016). As a result, this research focuses on investigating the tolerance strategies of mulberry plants in response to salinity stress by integrating 'ecophysiology' and 'omics'. This approach contributes to the emerging field of 'ecophysiolomics' and enhances our understanding of how plants respond to salinity stress. Although the literature has thoroughly documented how plants respond to high salt stress and the mechanisms behind their tolerance, investigations into the metabolites involved in the tolerance mechanisms of mulberry in response to salinity stress remain limited.

Mulberry (*Morus* spp.) exhibits a remarkable capacity for adapting to various environmental stresses, including salinity, drought, and waterlogging (Liu *et al.* 2017). This plant contains a wealth of secondary metabolites, including alkaloids, phenylpropanoids, and flavonoids, which play crucial roles in mitigating the stress of both abiotic and biotic factors, and are utilized in traditional Chinese medicine (Liu *et al.* 2019). Additionally, the leaves of the mulberry plant serve as the primary food source for silkworms (*Bombyx mori* L.), underscoring its significance in the sericulture industry. Besides its conventional application in silkworm farming, mulberry shows great potential for growth in less productive lands.

The integration of metabolomics and ecophysiology offers a new perspective on how plants adapt to stress. Salinity stress is believed to cause variations in proteins that respond to the stress as a defense mechanism. This research aimed to identify accessions that show tolerance to salinity stress using metabolomic and ecophysiological methods.

MATERIALS AND METHODS

Plant Materials and Experimental Design

Mulberry stems approximately 1.0 cm in length were first placed in polybags until roots developed. Following successful rooting, the cuttings were transferred to polybags with diameters of 30 cm and 20 cm, and a height of 20 cm. The planting medium comprised a 1:1 mixture of soil and manure. The plants were allowed to grow for a year and were trimmed to achieve a uniform height of 100 cm prior to the application of treatment. Plant selection and salinity treatment were conducted following the method of Yu *et al.* (2013).

Treatment 1 comprised seven levels of mulberry varieties, labelled as MB1 through MB7. Treatment 2 included four different concentrations of NaCl 0.0% (control), 0.2%, 0.3%, and 0.4%. This study used 2 sets of treatments, each set of treatments consisted of 3 groups as replications containing randomization of 7 mulberry accessions with 4 NaCl concentrations in the group, so that there were 28 plant combinations arranged randomly in each group.

The method for applying salinity stress was modified from the original source with specific adjustments. Irrigation was conducted daily using one liter of Hoagland solution, which contained 0.5% NaCl, until the desired solution chloride (NaCl) level was achieved. In contrast, the control plants received daily irrigation of one

liter of Hoagland solution without NaCl until they achieved the specified Electrical Conductivity (EC). Observations were conducted simultaneously, so the 0.5% NaCl treatment was administered first to the 0.4% NaCl treatment plants because it required approximately 16 weeks of watering. An average increase in EC of 0.5 required approximately one week of watering. The 0.3% NaCl treatment plants were watered four weeks after the 0.4% NaCl treatment, and the 0.2% NaCl treatment plants were watered 12 weeks after the 0.4% NaCl treatment. Watering with Hoagland's solution containing NaCl is done in polybags above the soil surface in the root area.

Assessment of Proline Content

The proline content was measured using the Bates method (1973), and the level of proline was evaluated based on the standard curve and computed in relation to the fresh weight of the sample as explained below:

$$\left[\text{Proline}\right] = \frac{\text{Concentration of proline (µg/ mL)} \times \text{Amount of Toluene (mL)}/115.5\text{Proline (µg/µ mol)}}{(\text{Amount of Sample (g)})/5}$$
$$= \frac{\text{Amount of Proline (µ mol)}}{\text{Weight of Fresh sample (g)}}$$

Assessment of Soluble Sugar Levels

The soluble sugar levels were analyzed utilizing the Ifmaily (2018) method. The determination of the level of soluble sugar content relied on the difference between the titration result of the blank sample and that of the actual sample, as illustrated by the following formula.

$$\text{Soluble sugar content}\left(\%\right) = \frac{\text{Amount of Glucose(mg)} \times \text{FP} \times 0.9}{\text{Amount of Sample(mg)}} \times 100\%$$

where:
Glucose (mg) = Luff Schoorl table number based on the difference in mL titration
FP = dilution factor (mL titration filtrate)

Assessment of Phenolic Levels

Determination of the total phenolic content was carried out using the Folin-Ciocalteu reagent, with gallic acid used as the reference standard. First, 10 µL of the extract was combined with 160 µL of distilled water (Aquadest). Then, 10 µL of the Folin-Ciocalteu reagent and 20 µL of 10% sodium carbonate (Na_2CO_3) solution were added to the mixture. This combination was allowed to incubate at room temperature for 30 minutes, and the absorbance was recorded with a microplate reader (Epoch Biotech, USA) at a wavelength of 750 nm. The total phenolic content was reported in milligrams of gallic acid equivalent per gram of dry weight (GAE (mg)/DW (g)).

Assessment of Flavonoid Levels

The total concentration of flavonoid was assessed using the technique outlined by Khumaida *et al.* (2019), which employed an aluminum chloride reagent along with quercetin as the standard reference. In this procedure, a solution mixture was created in a 96-well microplate, which included 60 µL of the sample extract solution, 10 % aluminum chloride, 10 µL of 1 M potassium acetate, and 120 µL of deionized water (aquadest). After a 30-minute incubation at room temperature, the absorbance was recorded with a microplate reader (Epoch Biotech, USA) at a wavelength of 415 nm. The outcome was quantified and reported as quercetin equivalents (QE (mg)/DW (g)).

Sample Preparation for Metabolomic Analysis

The phenol and flavonoid assay indicated that accessions with the highest levels of proline were MB2-3, MB4-2, and MB6-3. The sample MB2 (all concentrations) and sample MB4-2 (along with the control) were selected because they showed high phenol and flavonoid activity in the 0.4% NaCl treatment, while sample MB6-3 was selected because it showed high proline content in the 0.4% NaCl treatment. This samples were selected for metabolomic analysis as shown in Table 1.

Table 1 Accession and sample code for metabolic analysis

No.	Accession code	NaCl concentration (%)	Sample codes
1	MB2	0.0	2-0
		0.2	2-1
		0.3	2-2
		0.4	2-3
2	MB4	0.0	4-0
		0.3	4-2
3	MB6	0.4	6-3

Analysis of UHPLC-Q-ORBITRAP HRMS

The UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS, equipped with a 100 × 2.1 mm Accucore C18 column (1.5 µm particle size from Thermo Scientific™, Waltham, MA, USA), was used for the chromatographic separation. The mobile phase consisted of water with 1% formic acid denoted as (Component A), and acetonitrile (Component B). The solvent gradient followed the protocol established by Choi *et al.* (2021) and was operated at a flow rate of 200 µL/min.

The gradient procedure was executed as follows: it started at 5-95% B from 0 to 20 minutes, switched to 100% B for 20 to 20.1 minutes, transitioned to 100% A from 20.1 to 22 minutes, reverted to 100% B from 22 to 22.1 minutes and finally stabilized at 5% B from 22.1 to 24 minutes. An injection volume of 2 µL was utilized, and eluted compounds were detected in positive ion mode across a mass-to-charge ratio (m/z) range of 100 to 1,500. The flow rates for the auxiliary and sweep gases were set at 35 mL/min, 15 mL/min, and 0 mL/min, respectively.

Operating conditions included a spray voltage of 3.8 kV, a capillary temperature at 320 °C, an S-lens RF level of 50, and an auxiliary gas heater temperature of 38 °C. Acetonitrile, formic acid, and methanol used in the procedure were of HPLC-grade quality from Merck, Darmstadt, Germany. Data acquisition and multivariate analysis were carried out using Xcalibur 2.2 software from Thermo Scientific.

Compound identification and characterization were conducted using Compound Discoverer 2.1 software, followed by a comparison against information from online databases. The mass error expressed in parts per million (ppm), was calculated by comparing the theoretical monoisotopic mass from the database with the observed mass, applying a filtering criterion of 10 ppm for effective mass screening.

Fragment mass spectroscopy (MS2) validation was performed using the online Competitive Fragment Modeling (CFM-ID 4.0) software. Finally, the heatmap and hierarchical clustering analysis were executed with MetaboAnalyst 5.0 based on peak area data.

Clusters was made using a dendrogram model with Euclidean distance parameters, a complete clustering algorithm, and autoscale features standardization between groups. Putative metabolites used for metabolomics analysis were used for identification of metabolite roles and clusters using PubChem (https://pubchem.ncbi.nlm.nih.gov) and ChemSpider (https://chemspider.com) software.

Metabolite Abundance Percentage

After clustering the putative metabolite identities using PubChem and ChemSpider software to obtain their phytochemical classes, then all phytochemical classes were averaged to obtain the percentage abundance of their metabolites based on peak area.

Statistical Analysis

The analysis of the obtained data was carried out using SPSS software version 24.0 (IBM SPSS Statistics, Chicago, IL, USA). The data were evaluated at a 5% significance level utilizing Duncan's new multiple-range test. Proline content data was presented as mmol/g, soluble sugar concentration was presented as percentage (%), total phenolic content was presented as mg GAE/g, and total flavonoid content was presented as mg QE/g. Total phenolic and flavonoid values were presented as mean ± SD.

RESULTS AND DISCUSSION

Analysis of UHPLC-Q-ORBITRAP HRMS

Proline Levels in Response to Salt Stress

The proline levels in various leaves of

mulberry accessions were influenced by the interaction between the type of accession and the NaCl concentration ($P < 0.05$). In particular, accessions MB5, MB6, and MB7 exhibited significant differences ($P < 0.05$) at a 0.4% NaCl concentration, along with MB6 and MB7 at a 0.3% NaCl concentration, as shown in Figure 1. The level of proline exhibited a significant increase in response to sodium chloride (NaCl), a finding that aligns with previous research conducted on tomato (Gharsallah *et al.* 2016) and Solanum species (Brenes *et al.* 2020). Among the accessions assessed, MB6 exhibited the highest proline concentration at a NaCl level of 0.4%. The proline concentration of accession MB6 in 0.4% NaCl treatment was higher than that in accession MB2. However, accession MB2 showed a 2 – 3 fold increase in proline concentration compared to the control, while accession MB6 experienced a decrease in proline concentration in the 0.3% to 0.4% NaCl concentration treatment (Fig. 1). It has been documented that salt-tolerant tomato genotypes accumulate substantial levels of proline when subjected to salinity stress (Mishra & Singhal 1993).

Salt-tolerant varieties of *Salicornia europea* contain higher levels of proline compared to those that are sensitive. Moreover, research indicated that the proline metabolism in two cultivars of *Morus alba* L. exposed to NaCl underwent significant changes, with the tolerant cultivars exhibiting greater proline accumulation (Kumar *et al.* 2003; Cárdenas-Pérez *et al.* 2022).

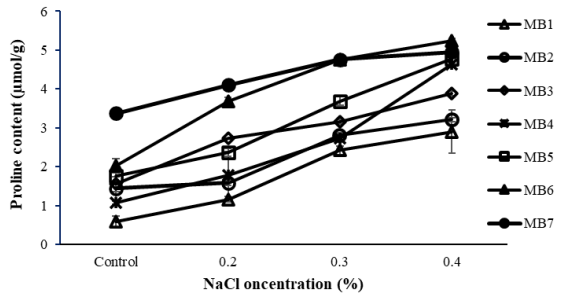

Figure 1 The proline content of mulberry accession as a response to salinity stress at the age of 16 WAT

The increased accumulation of proline plays a role in controlling turgor by reducing water loss and mitigating ion toxicity, thus acting as a strategy for coping with salinity stress. To alleviate the osmotic pressure caused by salinity, mulberry produces osmoprotective substances, including proline. The roles of this osmoprotectant compound involve counteracting reactive oxygen species (ROS), maintaining the stability of subcellular structures, managing redox balance within cells, supplying energy, and acting as signaling molecules.

Soluble Sugar Content in Response to Salinity Stress

The levels of dissolved sugar were significantly influenced by accession, as illustrated in Figure 2. Accession MB4 exhibited the highest level of dissolved sugar at 4.0%, which did not show a statistically significant difference from the concentrations observed in accessions MB3 and MB6 ($P < 0.05$). Additionally, the concentration of sodium chloride (NaCl) influenced the dissolved sugar levels, as presented in Figure 3. The highest sugar levels in the leaves were observed at a concentration of 0.4% NaCl, reaching 4.43%, which was significantly different from the other concentrations ($P < 0.05$).

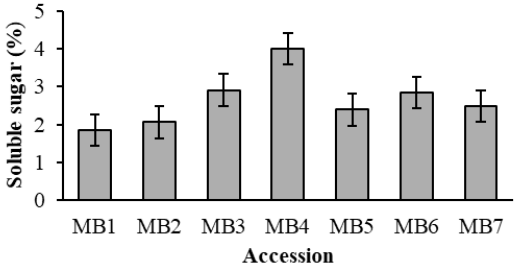

Figure 2 The concentration of dissolved sugar in different mulberry accession leaves in 16 WAT

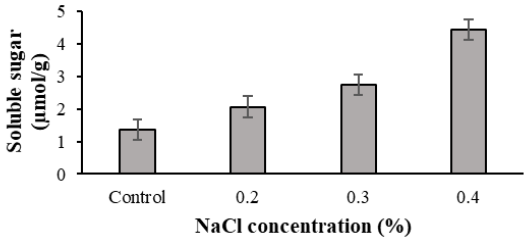

Figure 3 Concentration of dissolved sugar in different accession mulberry leaf at NaCl concentration at 16 WAT

The control of soluble sugar concentration in leaves is linked to reduced growth when exposed to salt stress conditions. Chickpea genotypes that are tolerant, such as SG-11 and DHG-84-11, exhibit a greater accumulation of soluble sugars compared to those that are sensitive, especially at a concentration of 0.4% (Singh 2004). Tolerant

varieties continually accumulate higher levels of soluble sugars in their leaves and growing tissues compared to those that are sensitive.

Studies showed that the salt-resistant sugarcane variety CP66-346 gathers a greater amount of soluble sugars compared to the more sensitive type CP65-357. Of all the varieties examined, MB4 exhibited the highest levels of soluble sugars, however, there was no considerable difference when compared to MB3 and MB6.

The concentration of soluble sugars increased with increasing NaCl levels, as shown in Figure 3. Accumulation of soluble sugars in the leaves attributed to NaCl levels has been reported in Physalis angulata L., Pistacia atlantica, and Saccharum sp. In response to salt stress, plants typically accumulate compatible osmolytes, such as proline and soluble sugars.

Phenolic Levels in Response to Salt Stress

In response to salinity, every accession showed a statistically significant total phenolic content ($P < 0.05$). The highest measurement was observed in accession MB4-2, which exhibited a total phenolic content of 154.6 mg/GAE/g extract. This value was not significantly different from that of accession MB2-3, which recorded an activity value of 141.9 mg GAE/g extract ($P > 0.05$). Conversely, the lowest total phenolic content was found in accession MB1-3, which had a measurement of 8.57 mg/GAE/g extract.

The selected accession MB2-3, along with the positive control, contains phenolic compounds (Table 2). Accession MB2-2 exhibited the highest total phenolic compounds, at 154.6 ± 27.45 mg/GAE/g extract, and this measurement showed no significant difference compared to MB2-3, which had a phenolic content of 141.9 ± 5.72 mg/GAE/g extract.

Most of the accessions showed a decrease in phenolic content compared to the control, which corresponds with findings in lettuce plants where 40 mM NaCl was associated with a reduction in phenolic content. The reduction in phenolic compounds is associated with inadequate antioxidant production, which fails to counter the stress of oxidative (Zhang *et al.* 2021). In comparison, the reduction observed within NaCl treatment is not as significant as that of the control group as evidenced by MB1, MB5, and MB6. A previous study had indicated that the total phenolic content decreases in the presence of NaCl, leading to metabolic changes (Ahmed *et al.* 2019). Research on lettuce plants exposed to 100 mM NaCl showed limited enzyme activity for multiple enzymes that play a role in the synthesis of phenolic compounds (Blasco *et al.* 2013). The enzymes examined include phenylalanine ammonia-lyase, 4-coumarate A ligase, and shikimate dehydrogenase.

The phenolic levels in plants are affected by various factors, including different plant varieties, environmental conditions, and stressors such as salinity. The differences in secondary metabolism across various species indicate their mechanisms for adaptation (Sanchez *et al.* 2008). In particular, exposure to salt stress leads to the accumulation of proline, an antioxidant compound that plays a crucial role in alleviating and minimizing oxidative stress in the plant system (Mahmoudi *et al.* 2010).

Flavonoid Levels in Response to Salt Stress

Total flavonoid content is affected by both the plant accession and the NaCl concentration ($P < 0.05$). Accession MB2-3 showed the highest flavonoid levels, achieving an activity of 619.56 mg QE/g DW (Table 3). However, the observed value was not significantly distinct from that of accession MB4-2, which recorded an activity of 605.11 mg/QE/g DW extract ($P < 0.05$). Additionally, the total flavonoid content of MB2 rose with the increase in NaCl concentration.

Table 2 Comparison of total phenolic content (mg GAE/g) mulberry leaf extract as a response to the interaction of accession and NaCl concentration

Accession	NaCl concentration (%)			
	0.0	0.2	0.3	0.4
MB1	65.71 ± 12.99^{c-e}	62.54 ± 11.11^{c-e}	14.92 ± 4.20^{h}	8.57 ± 1.59^{h}
MB2	24.44 ± 1.59^{gh}	54.60 ± 8.25^{d-f}	13.34 ± 1.59^{h}	141.90 ± 5.72^{a}
MB3	116.51 ± 0.0^{b}	57.78 ± 4.20^{d-f}	116.51 ± 4.76^{b}	86.35 ± 4.20^{c}
MB4	45.08 ± 2.75^{e-g}	30.79 ± 2.75^{f-h}	154.6 ± 27.49^{a}	10.16 ± 1.59^{h}
MB5	73.65 ± 5.5^{cd}	60.95 ± 4.2^{c-e}	60.95 ± 12.4^{c-e}	41.91 ± 15.14^{e-g}
MB6	57.78 ± 8.84^{d-f}	45.08 ± 5.5^{e-g}	18.10 ± 1.59^{gh}	26.03 ± 2.75^{gh}
MB7	67.30 ± 4.2^{c-e}	30.79 ± 2.75^{f-h}	41.91 ± 4.2^{e-g}	54.60 ± 7.27^{d-f}

Notes: Means values with different superscript letters in the same column denote significant ($P < 0.05$) differences between groups.

Table 3 Comparison of total flavonoid content (mg QE/g) mulberry leaf extract as a response to the interaction of accession and NaCl concentration

Accession	NaCl concentration (%)			
	0.0	0.2	0.3	0.4
MB1	295.11 ± 2.22^{de}	242.89 ± 11.76^{fg}	205.11 ± 4.84^{g-j}	184.00 ± 3.85^{j}
MB2	184.00 ± 3.85^{j}	217.33 ± 1.93^{f-j}	187.33 ± 16.78^{ij}	619.56 ± 2.94^{a}
MB3	376.22 ± 5.55^{c}	294.00 ± 13.88^{de}	336.22 ± 17.88^{cd}	492.89 ± 23.91^{b}
MB4	292.89 ± 14.57^{de}	208.44 ± 6.76^{f-j}	605.11 ± 34.92^{a}	176.22 ± 12.37^{j}
MB5	256.22 ± 13.10^{ef}	346.22 ± 33.90^{c}	201.78 ± 14.45^{g-j}	212.89 ± 25.56^{e}
MB6	215.11 ± 9.49^{f-j}	237.33 ± 8.39^{f-h}	182.89 ± 9.49^{j}	197.33 ± 1.93^{g-j}
MB7	298.45 ± 7.78^{de}	214.00 ± 10.00^{f-j}	234.00 ± 1.92^{f-i}	189.55 ± 4.00^{h-j}

Notes: Means values with different superscript letters in the same column denote significant ($P < 0.05$) differences between groups.

Flavonoid compounds were also found in the selected accession of MB2-3, along with the positive control (Table 3). Total flavonoid content reached its peak in MB2-3, with an activity value of 619.56 ± 2.94 mg/QE/g DW extract, which exceeds that of the control. There was no notable difference between accession MB2-3 and accession MB4-2, which had an activity value of 605.11 ± 34.92 mg QE/g DW extract. The increased levels of flavonoid observed under high salt conditions may be attributed to a rise in antioxidants, such as flavonoids, which help mitigate oxidative stress caused by salinity.

Certain accessions, like MB1 and MB7, demonstrated a reduction in total flavonoids. The plants' responses differ significantly and may stem from variations in salt sensitivity among species. Furthermore, this variation is influenced by factors such as the length of the stress treatment, cultivation techniques, and the impact of various stressors, including osmotic pressure, drought, and nutrient availability.

Analysis of Metabolite Build-up in Response to Salinity using UPHPLC-Q-orbitrap HRMS

The heat map representing all samples is illustrated in Figure 4, emphasizing two main clusters of metabolites. Cluster I features a significant presence of carboxylic acids and amino acids, whereas Cluster II is mainly made up of lipids and alkaloids.

Metabolite indicators in Cluster I included maraniol compounds (accession MB4-0), shikimic acid, and 4-O-(α-D-glucopyranosyl) moranoline (accession MB2-0). Other markers included kuwanon C, 1-DNJ (accession MB2-1), ageratriol, (+)-ar-tumerone (accession MB2-2), kuwanon G, octadecanedioic acid, gingerdione, ferulic acid, eugenitin, myristoleic acid, and coniferyl ferulate (accession MB2-3). Furthermore, α-tocopherol was suggested to serve as a marker metabolite for accession MB6-3.

Figure 4 shows that certain metabolites, such as corchorifatty acid, erucamide, hexadecanamide, oleic acid alkyne, lauroylcarnitine, capryoylglycine, palmitoleic acid, octadecanedioic acid, and suberic acid, exhibited higher concentrations in comparison to the control. Oleic acid functions as a signaling molecule that activates the enzyme phospholipase D, which plays a role in preventing cell death (Zhang *et al.* 2003). The levels of the alkyne group seem to rise in samples MB4-0 and MB2-3. Extracellular lipid polymers like cutin and suberin, which are important fatty acids, play a crucial role in preserving membrane fluidity in harsh environmental conditions.

Furthermore, it has been observed that the levels of unsaturated fatty acid in *Solanum tuberosum* L. rise under salt stress (Hamooh *et al.* 2021). This phenomenon has also been reported in *Brassica oleraceae* (Lopez-Perez *et al.* 2009), Arabidopsis (Gigon *et al.* 2004), safflower (Harrathi *et al.* 2012), and *Suaeda salsa* L. (Hanooh *et al.* 2021). Unsaturated fatty acids, including erucamide, corchorifatty acid F, palmitleic acid, and oleic acid alkyne, show elevated levels when subjected to salinity stress. Moreover, octadecanedioic acid and myristoleic acid are recognized as indicators for accession MB2-3, as shown in Figure 4. The elevated levels of these compounds may contribute to the high salt tolerance of accession MB2-3, as their accumulation can act as stabilizers for their membrane.

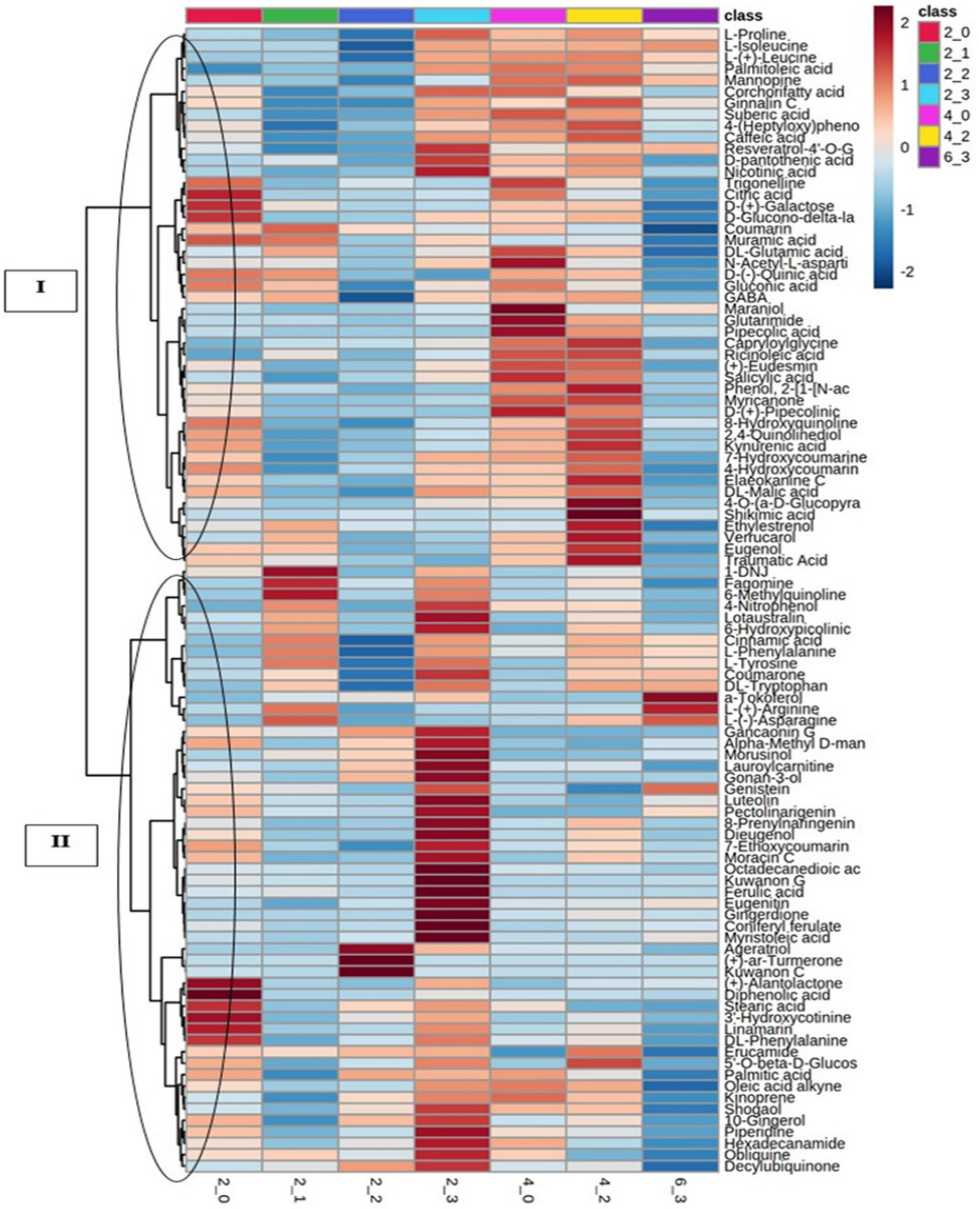

Figure 4 Heat map with cluster analysis based on metabolites
Notes: The color in the legend shows a range from blue to red, reflecting the abundance of metabolites from low to high concentrations.

Lipids represented the most abundant class of phytochemicals, comprising 51.28% of the total detected metabolites (Table 4). A similar finding was reported by Ackah *et al.* (2021), which noted that lipids accounted for 47.7% of the total metabolites identified using LC-MS.

Alongside the rise in metabolite levels, there was a reduction in traumatic acid as a reaction to salinity stress. A Significant decrease in fatty acids may occur due to damage to membranes. A considerable drop in fatty acid, including traumatic acid was also noted in the drought stress treatment of the mulberry variety Yu-711 (Ackah *et al.* 2021). Fatty acids, such as linoleic acid, are essential for maintaining membrane integrity and play a functional role in the proteins involved in the photosynthetic process. Thus, a notable decline in fatty acid levels has a considerable effect on photosynthesis. The breakdown of linoleic acid and other polar lipids through hydrolysis can lead to the release of free fatty acids and hydroperoxide lipids, triggering the aging process (Upchurch 2008).

The salinity treatment applied to mulberry plants resulted in alternation in various lipid classes, including steroid and their derivatives. This study identified the production of two types of steroids-ethylestrenol and gonan-3-ol with

Table 4 Percentage of metabolite abundance of all accessions that have been grouped into each phytochemical class based on peak area

Phytochemical class	Metabolite abundance (%)
Lipid	51.28
Carboxylic Acid	15.99
Terpene	8.46
Amino Acid	7.96
Alkaloid	6.38
Vitamine	3.93
Carbohydrate	3.91
Phenol	1.46
Phenylpropanoid	0.22
Sesquiterpene	0.16
Flavonoid	0.15
Steroid	0.10

their concentration increasing with increasing NaCl levels, especially in the accession MB4-2. Phytosterols, which are vital components of the lipid bilayer membrane, play a role in shielding plants from stress, maintaining membrane fluidity, and affecting the arrangement, function, and structure membrane (Rogowska & Szakiel 2020). Several studies have indicated that the phytosterol levels increase during drought stress periods (Kumar *et al.* 2015; Moradi *et al.* 2017), the rise in sterol content serves as an adaptive mechanism to environmental stress, attributed to the heightened activity of terpene synthase (Antunes *et al.* 2019).

Prenol lipids, encompassing isoprenoids and terpenoids, showed an elevated concentration of metabolites when subjected to increased salt stress. The identified terpenoid compounds include linamarin, kinoprene, and (+)-ar-tumerone (refer to Fig. 4), while the sesquiterpenes found are ageratriol, verrucarol, and (+)-alantolactone. As illustrated in Figure 4, both ageratriol (a type of sesquiterpene) and (+)-ar-tumerone (a type of terpene) act as marker metabolites for the accession MB2-2, in addition to kuwanon C (a flavonoid). Furthermore, variations in prenol lipid levels have also been noted in soybeans experiencing drought stress (Nam *et al.* 2019).

CONCLUSION

Accession MB2-3 exhibited physiological changes associated with salinity stress. Proline, soluble sugars, phenolic, and flavonoids compounds increased with increasing NaCl concentration. The impact of salinity stress influenced the amount of various metabolite classes in the leaves of mulberry. Specifically, at the highest salt concentration, the levels of flavonoids, phenolic compounds, carboxylic acid, and lipids increased in the accession MB2-3. The metabolites kuwanon G, eugenitin, ferulic acid, gingerdione, coniferyl, ferulate, myristoleic acid, and octadecanedioic acid showed improvement compared to control. Furthermore, lipid compounds were found to be the most prevalent metabolites, as indicated by UHPLC-Q-Orbitrap-HRMS analysis results. These substances assist in shielding cells and chloroplast membranes, maintaining their integrity and functionality against potential damage. In summary, the interplay among flavonoids, phenolic compounds, carboxylic acids, and lipids plays a crucial role in mediating tolerance to salinity stress.

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