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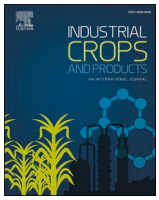

Integrated microbiological and metabolomic analysis reveals the mechanisms by which biochar and wood vinegar enhance the cold tolerance of alfalfa

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ABSTRACT

Freezing stress limits the sustainable production of alfalfa in northern China. While biochar and wood vinegar enhance crop stress tolerance, their potential to improve alfalfa cold resistance remains unknown. This field study compared the effects of a control group (CK), biochar (B, 10 t/ha), wood vinegar (W, diluted 200-fold), and a combined treatment (BW). Results indicate that B, W, and BW significantly enhanced cell membrane stability and alleviated oxidative stress, as evidenced by increases in root soluble protein (SP), soluble sugar (SS), and proline (Pro) content by 7.74 %-11.21 %, 12.81 %-51.54 %, and 3.50 %-43.00 %, respectively ($p < 0.05$). Superoxide dismutase (SOD) and catalase (CAT) activities increased by 20.13 %-46.18 % and 163.54 %-235.16 %, respectively ($P < 0.05$); Maldodialdehyde (MDA) content and relative electrical conductivity in BW were significantly reduced by 22.55 % and 16.56 %, respectively, compared to CK ($p < 0.05$). Biochar and wood vinegar increased the contents and availability of nutrients such as nitrogen, phosphorus, and potassium in the soil and improved the structure of the rhizosphere bacterial community (for example, bacteria of the phyla Bacteroidetes, Actinobacteria, and Acidobacteria were significantly enriched in B, W, and BW, and the community structure became more complex). They also activated metabolic pathways such as flavonoid and isoflavone biosynthesis in the roots, thereby enhancing the cold tolerance of alfalfa and increasing the yield of alfalfa after overwintering by 13.63 %–19.91 %. The findings of this study suggest that the concurrent utilisation of wood vinegar and biocharcan effectively augment the cold tolerance of alfalfa, thereby facilitating the sustainable production of this crop.

1. Introduction

Alfalfa (*Medicago sativa* L.) is cultivated on a wide scale across the globe. This is primarily due to its high nutritional value, which includes proteins, dietary fibre, minerals and vitamins. Its extensive root system confers it with desirable agronomic traits such as strong regenerability, high yield, and strong adaptability (Noori et al., 2018; Hadidi et al., 2018). Despite the greater resilience of alfalfa, the alpine region in the north of China experiences colder winters and less snowfall, with extreme low temperatures often occurring in winter and spring due to cold spells. As a result, extreme low-temperature stress has emerged as an important issue restricting sustainable alfalfa cultivation in China (Wang et al., 2022; Xu et al., 2024). Consequently, enhancing the cultivation and management techniques of alfalfa is crucial to alleviate the detrimental impacts of extreme low temperatures on its sustainable production and to foster the robust growth of the alfalfa cultivation sector in northern China.

The detrimental effects of low temperature on plants can be categorized into two types, namely chilling injury and freezing injury, based on the degree of low temperature and the extent of damage. Low-temperature stress, defined as occurring between 0 and 15°C, is termed chilling injury, but temperatures below 0°C may lead to freezing

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injury (Satyakam et al., 2022). During the process of freezing injury, the arrangement of membrane lipids in plant cells is subject to alteration, resulting in the transition of the cell membrane from a fluid state to a rigid gel phase. This transition results in increased membrane permeability and a reduced capacity to maintain ionic homeostasis. And the formation of ice crystals within the apoplast during the process of freezing injury has been demonstrated to induce alterations in the water potential within and without the cells, thereby precipitating cellular dehydration or even rupture (Ding et al., 2019). Moreover, low-temperature stress results in an elevation of reactive oxygen species (ROS) levels in plant cells. Elevated levels of ROS can induce lipid peroxidation, protein oxidation, and DNA damage (base loss and alteration), thereby impairing normal cellular functions and directly causing oxidative damage to cells and reducing membrane fluidity (Mittler, 2017; Zhang et al., 2022a). To maintain ROS homeostasis and stabilize cell membrane and protein structures, plants have evolved a highly effective enzymatic antioxidant defense system, which mainly includes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). These enzymes can effectively scavenge ROS and alleviate the damage caused by ROS (Edas and Eroychoudhury, 2014; Amna et al., 2010; Uarrota et al., 2016). Furthermore, osmotic regulatory substances within plants, such as soluble sugars (SS), soluble proteins (SP), and free proline (Pro), also play important roles in coping with extreme low temperatures. The accumulation of soluble sugars can prevent intercellular freezing at low temperatures, enhance cell membrane stability, and participate in the removal of ROS from cells (Lin et al., 2023). Elevated concentrations of soluble protein can enhance cellular water retention and reduce their freezing point (Han et al., 2022). Free proline preserves the structural integrity of biomembranes and enzyme proteins, mitigates the excessive production of reactive oxygen species under stress, and elevates cell sap concentration to reduce its freezing point, thus averting cellular dehydration (Wang et al., 2021). It is worth noting that the rhizosphere of crops is a hotspot for soil microbial activity and has a significant impact on the absorption of soil nutrients by crops and their resistance to external environmental stresses (Li et al., 2021).

Biochar is a carbon-dense and very stable solid material produced from the pyrolysis of biomass in oxygen-limited or anoxic conditions at temperatures exceeding 250℃. Due to its elevated porosity, substantial specific surface area, excellent stability, and significant cation exchange capacity, biochar is frequently utilized to augment soil fertility, enhance soil structure, regulate microbial populations, stimulate crop growth, and alleviate abiotic pressures (Johannes and Stephen, 2015; Shalini et al., 2020). The phosphorus content and availability in soil significantly influence alfalfa's cold tolerance (Wang et al., 2024). Research by Jing et al. revealed that applying biochar effectively increases soil total phosphorus and alkali-hydrolyzable phosphorus content (by 21.95 % and 28.91 %, respectively, after adding 15 t/ha biochar; $p < 0.05$), thereby promoting alfalfa growth (Jing et al., 2025). However, Jing et al. did not further investigate the effect of biochar on the cold tolerance of alfalfa during overwintering. Furthermore, Yuan et al. discovered that organic compounds on biochar's surface can affect the ZAP1 protein, hence improving crop cold tolerance (Yuan et al., 2017). However, there are currently no reports on whether biochar can enhance alfalfa's cold tolerance under field conditions.

Wood vinegar is a liquid generated by the condensation and separation of gases released during the pyrolysis of biomass. The primary constituents comprise acids, phenols, aldehydes, ketones, alcohols, esters, and trace elements such as potassium, calcium, magnesium, zinc, germanium, manganese, and iron (Wu et al., 2015). Wood vinegar's organic acids and phenolic components can influence plant physiological activities, significantly augment the antioxidant capacity of crops, enhance stress resistance, and facilitate plant growth and development in adverse situations (Zhu et al., 2022). Zhu et al. discovered that butyrolactone in wood vinegar is a crucial compound for augmenting low-temperature stress tolerance in oilseed rape, elevating the levels of SS and Pro, and promoting the activity of antioxidant enzymes (Zhu et al., 2024). The research by Zhu et al. primarily focused on the metabolites produced by crops under low-temperature stress. The effects of wood vinegar on crop rhizosphere bacterial communities and whether it exerts similar effects in enhancing alfalfa's tolerance to low-temperature stress remain unknown.

Although previous studies have demonstrated the potential of biochar and wood vinegar in enhancing crop stress resistance, several key knowledge gaps remain. First, their effects on perennial leguminous forages such as alfalfa under field freezing stress have not been extensively explored. Second, while the role of biochar in soil improvement and the function of wood vinegar as an antioxidant are known, their impacts on key cold-tolerance metabolic pathways in alfalfa roots have not been elucidated. Third, most existing studies focus on either soil properties, microbial communities, or plant physiological responses, with few integrating these key elements to systematically reveal the mechanisms by which biochar and wood vinegar enhance crop cold tolerance. Therefore, we hypothesize that biochar and wood vinegar will reshape the rhizosphere microenvironment of alfalfa, thereby altering root metabolism and ultimately enhancing cold tolerance. To our knowledge, this study is the first to systematically dissect the mechanisms by which biochar and wood vinegar enhance alfalfa cold tolerance under field conditions using an integrated microbiome and metabolomics analysis approach. Our objectives are to quantify their impacts on alfalfa physiological performance and post-overwintering yield, identify key changes in root metabolites and their associated biosynthetic pathways, characterize changes in rhizosphere bacterial community structure and network complexity, and ultimately establish correlations between soil properties, microbial communities, root metabolism, and plant cold tolerance.

2. Materials and methods

2.1. Experimental design

The trial site was situated in Hailutu Science and Technology Park, Hohhot (40°41′30″ N, 111°2′30″ E). The experimental location experiences a temperate continental monsoon climate, with an average annual frost-free period of 133 days. The precipitation in 2024 reached 628.5 mm. The average temperature in December 2024 was between −2.7 ℃ and −14 ℃, with the lowest temperature reaching −19℃ (The minimum/maximum temperature records during the study period can be seen in Fig S1 of the Supplementary Materials.). The soil texture of the research region is silt loam, characterized by the following properties: pH: 8.22; electrical conductivity: 0.56 μS/cm; total nitrogen: 0.7 g/kg; ammonium nitrogen: 6.32 mg/kg; nitrate nitrogen: 2.075 mg/kg; available phosphate: 29.26 mg/kg; available potassium: 107.85 mg/kg. The biochar utilized in this research was acquired from Zhengzhou Haosen Environment Protection Technology Co., Ltd. It was produced by hydrothermal carbonization of corn straw, and its specific properties are shown in Table S1. Wood vinegar was obtained from Hongsen Carbon Activated Co., Ltd., situated in Shijiazhuang City. The primary basic source for wood vinegar manufacture is peach shells. The pH of wood vinegar is 3.4, with the total nitrogen level at 8.81 g/L, the total phosphorus level at 0.28 g/L, and the total potassium level at 0.57 g/L. Additional chemical properties are included in Table S2.

The alfalfa variety used in this study is "WL363" (purchased from Beijing Zhengdao Seed Industry Co.), which is characterized by its strong cold tolerance and high yield, making it suitable for cultivation in this region. Alfalfa was sown on May 22, 2024, at a seeding rate of 22.5 kg/ha. Based on our previous research results, it is believed that adding 10 t/ha of biochar and diluting wood vinegar 200 times can more effectively improve the production performance of alfalfa and enhance its nutritional quality (Yan et al., 2024) (The results of this study can be seen in Fig S1 and S2 in the supplementary materials). Therefore, we set up the following four treatment groups in this study: CK (no biochar or wood vinegar applied), B (application of 10 t/ha biochar), W

(application of wood vinegar diluted 200 times), and BW (application of 10 t/ha biochar + wood vinegar diluted 200 times). The biochar was uniformly distributed throughout the plots prior to sowing and subsequently integrated into the soil to a depth of 0–20 cm using a rotary tiller for comprehensive mixing. This study employed a split-plot experimental design, with biochar as the main plot and wood vinegar as the subplot. Each treatment was replicated in three plots, totaling 12 plots. Each plot had an area of 9 m^2 (3 m × 3 m), and a 1 m wide isolation belt was established between plots. To prevent the influence of weeds, manual weeding was conducted during the experiment to remove weeds from both the isolation belts and the plots. All plots were fertilized with 400 kg/ha compound fertilizer (N-P_2O_5-K_2O, 22–11–10, NO_3-N ≥ 9 %) before sowing. Other management practices, including irrigation, were consistent across all treatment groups. The second cutting of alfalfa was used as the experimental material. After the alfalfa was harvested, the wood vinegar was sprayed. For the W and BW treatment groups, a 200-fold diluted wood vinegar solution was sprayed, while the CK and B treatment groups were sprayed with an equal amount of water. The application rate was 9 L per plot. The fresh forage yield of the first cutting of alfalfa after greening was measured on June 5, 2025. Three 1 m * 1 m quadrats were randomly selected from each plot for harvesting and weighing, with a total of nine replicates for each treatment.

2.2. Sample sampling

Sampling was conducted on December 26, 2024, when the study area entered the coldest period of the year. This period is also the critical stage that determines whether alfalfa can safely survive the winter. Under these conditions, alfalfa had already experienced typical and sustained freezing stress and exhibited a clear state of freezing response. Therefore, we chose this representative time point for sampling. Five alfalfa plants were randomly selected from each plot and their root systems were completely excavated. Following the method of Prendergast-Miller et al., the soil loosely attached to the roots was removed. The soil near the roots was gently brushed off with a brush, collected, and then the detached roots were removed. This soil was used as the rhizosphere soil (Prendergast-Miller et al., 2011). The collected rhizosphere soil was divided into two portions. One portion was stored in a refrigerator at −80°C for DNA extraction of soil microorganisms, and the other portion was air-dried for the analysis of soil chemical properties. After removing the surface soil, the root tissues were also preserved in liquid nitrogen for the assessment of various cold tolerance indicators.

2.3. Physiological indicators and measurements

The activities of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) were evaluated using enzyme-linked immunosorbent assay (ELISA) kits provided by Jiangsu Aiding Bio-Tech Co., Ltd. The anthrone-sulfuric acid method was employed to ascertain the concentration of soluble sugars (Ai et al., 2024). The Coomassie brilliant blue method was employed to determine the concentration of soluble proteins. The ninhydrin method was employed to ascertain the proline content (Zhao et al., 2018). The thiobarbituric acid (TBA) method was employed to quantify the concentration of malondialdehyde (MDA). The method described by Jun et al. was employed to measure the relative electrolyte leakage (Cui et al., 2018).

2.4. Metabolomics analysis

Metabolomics analysis was conducted using ultra-performance liquid chromatography coupled with Fourier transform mass spectrometry (UPLC-MS) (UHPLC-Q Exactive, Thermo Fisher Scientific). A 100 mg solid sample was placed into a 2 mL centrifuge tube, to which a 6 mm diameter grinding bead was added. An extraction solution (800 μL) consisting of methanol and water in a 4:1 (v:v) ratio, along with four internal standards (including L-2-chlorophenylalanine at 0.02 mg/mL), was employed for metabolite extraction. The sample was milled for 6 min at −10℃ and 50 Hz using a cryogenic tissue grinder, then subjected to low-temperature ultrasonic extraction for 30 min at 5℃ and 40 kHz. Thereafter, the sample was preserved at −20℃ for 30 min, centrifuged for 15 min at 4℃ and 13000 g, and the supernatant was transferred to an autosampler vial with an insert for analysis. The LC-MS analysis utilised the Thermo Fisher Scientific UHPLC-Q Exactive system (Shanghai Megi Biomedical Technology Co., Ltd.). Upon completion of onboarding, LC-MS raw data were processed via Progenesis QI (Waters Corporation, Milford, USA), and Meggie's proprietary database of plant metabolites, MJDBPM, was aligned with MS and MS/MS mass spectrometry data.

Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were conducted utilizing the ropls package in R (Version 1.6.2). Metabolites with VIP> 1, $p < 0.05$ were considered significantly different metabolites. Furthermore, The KEGG database was used to annotate metabolic pathways for different metabolites (https: //www.kegg.jp/kegg/pathway.html)., and pathway enrichment analysis was performed employing the scipy.stats package in Python.

2.5. Soil chemical properties analysis

Soil and CO_2 - free water were mixed at a ratio of 1:2.5. After 30 min, the soil pH was measured using a pH meter (STARTER 300, Parsippany, USA). The total carbon (TC) and total nitrogen (TN) contents of the soil were determined using an elemental analyzer (FlashSmart CN/CNS, Thermo Fisher, USA). The total phosphorus (TP) content was determined by spectrophotometry. The concentration of potassium ions in the solution was measured using atomic absorption spectrophotometry and then converted to the total potassium (TK) content of the soil. The available nitrogen (AN) content was determined by the alkali - hydrolysis diffusion method. The available phosphorus (AP) content in the soil was determined by the molybdenum - antimony - resistance colorimetric method. The available potassium (AK) content in the soil was determined by atomic absorption flame spectrophotometry.

2.6. Determination of rhizosphere soil bacterial community

After being gathered, the rhizosphere soil samples were put into sterile centrifuge tubes, which were subsequently delivered to the lab in liquid nitrogen and kept at −80°C. The E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) was used to extract the total DNA from the microbial communities. Using the extracted DNA as a template, the barcoded primers 27 F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492 R (5′-RGYTACCTTGTTACGACTT-3′) were used to amplify the full-length 16S rRNA gene by PCR. Following 2 % agarose gel electrophoresis detection, magnetic beads were used to purify the products, and a Qubit 4.0 (Thermo Fisher Scientific, USA) was used for quantification. The RDP Classifier (http://rdp.cme.msu.edu/, version 2.11) was used to align with the Silva 16S rRNA gene database (v138) for OTU taxonomic annotation, with a confidence threshold of 70 %, and the community composition of each sample was statistically analyzed at different taxonomic levels. The Mothur program was employed to calculate alpha diversity measurements, specifically the Shannon and Chao indices. To evaluate the similarities in microbial community compositions among samples, we performed a principal coordinate analysis (PCoA) utilising the Bray-Curtis distance metric. Additionally, we performed a linear discriminant analysis (LEfSe) to pinpoint bacterial taxa with considerable abundance disparities between groups, with a threshold of LDA> 3.5 and a p-value below 0.05 for significance. Correlation network analysis was performed for the top 200 most abundant OTUs at the taxonomic level, using Pearson correlation coefficients with a threshold of $|r| > 0.5$ and $P < 0.05$.

2.7. Statistical analyses

One-way ANOVA was conducted utilising IBM SPSS Statistics 26. Multiple range tests were conducted utilising Duncan's test to evaluate disparities among treatments. Visualisation was conducted utilising Origin 2021. Based on the correlations among differential metabolites, rhizosphere bacterial communities, soil properties, and alfalfa physiological indicators, co-occurrence network analysis was performed using the Majorbio Cloud Platform (www.majorbio.com) and visualized using Gephi. The Mantel test was completed using the Chiplot online tool (https://www.chiplot.online/). The main predictors affecting alfalfa yield after overwintering were determined using the "randomForest" package in R (4.1.0).

3. Results and discussion

3.1. Physiological parameters of alfalfa roots

The integrity and restoration of cell membranes, the buildup and elimination of reactive oxygen species, and osmoregulation are intricately associated with low-temperature tolerance in plants (Zhang et al., 2021). Fig. 1 shows the changes in Osmotic regulation systems, Enzymatic antioxidant systems and Cell membrane lipid systems in alfalfa root system. Significant changes were observed in SP, SS and Pro content of alfalfa roots after application of wood vinegar and biochar ($P < 0.05$). The maximum SP level of alfalfa roots in group B attained 7.28 mg/g, representing an increase of 11.21 % compared to CK. The SS and Pro contents in the BW group were the highest, with increases of 51.54 % and 43.00 % compared to the CK group, respectively. In response to stress, plants store chemicals such as SP, SS, and Pro within their cells. These substances can maintain the concentration of the cell sap, reduce the osmotic potential, and are important osmotic regulatory solutes for enhancing plant stress tolerance (Delauney and Verma, 1993; Li et al., 2015). Yuan et al. also posited that organic compounds on biochar surfaces may infiltrate plants and engage with stress-related proteins, hence facilitating the synthesis of osmotic regulating solutes such as SS and Pro, which augment plant cold tolerance (Yuan et al., 2017), this resembles the findings of this investigation.

The application of biochar and wood vinegar did not significantly affect the activity of POD ($P > 0.05$), but significantly increased the activities of CAT and SOD by 162.91 %–235.16 % and 20.13 %–46.18 %, respectively, compared with the CK treatment group ($P < 0.05$). Under stress, plants generate reactive oxygen species (ROS) that oxidise membrane lipids, resulting in the formation of MDA. Furthermore, plants mitigate ROS and diminish cellular damage by enhancing the activity of antioxidant enzymes, including SOD, POD, and CAT (Shri et al., 2008; Xu et al., 2021). This also alleviated the damage to the cell membrane lipid system of alfalfa roots in the B, W, and BW groups during overwintering, which was primarily evidenced by significant reductions in MDA concentration and relative conductivity. The effect of the BW treatment group was the best, with MDA content and relative conductivity significantly reduced by 22.55 % and 16.56 % compared with CK. Rizwan et al. discovered that the use of 5 % biochar could decrease MDA concentration in rice under abiotic stress by 31 %, hydrogen peroxide levels by 40 %, and electrolyte leakage by 34 % (Rizwan et al., 2018). Moreover, water is a crucial element influencing the effectiveness of alfalfa overwintering. Water can protect cells from damage by changing root configuration and altering physiological and metabolic pathways, thereby enhancing the cold resistance of alfalfa (Susanne et al., 2003; Castonguay et al., 2006). Duan et al. posited that the distinctive physicochemical features of biochar augmented the hydrophilicity and water retention capacity of the soil. The augmentation

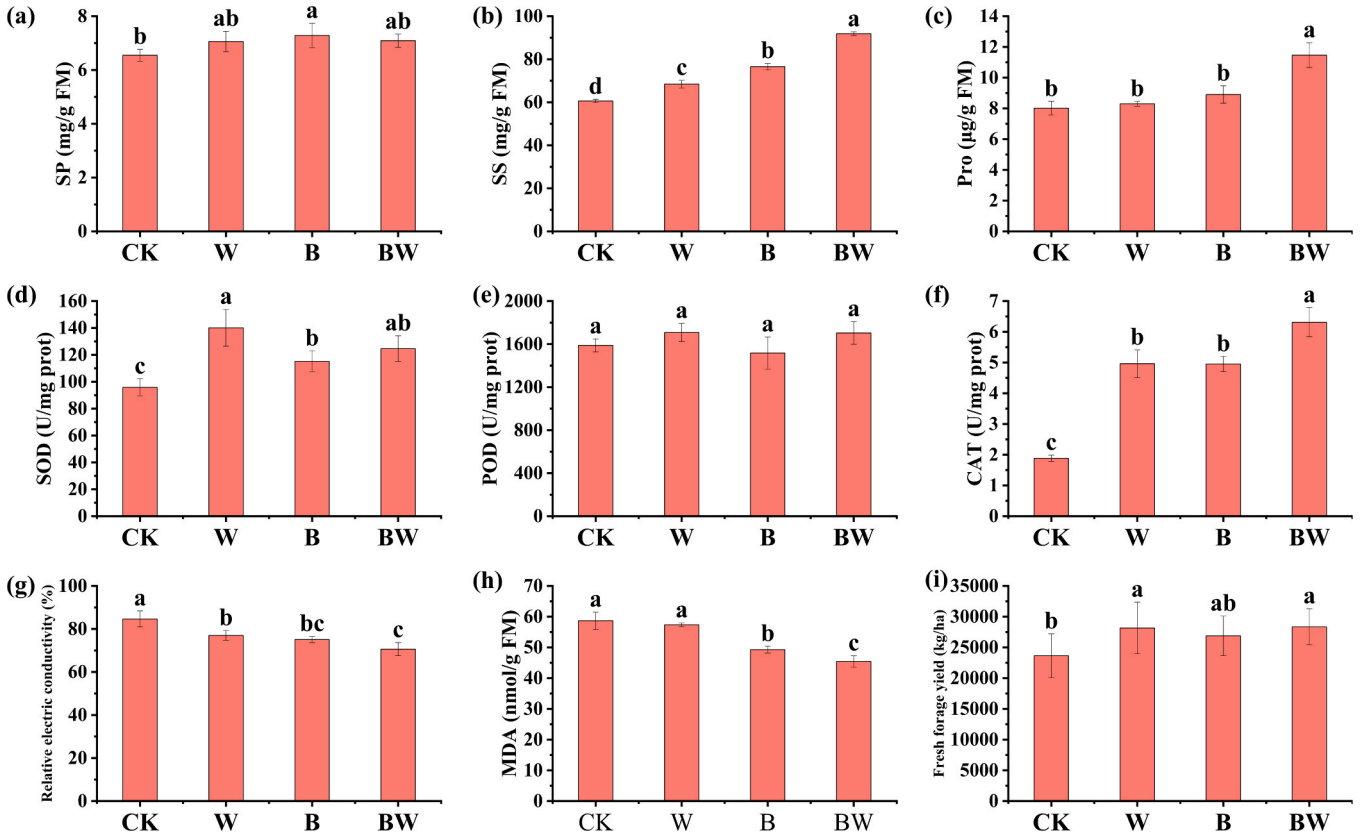

Fig. 1. The effects of biochar and wood vinegar on the contents of SP (a), SS (b), Pro (c), and the activities of SOD (d), POD (e), and CAT (f) in alfalfa roots, as well as relative conductivity (g), MDA content (h) and Fresh forage yield of the first cutting of alfalfa in the next year (i). Distinct letters signify substantial differences among treatment groups ($P < 0.05$).

of soil water content and the reduction of salt modified plant metabolites and mitigated the detrimental impacts of abiotic stress on crops (Duan et al., 2023). This may be a primary mechanism by which biochar enhances the cold tolerance of alfalfa in our study. The availability of phosphorus influences the concentrations of SS and SP in the root crown, which is crucial for enhancing the cold tolerance of alfalfa (Wang et al., 2023). Zhao et al. discovered that the introduction of wood vinegar and biochar to saline soils drastically modified the makeup of the soil inorganic phosphorus bacterial community, hence enhancing phosphorus efficacy (Zhao et al., 2024). Thus, the increase in phosphorus availability may be another crucial component enhancing the cold tolerance of alfalfa. Acids and phenolic compounds constitute the primary components of wood vinegar (Wu et al., 2015). The acids in wood vinegar can enhance the production of Pro and SS, diminish electrolyte permeability and MDA levels in crop under low-temperature stress, while the phenols can significantly elevate the activities of antioxidant enzymes such as CAT, POD and SOD (Chen, 2019). Zhu's research also found that the low-temperature tolerance of rapeseed was significantly enhanced after spraying wood vinegar, with the Pro content in leaves significantly increased by 103.98 %, SOD enzyme activity enhanced by 20.93 %, and MDA content reduced by 6.22 % (Zhu et al., 2021). Furthermore, the present study demonstrated that the amalgamation of biochar and wood vinegar exerted the most significant influence on augmenting SS and Pro content, as well as CAT activity, a finding that aligns with the observations reported by He et al. (2024).

The extent of damage caused by low-temperature stress to the alfalfa root system also significantly affects its productive performance in the following year (Fig. 1i). Measurements of fresh forage yield from the first cut after regrowth showed that the BW, W, and B treatments all yielded higher than the CK group. Among these, the BW treatment demonstrated the most pronounced improvement, with a fresh forage yield reaching 28,365 kg/ha, representing a significant increase of 19.91 % compared to CK ($p < 0.05$).

3.2. Metabolites of alfalfa roots

An untargeted metabolomics investigation was performed to analyze the alterations in metabolic levels of alfalfa roots subjected to various treatments. Among all the samples, the metabolites we identified were mainly categorized as Terpenoids, Lipids, Flavonoids, Amino acids and derivatives and Carbohydrates and derivatives, which accounted for 11.92 %, 9.31 %, 6.67 %, 4.39 % and 3.62 % of the metabolites, respectively (Fig. 2a). The PCA analysis (Fig. 2b) demonstrates the presence of four distinct groups, each exhibiting a significant separation. PC1 of the OPLA-DA study results indicates the extent of heterogeneity among each treatment group compared to the CK group. Fig. 2c-e illustrates that the most significant group disparities were evident in the BW group.

In the B_vs_CK comparison, a total of 880 differential metabolites were identified, with 441 upregulated metabolites primarily consisting of Ala-Trp and Val-Hyp, and 439 downregulated metabolites mainly including Plazomicin and Coumaroyl hexoside (Fig. 3a-b). In the W_vs_CK comparison, there were 896 differential metabolites, with 457 upregulated metabolites mainly consisting of Ala-Trp, and 439 downregulated metabolites primarily including Pavetannin B6 and Plazomicin (Fig. 3c-d). In the BW_vs_CK comparison, a total of 891 differential metabolites were detected, with 464 upregulated metabolites mainly consisting of Val-Hyp, 2-Butylisothiocyanate, and EPTC, and 427 downregulated metabolites primarily including Fulvic acid, Bissalicyl fumarate, and Plazomicin (Fig. 3e-f). Studies have shown that Ala-Trp has strong antioxidant activity, can regulate the intracellular content of MDA and the activity of CAT, and provides protection against cell toxicity induced by H_2O_2 (Xia et al., 2020).

3.3. KEGG enrichment analysis of DAMs

To enhance comprehension of metabolic level alterations, the pathway enrichment analysis of differentially expressed metabolites (DEMs) was conducted by mapping DEMs to the metabolic enrichment

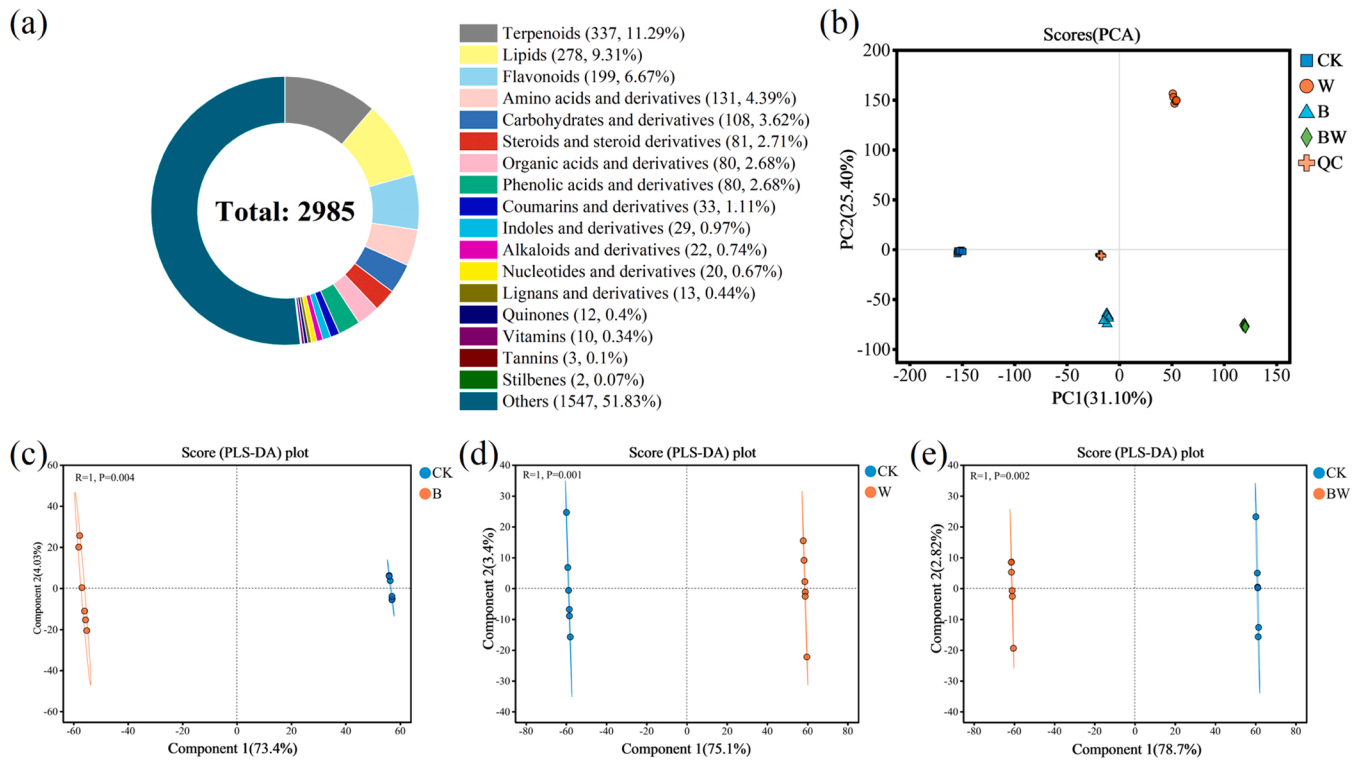

Fig. 2. Quantity of each metabolite (a). Principal Component Analysis (b). Partial least squares discriminant analysis was performed for the B (c), W (d), and BW (e) treatment groups compared to CK.

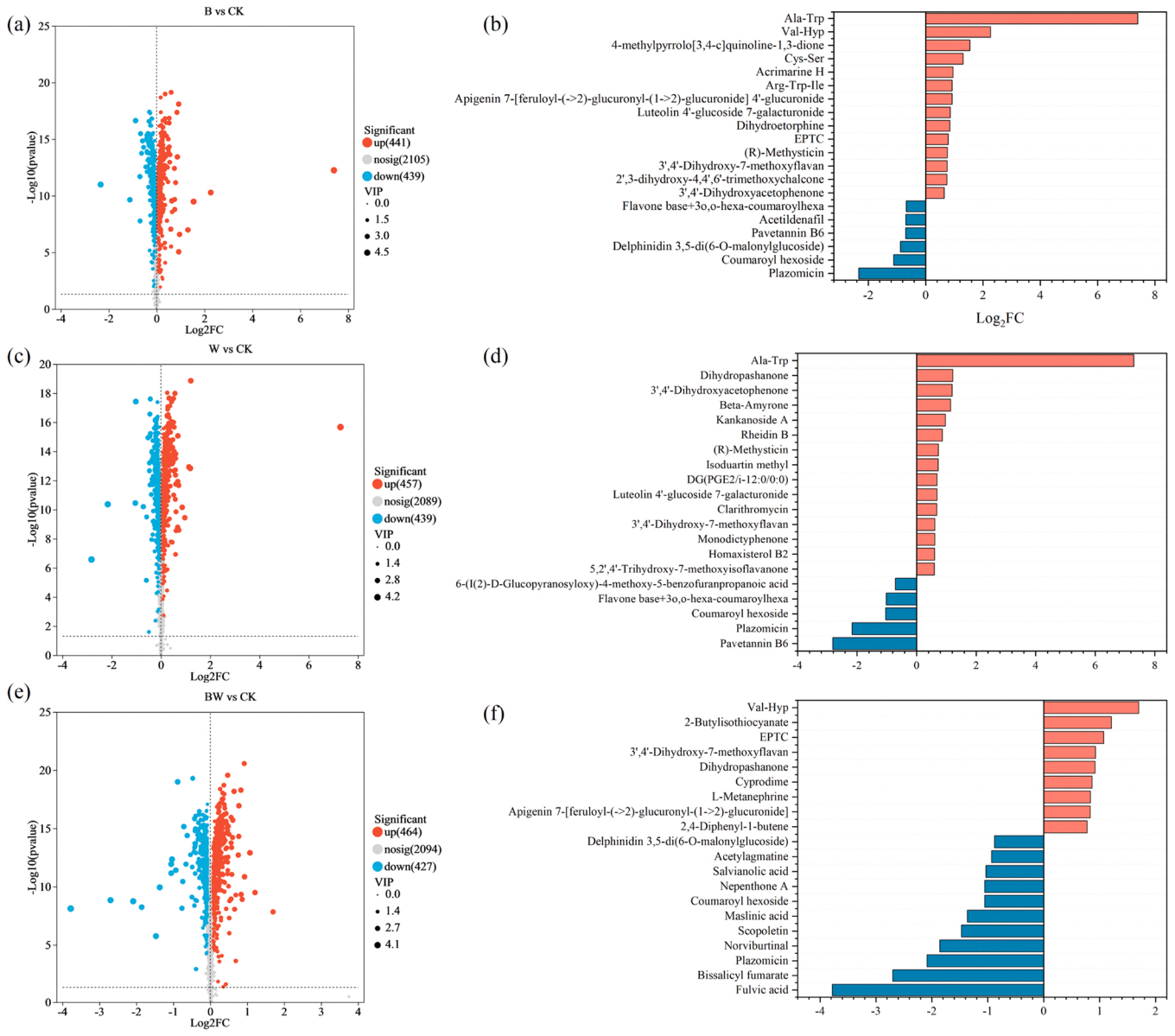

Fig. 3. Volcano plots of differential metabolites between the treatment and control groups, as well as the upregulation and downregulation of the top 20 metabolites in each comparison group (red indicates upregulation, blue indicates downregulation). a, b represent B vs CK; c, d represent W vs CK; e, f represent BW vs CK.

pathways in the KEGG database (Fig. 4). Each group's distinct metabolites were combined to create a variety of metabolic pathways. The bubble map was generated from the top 20 pathways based on significance scores, with each bubble representing a distinct KEGG pathway. In the B_vs_CK comparison, the metabolic pathways of Isoflavonoid biosynthesis, Flavonoid biosynthesis, Valine, leucine and isoleucine biosynthesis, and Biosynthesis of various plant secondary metabolites were significantly enriched (Fig. 4a, b). In the W_vs_CK comparison, the pathways of Isoflavonoid biosynthesis, Flavonoid biosynthesis, Flavone and flavonol biosynthesis, and Biosynthesis of various plant secondary metabolites were significantly enriched (Fig. 4b, c). In the BW_vs_CK comparison, the pathways of Isoflavonoid biosynthesis, Flavonoid biosynthesis, Flavone and flavonol biosynthesis, and Steroid biosynthesis were significantly enriched (Fig. 4d, e). Notably, the KEGG pathway differential abundance scores for the three comparisons indicated a trend of up-regulation in all three pathways: isoflavone biosynthesis, flavonoid biosynthesis, and flavonoid and flavonol biosynthesis. Sardar et al. discovered that the incorporation of biochar markedly enhanced the total phenolic content (49.4 %), flavonoid content (40.2 %), and antioxidant activity (52.3 %) in tomato plants (Sardar et al., 2025). Zhang's research also indicated that under drought stress, the treatment group with wood vinegar application, compared to CK, had metabolic pathways enriched in flavonoid and flavonol biosynthesis and phenylpropanoid biosynthesis, thereby effectively mitigating the detrimental effects of abiotic stress on crops compared to the control group (Zhang, 2024). Extensive research has shown that flavonoids, encompassing flavonols, flavones, and isoflavones, augment antioxidant activity to eradicate or alleviate damage induced by reactive ROS accumulation resulting from detrimental factors such as cold, drought, and heavy metal ions, thereby serving a vital regulatory function in plant growth and development to safeguard plants from or lessen various biotic and abiotic stresses (Nakayama et al., 2019; Peng et al., 2019; Zhang et al., 2022b). Therefore, we assert that wood vinegar and biochar primarily mitigate the adverse effects of low-temperature stress on alfalfa by enhancing the production of flavonoids, thereby improving the production performance of alfalfa after overwintering. This finding is consistent with previous research. Liu et al. found that under combined low-temperature and saline-alkali stress, cold-tolerant

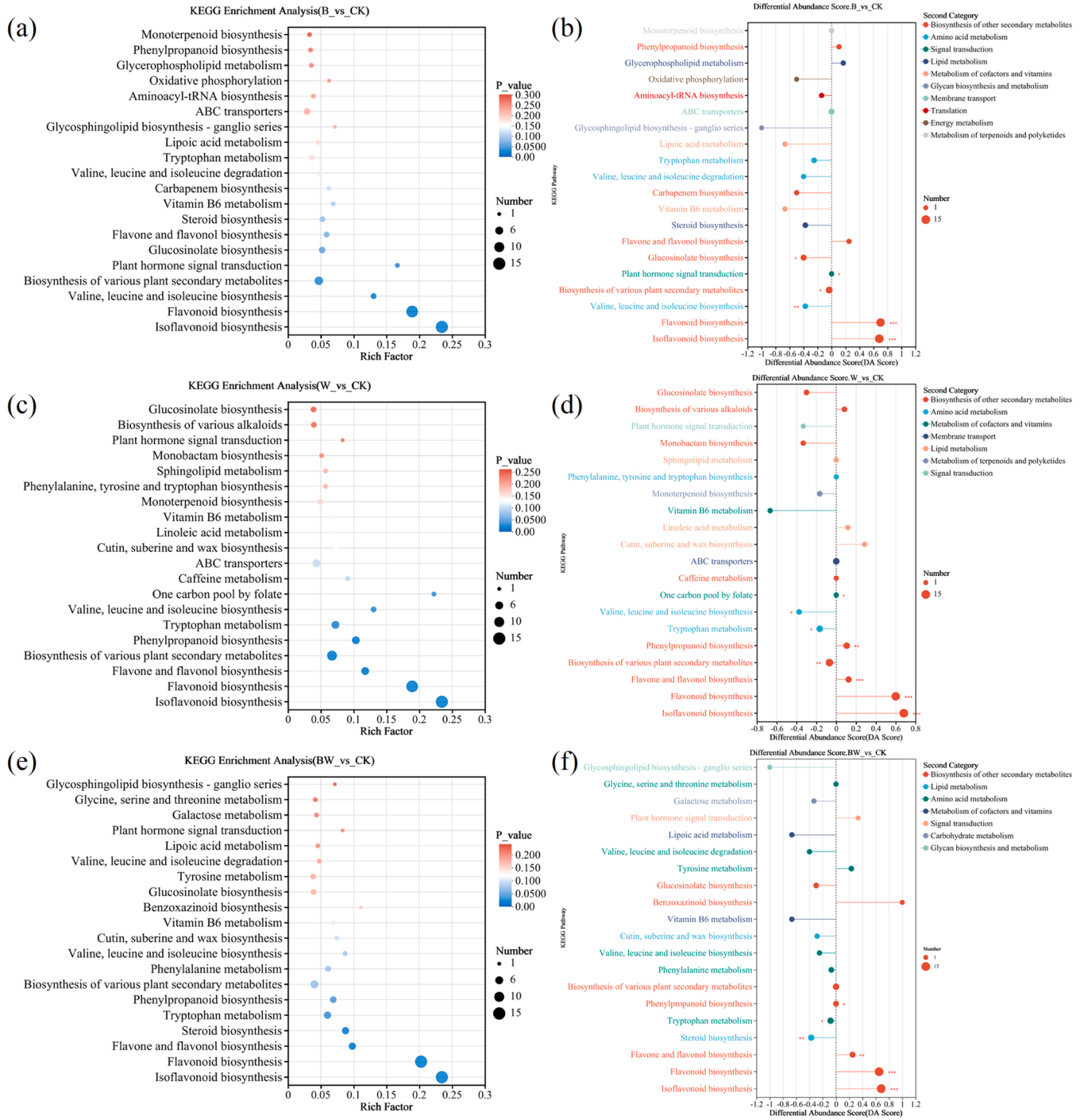

Fig. 4. KEGG enrichment analysis and KEGG pathway differential abundance scores. a, b represent B vs CK; c, d represent W vs CK; e, f represent BW vs CK. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

alfalfa varieties exhibited significantly higher expression of genes in the flavonoid biosynthesis pathway and flavonoid content, which enhanced flavonol accumulation and antioxidant capacity. This, in turn, increased the tolerance of alfalfa to combined stress and the biomass of alfalfa after stress (Liu et al., 2024).

Fig. 5 illustrates the specific effects of wood vinegar and biochar application on the metabolic processes of Isoflavonoid biosynthesis and Flavonoid biosynthesis in alfalfa roots. In the metabolic pathway of Isoflavonoid biosynthesis, compared with CK, 15 metabolites, including Pisatin, Pseudobaptigenin, Glycitein, Inermin, Malonylglycitin, and Vestitone, were upregulated in the B, W, and BW treatment groups. The increase in Pisatin levels was particularly significant in the B-CK and BW-CK comparisons, indicating that biochar has a more pronounced effect on promoting the synthesis of Pisatin in alfalfa. Fan's study also found that the metabolic level of Pisatin in alfalfa is significantly upregulated under abiotic stress (Fan, 2024). Pisatin has been proven to be an important compound involved in plant defense against pathogen infection (Morkunas et al., 2024), but its function in plant responses to abiotic stress necessitates additional comprehensive investigation. Compared with biochar, wood vinegar application more effectively promotes the synthesis of Daidzein in alfalfa roots. The expression level of Pseudobaptigenin in BW was the most significantly elevated

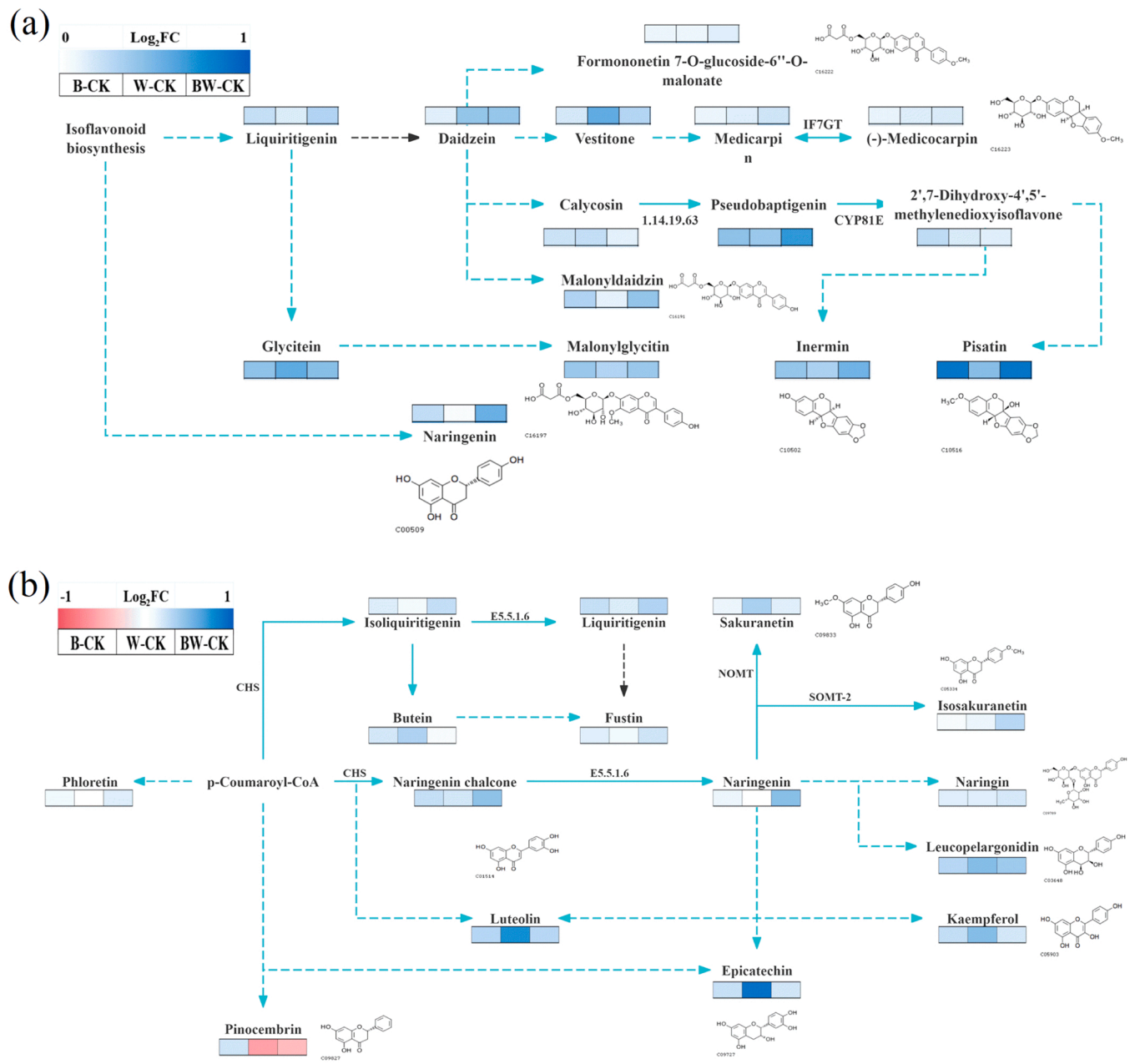

Fig. 5. Changes in the metabolic pathways of Isoflavonoid biosynthesis (a) and Flavonoid biosynthesis (b) among different treatment groups. Both upward and downward adjustments are based on CK as a reference.

compared to CK, indicating that the combined application of biochar and wood vinegar has a better effect on the synthesis of this substance (Fig. 5a).

In the metabolic pathway of Flavonoid biosynthesis, compared with CK, 14 metabolites, including Epicatechin, Fustin, Isoliquiritigenin, Leucopelargonidin, Luteolin, Naringenin, and Naringenin chalcone, were upregulated in the B, W, and BW treatment groups, while Pinocembrin was downregulated in the W and BW treatment groups. The upregulation of Epicatechin and Luteolin was the most significant in the W-CK comparison, suggesting that the usage of wood vinegar alone may be more conducive to the synthesis of these two substances in alfalfa roots. It is noteworthy that the application of wood vinegar may inhibit the synthesis of Pinocembrin in alfalfa roots, as evidenced by the significant downregulation of Pinocembrin in both W-CK and BW-CK (Fig. 5b).

3.4. Rhizosphere soil chemical properties

Fig. 6 shows the effects of biochar and wood vinegar on the chemical properties of the rhizosphere soil of alfalfa. The addition of biochar and wood vinegar did not significantly change the pH value of the rhizosphere soil (Fig. 6a), but significantly increased the contents of TC, TN, TP, and TK in the soil (Fig. 6b-e). Compared with CK, the contents of TC, TN, TP, and TK in BW increased by 78.43 %, 91.94 %, 63.57 %, and 47.32 %, respectively ($p < 0.05$). In terms of nutrient availability, the availability of nitrogen, phosphorus, and potassium also changed significantly after the addition of biochar and wood vinegar (Fig. 6f-h). BW had the highest contents of AN, AP, and AK, reaching 112.07 mg/kg, 33.94 mg/kg, and 112.63 mg/kg, respectively, with significant differences compared to CK ($p < 0.05$). Soil nutrients, as the material basis for plant growth and development, directly affect the physiological metabolism and stress resistance of plants (Xu et al., 2025; Gloria et al.,

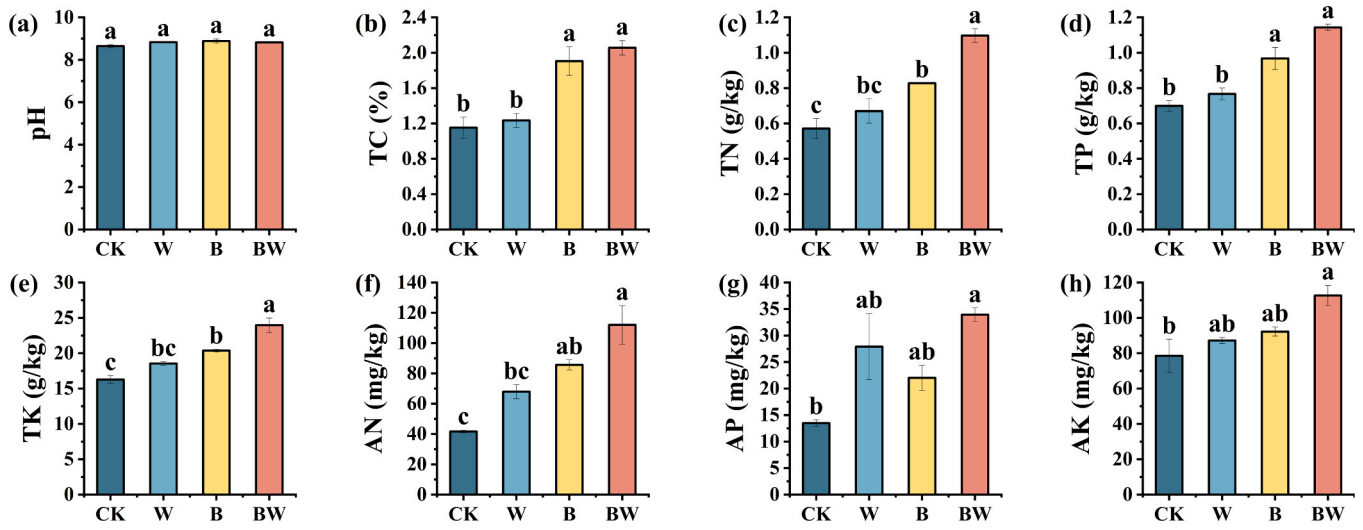

Fig. 6. The effects of biochar and wood vinegar on rhizosphere soil chemical properties. a, pH value; b, total carbon (TC); c, total nitrogen (TN); d, total phosphorus (TP); e, total potassium (TK); f, available nitrogen (AN); g, available phosphorus (AP); h, available potassium (AK).

2020). In this study, the contents and availability of nutrients in the rhizosphere soil of alfalfa significantly increased after the application of biochar and wood vinegar, which is similar to the results of many previous studies (Zhou et al., 2023; Zhao et al., 2024; Yan et al., 2024). The porous structure of biochar provides abundant adsorption sites for nutrients, and the functional groups on the surface of biochar can further enhance adsorption performance through mechanisms such as hydrogen bonding, electrostatic attraction, and complexation, effectively preventing the loss of nitrogen, phosphorus, potassium, and other nutrients in the soil (Lu et al., 2024; Zhao et al., 2023). In addition, biochar and wood vinegar can enhance the bioavailability of phosphorus by changing the community composition of inorganic phosphorus bacteria in saline-alkali soils (Zhao et al., 2024). The significant improvement in soil nutrient conditions is also an important reason why biochar and wood vinegar can enhance the cold resistance of alfalfa.

3.5. Rhizosphere bacterial communities of alfalfa roots

A total of 1057,647 optimized sequences were obtained from the samples. The diversity and species composition of the alfalfa rhizosphere bacterial community are illustrated in Fig. 7. The Shannon diversity index and the Simpson index were employed to assess the diversity of the bacterial community (Fig. 7a, b). The Chao index was employed to assess the bacterial richness (Fig. 7c). The results indicated that the Shannon index did not undergo significant changes following the addition of biochar and wood vinegar. However, the Simpson index significantly decreased, with the B treatment group exhibiting the smallest Simpson index. The Chao index was diminished in the B, W, and BW treatment groups relative to CK, with a significant difference observed between the BW treatment group and CK ($P < 0.05$). This indicates that the incorporation of biochar and wood vinegar resulted in a decrease in the richness of the rhizosphere bacterial community while enhancing its diversity. This aligns with the findings of Zheng et al. in *Brassica chinensis* L. (Zheng et al., 2021). The dissimilarity in microbial communities among samples was determined using the beta diversity metric. In the principal coordinate analysis (PCoA), samples from each treatment group were distinctly separated from those of CK, particularly the B and BW treatment groups. (Fig. 7e). The results of α and β diversity analyses indicate that, compared with CK, the rhizosphere bacterial communities in B, W, and BW underwent significant changes.

The Upset plot revealed that CK had the highest number of OTUs (3899), followed by W (3842), B (3829), and BW (3751). CK, B, W, and BW had 123, 118, 117, and 87 unique OTUs, respectively (Fig. 7d). Pseudomonadota and Acidobacteriota were the predominant phyla in all samples, exhibiting relative abundances between 21.72 % and 27.23 % and 16.88 % and 23.31 %, respectively (Fig. 7f). At the genus level, *Aridibacter* and *norank_p_Acidobacteriota* exhibited the largest relative abundances, comprising 5.07 %–8.02 % and 4.88 %–6.29 %, respectively (Fig. 7g). Linear discriminant analysis identified a total of 51 biomarkers, with the BW treatment group having the most, at 24 (Fig. 8a, b). The phyla Planctomycetota, Bacteroidota, Actinomycetota, and Acidobacteriota were significantly enriched in CK, B, W, and BW, respectively. The ecological functions of the phylum Actinomycetota include carbon fixation, nitrogen fixation and phosphate solubilisation. In addition, it participates in nutrient mineralisation and accelerates the decomposition of soil organic matter (Chen et al., 2019). The phylum Acidobacteriota performs a pivotal function in the cycling of nutrients within terrestrial ecosystems (Munk et al., 2021). The abundance of these phyla is closely related to soil nutrient status (Li et al., 2025). The phylum Bacteroidota is associated with plant stress resistance. Research indicates that it can trigger stomatal closure in plants, enhance the expression of stress-responsive genes, and facilitate lateral root formation, allowing plants to more efficiently acquire water and nutrients, thus mitigating the detrimental impacts of abiotic stress (Pan et al., 2023). Some members of the phylum Bacteroidota can also produce plant hormones such as gibberellins, cytokinins, and auxins, thereby regulating plant root structure under abiotic stress (Maheswari et al., 2013). Therefore, we propose that the addition of biochar and wood vinegar may enhance stress resistance by altering the bacterial community in the alfalfa rhizosphere, promoting soil nutrient cycling, and stimulating root growth and development.

3.6. Co-occurrence network analysis

Co-occurrence network analysis was conducted on the top 200 operational taxonomic units (OTUs) of each treatment group to examine the impact of wood vinegar and biochar on the network structure of the rhizosphere bacterial community associated with alfalfa (Fig. 9). Compared with CK, the networks of the B, W, and BW treatment groups had more edges, greater average degree, and shorter average path length. This indicates that the rhizosphere bacterial communities of alfalfa in B, W, and BW formed more connected and more complex network structures (Jiao et al., 2021). Our prior study indicates that the incorporation of biochar can enhance the complexity and stability of bacterial communities in the alfalfa rhizosphere. Li et al. discovered that during winter's low-temperature stress, the bacterial communities in the

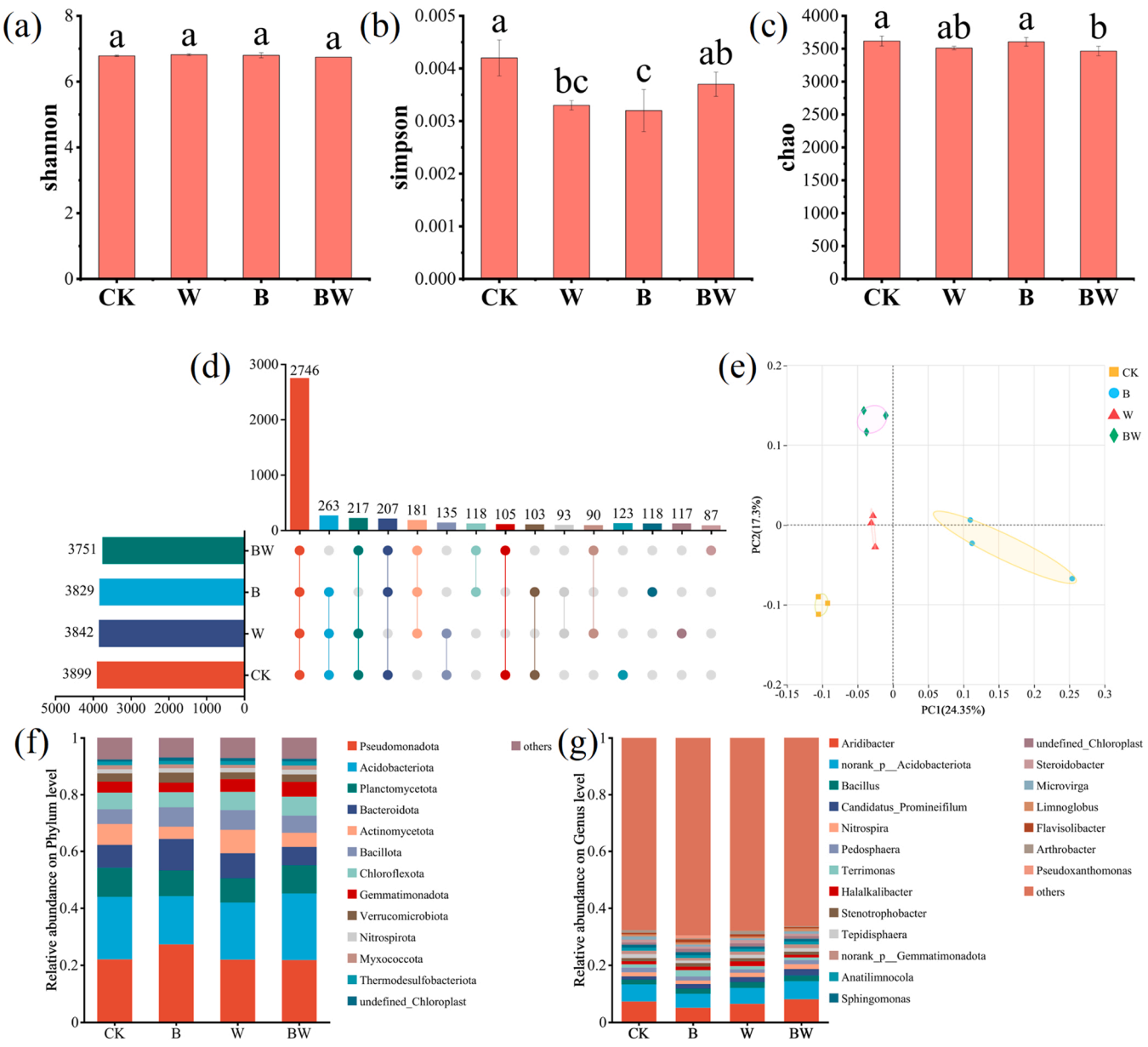

Fig. 7. The impact of wood vinegar and biochar on the alpha diversity of the rhizosphere bacterial community of alfalfa (a, Shannon; b, Simpson; c, Chao); an Upset diagram illustrating shared and unique OTUs (d); principal coordinate analysis (e); bacterial composition at the phylum (f) and genus (g) levels. Distinct letters denote substantial differences among treatment groups ($P < 0.05$).

rhizosphere of cold-tolerant varieties exhibited greater complexity and stability compared to those of non-cold-tolerant kinds (Li et al., 2024). The increased complexity of the rhizosphere bacterial community structure may indirectly enhance the cold tolerance of alfalfa, but the specific mechanisms underlying this effect require further investigation.

3.7. Relationship between alfalfa yield, cold resistance, metabolites, and the rhizosphere microenvironment

We employed Mantel tests to determine correlations among key factors influencing alfalfa cold tolerance and yield (Fig. 10a). Correlation analysis revealed that SOD and CAT activities, SS content in roots, along with TN, TP, TK, AN, AP, and AK concentrations in rhizosphere soil, as well as pH levels, all showed significant positive correlations ($p < 0.05$) with yield of alfalfa after overwintering. Mantel analysis revealed significant positive correlations ($p < 0.01$) between alfalfa yield and two metabolic pathways: isoflavone and flavonoid biosynthesis in alfalfa roots. Furthermore, isoflavone and flavonoid biosynthesis significantly enhanced antioxidant enzyme activity, with correlation coefficients (r) of 0.92 and 0.82 for CAT activity, respectively. Sun et al. also observed that kiwifruit specifically upregulates the flavonoid metabolic pathway under low temperatures to enhance ROS scavenging capacity (Sun et al., 2021). Similarly, soybeans regulate ROS levels by activating antioxidant enzymes through isoflavones, with key enzymes like GmIMaT1 and GmIMaT3 providing cold protection by modulating isoflavone glucoside levels (Tan et al., 2024; Ahmad et al., 2017). Furthermore, flavonoid and isoflavone biosynthesis showed significant positive correlations ($p < 0.05$) with soil TC, TN, TP, TK, AN, AP, and AK content. This aligns with findings by Duan et al., indicating that flavonoid compound content in plants increases with enhanced soil fertility (Duan et al., 2024). The composition and diversity of rhizosphere bacteria showed significant correlations with soil TP, TK, AN, and AP content. This suggests that biochar and wood vinegar may primarily alter rhizosphere bacterial communities by enhancing soil nutrient

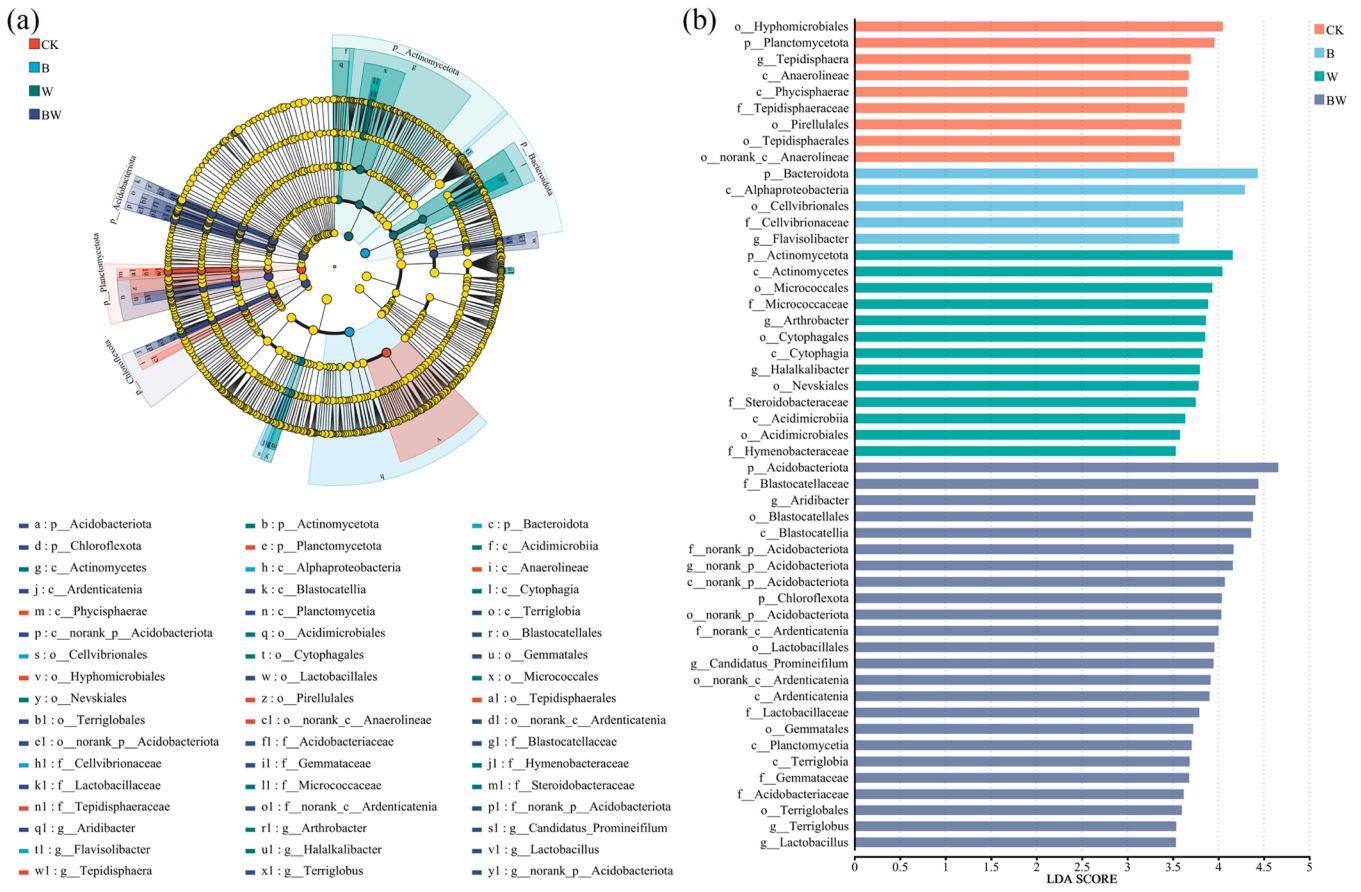

Fig. 8. Results of LEfSe (a) and LDA (b) analyses of the rhizosphere bacterial community of alfalfa.

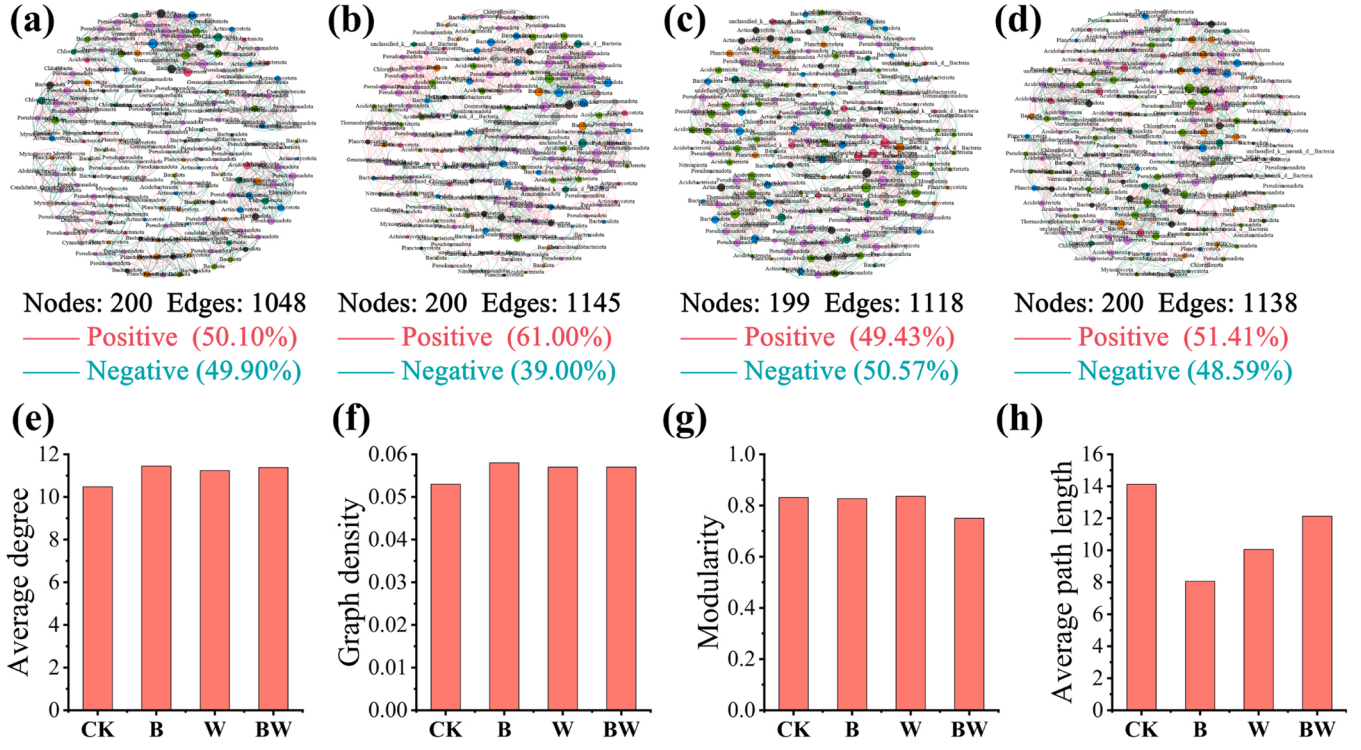

Fig. 9. Co-occurrence networks of the CK (a), B (b), W (c), and BW (d) treatment groups, as well as the main characteristics of the networks (e, Average degree; f, Graph density; g, Modularity; h, Average path length).

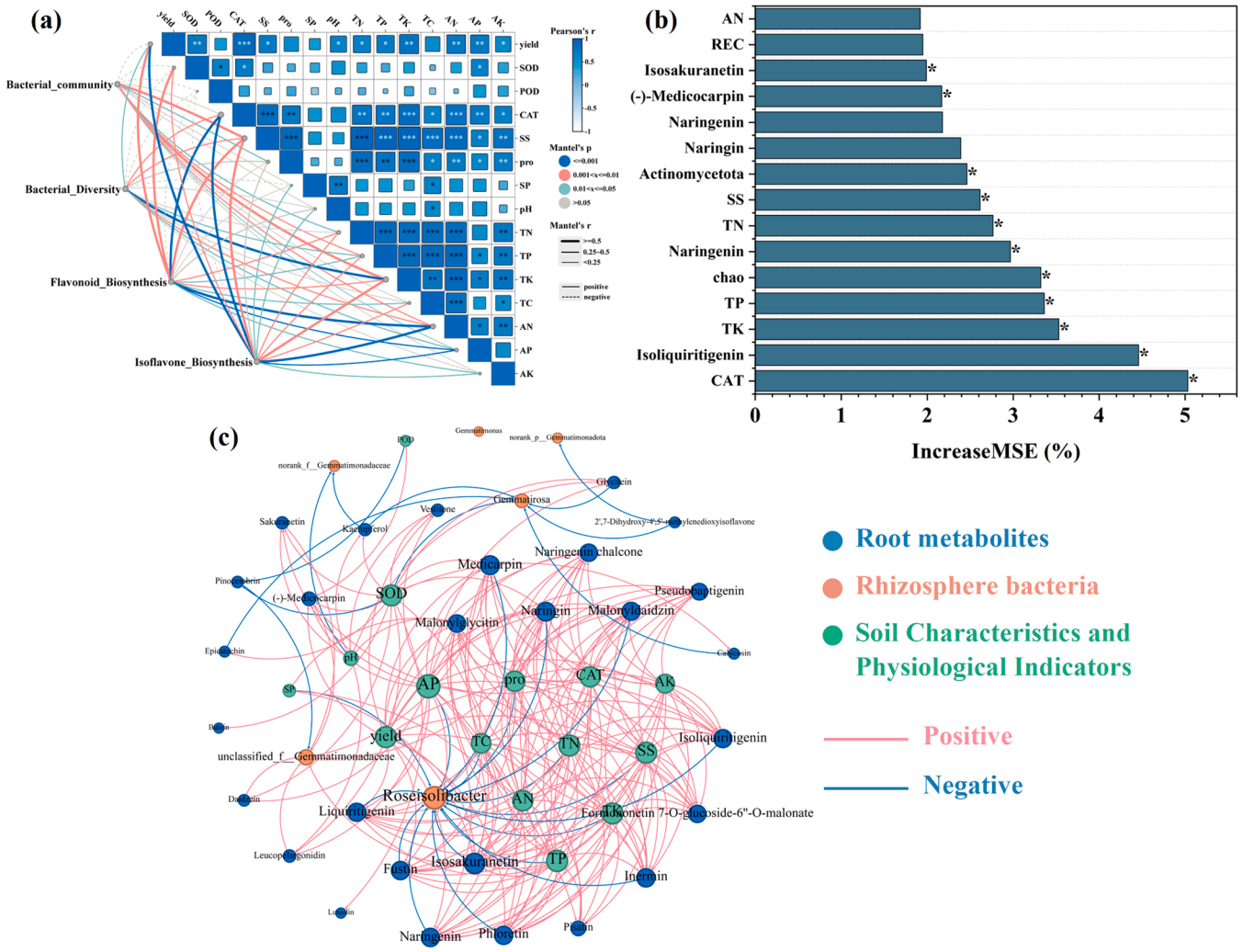

Fig. 10. Using the Mantel test to calculate the relationships between rhizobacteria, key differential metabolites, alfalfa yield, cold resistance, and rhizosphere soil properties (a). Evaluating the random forest prediction model for the contribution of each factor to alfalfa yield after overwintering (b).Co-occurrence network analysis of metabolites, rhizobacteria, soil properties, and alfalfa physiological indicators.Links indicate correlations among nodes ($|r| > 0.6$, $p < 0.05$) (c). Red lines and blue lines represent positive correlation and negative correlation, respectively. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

content and availability, particularly for N, P, and K. Random forest model results indicate that CAT activity, Isoliquiritigenin expression, TK content, TP content, and the Chao index are the primary factors influencing alfalfa yield after overwintering (Fig. 10c). Fig. 10c displays correlations among soil properties, alfalfa physiological indicators, key differential metabolites in the isoflavone and flavonoid biosynthetic pathways within roots, and the top 50 OTUs by relative abundance. The network comprises 49 nodes and 225 edges, with positive and negative correlations accounting for 86.67 % and 13.33 % of edges, respectively. Notably, most negative edges point toward rhizosphere bacteria. Among these, Roseisolibacter exhibited negative correlations with 10 key isoflavone and flavonoid biosynthetic products, including Isosakuranetin, Naringin, Isoliquiritigenin, and Medicarpin. Sixteen differentially expressed metabolites, including formononetin 7-O-glucoside-6''-O--malonate, isoliquiritigenin, and naringenin chalcone, showed significant positive correlations with alfalfa yield ($p < 0.05$).

4. Conclusion

This study investigated the effects of biochar and wood vinegar application on alfalfa cold tolerance and their underlying mechanisms. Biochar and wood vinegar application significantly increased SP, SS, and Pro content, as well as SOD and CAT activity, while reducing relative electrical conductivity and MDA content. Among these treatments, BW demonstrated the most pronounced effects. Compared to the control (CK), BW increased SS and Pro content by 51.54 % and 43.00 %, respectively, elevated CAT activity by 235.16 %, and reduced relative electrical conductivity and MDA content by 16.56 % and 22.55 %. The upregulation of processes such as isoflavonoid biosynthesis and flavonoid biosynthesis in alfalfa roots likely constitutes the primary mechanism enhancing cold tolerance. Analysis of the rhizosphere microenvironment revealed that biochar and wood vinegar primarily enhance cold tolerance by increasing soil nutrient content and availability, including total carbon (TC), total nitrogen (TN), total phosphorus (TP), and total potassium (TK). Concurrently, changes in the rhizosphere bacterial community occurred. The enrichment of bacteria such as Bacteroidetes, Actinomycetota, and Acidobacteria, along with increased community complexity, directly or indirectly mitigated the adverse effects of freezing damage on alfalfa. In summary, applying 10 t/ha of biochar combined with wood vinegar diluted 200 times is a suitable method for alfalfa production, contributing to sustainable agriculture.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.122388.

Data Availability

Data will be made available on request.

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