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Uptake, metabolism, and underlying mechanisms of neonicotinoid insecticides in rice plants (*Oryza sativa* L.)

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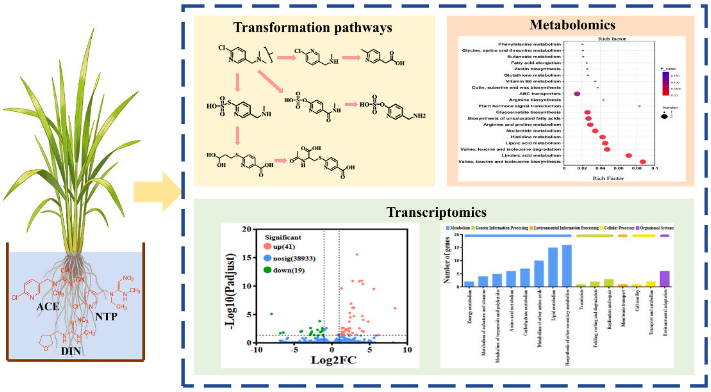
HIGHLIGHTS

- DIN and ACE exhibited higher upward mobility than NTP from roots to shoots.
- Antioxidant defense mechanism of rice was activated by exposing to NEOs.
- Seventeen novel transformation products of NEOs were identified in rice by HRMS.
- NEO exposure triggered systemic metabolic reprogramming in rice.
- NEOs altered the expression of genes involved in anabolism and signal transduction.

GRAPHICAL ABSTRACT



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ABSTRACT

Neonicotinoid insecticides (NEOs) are widely applied in agriculture but pose emerging ecological risks. Here, we investigated the uptake, translocation, and biotransformation of three representative NEOs in rice, including nitenpyram (NTP), acetamiprid (ACE), and dinotefuran (DIN). Results showed that NEOs were efficiently taken up and preferentially translocated to aboveground tissues, with ACE reaching the highest concentration at 669.1 mg/kg (dw) in shoots. Multiple transformation products were identified, resulting from hydroxylation, sulfonation, cyanation, dechlorination, oxidation, and bond cleavage reactions. NEO exposure significantly altered mineral nutrient homeostasis and induced phytotoxic effects, as evidenced by characterized by elevated oxidative stress and disrupted antioxidant enzyme activities. Non-targeted metabolomics revealed substantial perturbations in flavonoids, amino acids, and lipids. In parallel, transcriptomic analysis indicated that differentially expressed genes were primarily enriched in pathways related to secondary metabolite biosynthesis, as well as lipid, amino acid, and carbohydrate metabolism. Together, these findings provide mechanistic insight

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into the behavior and toxicity of NEOs in crop plants and advance our understanding of their environmental fate in agricultural systems.

1. Introduction

Neonicotinoid insecticides (NEOs) are among the most extensively used insecticides in modern agriculture, but their widespread application has drawn increasing attention due to their persistence, bioaccumulation potential, and toxicity [1]. To date, NEOs have been detected ubiquitously in diverse environmental matrices, including water, sediment, soil, and dust [2,3]. Furthermore, substantial evidence indicates that NEOs can accumulate in living organisms and exert endocrine-disrupting, reproductive, and neurotoxic effects [4]. Consequently, long-term exposure to NEOs poses potential risks to both ecosystems and human health. Rice (*Oryza sativa* L.) is a staple food for nearly half of the global population, making the protection of rice yield and food safety particularly critical [5]. Rice cultivation is characterized by prolonged flooding, which facilitates the accumulation of pesticide residues in paddy water and enhances their subsequent transfer into rice plants [6]. Under these conditions, understanding the uptake, translocation, metabolism, and accumulation of NEOs in rice is essential for evaluating their environmental fate and food safety risks.

Insecticide exposure can disrupt multiple physiological processes in non-target crops, including photosynthesis, transpiration, and cellular metabolism, thereby impairing plant growth and development [7,8]. In response to xenobiotic stress, plants activate complex defense mechanisms involving transcriptional regulation, metabolic reprogramming, and oxidative stress [9]. Oxidative stress represents a central physiological response to chemical exposure and is characterized by the excessive accumulation of reactive oxygen species (ROS) [10]. To counter this, plant cells mobilize their endogenous ROS scavenging systems to maintain cellular homeostasis. A key component of this system is the antioxidant enzyme network, which mainly includes peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) [11]. These antioxidant components effectively remove excess ROS through synergistic action and play an important role in regulating redox homeostasis and maintaining normal physiological activities of cells [12]. Studies have shown that higher doses of NEOs trigger a gradual decrease in biological performance, impair physiological and enzymatic activities, and even induce cellular death via oxidative stress in chickpeas [13]. However, few studies have considered the enzymatic reactions involved in neonicotinoid metabolism in rice.

The application of metabolomics and transcriptomics technology has facilitated the study of the response mechanism of plants to insecticide stress, including *Brassica rapa* L. [14], *Oryza sativa* L. [9], and *Lactuca sativa* [15]. Studies have shown that insecticides enter plant cells via the transmembrane symplastic pathway and accumulate mainly in the cytosol [14]. In response, plants upregulate the expression of genes encoding cytochrome P450 enzymes, POD, and glutathione S-transferases [16]. Furthermore, insecticide dramatically affected six amino acid metabolism pathways in *Oryza sativa* L., with key differentially expressed genes (DEGs) mainly enriched in aspartate and glutamate metabolism, leading to increased free amino acid content and degradation of soluble proteins [17]. Collectively, these studies provide a foundation for understanding the molecular mechanisms underlying plant responses to insecticides. However, detailed information on the molecular response of plants to NEOs remains limited.

Nitenpyram (NTP), acetamiprid (ACE), and dinotefuran (DIN) are the most widely used NEOs in agricultural production and exhibit substantial structural differences. In this study, these three compounds were selected as representative NEOs to systematically investigate their uptake, translocation, and accumulation in rice tissues. We further evaluated their effects on rice growth, mineral nutrient homeostasis, and antioxidant defense systems. In addition, NEO metabolites were identified and potential metabolic pathways in rice were proposed. Integrated metabolomics and transcriptomic analyses were employed to elucidate the underlying molecular regulatory mechanisms induced by NEOs. As such, this study provides critical mechanistic insight into the behavior and biological effects of NEOs in rice and offers a scientific basis for assessing their environmental risks and supporting sustainable pesticide management in agricultural systems.

2. Materials and methods

2.1. Chemicals and reagents

NTP (99%, CAS No.150824-47-8), ACE (99%, CAS No.135410-20-7), and DIN (99%, CAS No.165252-70-0) were obtained from ANPEL Co., Ltd. (Shanghai, China). Detailed information on the three NEOs is presented in Supporting information Table S1. Three insecticides were dissolved in deionized water to prepare the stock solution. All other solvents were of analytical or HPLC grade.

2.2. Rice cultivation and NEOs exposure

Seeds of Qingxiangyou-19 rice (*Oryza sativa* L.) were supplied by Guangdong Delicious Seed Stock Co., LTD. The plump rice seeds were disinfected with 3% H_2O_2 for 30 min, washed three times with deionized water, and then soaked for 48 h at 25°C in darkness, with the water renewed every 24 h. Rice seedlings were cultured in a greenhouse (25°C, 250 μmol photons/(m^2·s), 14 h light/10 h dark, 70% humidity). Exposure experiments were conducted in a sterile glass reactor containing five rice seedlings and 30 mL exposure solutions with the selected NEO concentration of 10 mg/L. Although this concentration exceeds typical environmental levels, it was necessary to facilitate the identification of biotransformation products. Detailed exposure procedures are provided in Supporting information Text S1. After 10 days of exposure, rice seedlings were harvested for further analysis.

2.3. Analysis of NEOs and rice morphology

Residual concentration of NTP, ACE, and DIN in rice tissues were determined following previously reported methods with minor modifications [9]. Degradation products of the three NEOs were identified using high-resolution mass spectrometry (QE-MS, ThermoFisher, Ltd.). Analyses were performed in positive electrospray ionization mode over a full-scan mass range of 50–750 *m/z*. Chromatographic separation was achieved on an Acquity BEH C18 column (50:50 methanol/5 mM ammonium acetate) at 30 °C, with a flow rate of 0.2 mL/min. Data were acquired using Orbitrap MS/MS and processed with Compound Discoverer software. The MS/MS spectra were subsequently analyzed using Mass Frontier 8.1 software. Detailed analytical methods for quantifying NEO concentrations and assessing rice morphology are provided in Supporting information Text S2.

2.4. Determination of mineral elements

After freeze-drying the rice seedling samples for 48 h to a constant weight, 0.2000 g of dry powder was accurately weighed into a digestion tank. Then, 5 mL of concentrated nitric acid was added, and the mixture was pre-digested on a 100°C controlled electric heating plate for 30 min. The digestion tank was then sealed and transferred to the microwave digestion instrument, where digestion was carried out using a ramped temperature program. Detailed procedures for mineral element analysis are provided in Supporting information Text S3.

2.5. Assay for oxidative damage and antioxidant enzyme activities

The activities of antioxidant enzymes, including SOD, CAT, and POD, as well as ROS and malondialdehyde (MDA) levels, were measured using the plant enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, China). A more complete description is available in Supporting Information Text S4.

2.6. Metabolomics analysis

Plant samples (100 mg) were flash-frozen in liquid nitrogen and ground with pre-cooled extraction solvent. The resulting mixture was kept on ice for 5 min before centrifugation at 4℃. To monitor analytical variability, a quality control (QC) sample was generated by combining equal volumes from all test samples. This pooled QC sample was used to assess and reduce potential errors introduced during sampling and analysis. Background interference was accounted for by including a blank sample. Chromatographic separation was performed on a BEH C18 column (100 × 2.1 mm, 1.7 µm, Thermo Fisher, Germany) using a UHPLC-Q Exactive System. The metabolomic data reported in this study have been deposited in the Genome Sequence Archive (accession: PRJCA059732). Further methodological details are available in Supporting Information Text S5.

2.7. Transcriptomic analysis

Total RNA was extracted from rice tissues using TRIzol reagent according to the manufacturer's instructions. RNA concentration and purity were determined using a NanoDrop 2000 (NanoDrop Technologies); RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent). Subsequently, 1 µg of total RNA was used to generate an RNA-seq transcriptome library with the Illumina TruSeq RNA sample preparation kit (Illumina, San Diego, CA). The library was sequenced on the Illumina NovaSeq X Plus platform. Five databases, including Non-Redundant Protein Sequence Database, Swiss Protein Database, Clusters of Orthologous Groups of proteins, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO), were used to annotate the assembled unigenes. DEGs were defined as those with an adjusted *p*-value ≤ 0.05 and $|\log_2 (\text{Fold Change})| \geq 1$. The transcriptomic data generated in this study have been deposited in the Sequence Read Archive under accession number PRJNA1434330. In addition, six genes were randomly selected for validation by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Detailed information on the transcriptomic analysis is provided in Supporting information Text S6.

2.8. Statistical analysis

All results are presented as means ± standard deviation (SD) from triplicate experiments. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine statistical differences among multiple groups. A *p*-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Uptake and translocation of NEOs by rice

The height of rice seedlings showed minor variations among treatments exposed to different NEOs compared with the control, ranging from 17.8 to 28.8 cm (Fig. S1). These results suggest limited effects of NTP, ACE, and DIN on rice seedling growth. Similar observations have been reported previously, with no significant alterations in the fresh biomass of lettuce leaves exposed to NEOs [15]. After 10 d of exposure, NTP, ACE, and DIN were detected in both the roots and shoots of rice seedlings, although their accumulation patterns differed markedly. NEO concentrations in roots initially increased during the early exposure period and reached maximum values of 7.2, 74.2, and 27.1 mg/kg (dw) for NTP, ACE, and DIN, respectively, before plateauing. Concurrently, NEO levels in shoots increased rapidly during the exposure period, with maximum concentrations of NTP, ACE, and DIN reaching 22.8, 669.1, and 236.8 mg/kg (dw), respectively (Fig. 1A–C). Notably, ACE exhibited substantially higher root and shoot concentrations than NTP and DIN. This finding may be explained by its relatively high log K_{ow} value (0.8), which can enhance interactions with root organic components and facilitate compound uptake [18]. Previous studies have similarly shown that compounds with higher hydrophobicity tend to associate more readily with root lipids, thereby promoting accumulation in roots [19].

As exposure progressed, the internal distribution of NEOs within rice seedlings shifted gradually. The proportion of NEOs retained in roots decreased, while accumulation in shoots increased correspondingly (Fig. 1D–F). To further evaluate translocation behavior, the translocation factor (TF) was calculated according to the method described in Text S7. DIN and ACE exhibited higher TF values than NTP, indicating a greater capacity for upward transport from roots to shoots (Table S2). This trend was consistent with previous reports showing higher NEO concentrations in aerial tissues compared with roots [20]. Collectively, these results demonstrated that NEO translocation in rice is compound-specific and likely governed by physicochemical properties such as molecular weight and water solubility. In addition, this dynamic transport and accumulation pattern likely reflects the coordinated physiological mechanisms underlying intraplant resource distribution [21,22].

3.2. Metabolic pathways of NEOs

A total of 12 transformation intermediates of NTP, 10 intermediates of ACE, and 2 intermediates of DIN were identified using ultra-high resolution mass spectrometry. Detailed information on these intermediates is provided in Table S3 and Fig. S2. NTP contains a chloropyridine ring linked to a nitro-substituted ethylenediamine moiety through a methyl group. Based on its structure characteristics and the identified intermediates, we propose the metabolic pathway of NTP in rice seedlings, as illustrated in Figs. 2A and 2D. Previous studies have reported that the nitromethyl group of NTP was susceptible to free radical attack, leading to the release of NO_2^- ions [23]. In the present study, NTP initially underwent cyanation to form P1 ($C_{11}H_{13}ClN_4$, *m/z* 237.08791), in which the nitromethyl group was replaced by a cyano group. Since the cyano group is readily attacked by free radicals, P1 subsequently underwent decyanation to yield P2 ($C_{10}H_{14}ClN_3$, *m/z* 212.09288). Alternatively, P1 could undergo hydroxylation and further oxidation to a carboxyl group to form P4 ($C_{11}H_{14}ClN_3O_2$, *m/z* 256.08279), which was further hydroxylated and decarboxylated to produce P5 ($C_{10}H_{14}ClN_3O$, *m/z* 228.08760). In addition, cleavage of C–N and C–C bonds in NTP generated a cleavage product P3 ($C_{10}H_{14}ClN_3$, *m/z* 212.09300).

ACE belongs to the N-cyanamidine class of NEOs and consists of a 6-chloropyridine ring connected to an acetamidine group via a methyl bridge, with cyano and methyl substituents. The proposed metabolic pathway of ACE in rice seedlings is shown in Figs. 2B and 2D. ACE metabolism was initiated by demethylation to form intermediate M1 ($C_9H_9ClN_4$, *m/z* 209.05948). This reaction pathway is consistent with earlier studies showing that acetylamidines can be demethylated by microorganisms such as *Stenotrophomonas maltophilia* [24]. Subsequently, M1 underwent further degradation, during which the 6-chloropyridine ring was cleaved to form M2 ($C_4H_7N_3$, *m/z* 98.07133) [25]. This step likely involved hydrolysis or oxidation, resulting in ring opening and chlorine removal. M2 was then further demethylated to produce the final product M3 ($C_3H_5N_3$, *m/z* 84.05543), potentially through enzymatic or oxidative processes.

Due to the structural similarities between NTP and ACE—notably the presence of pyridine rings, amino groups, and chlorine

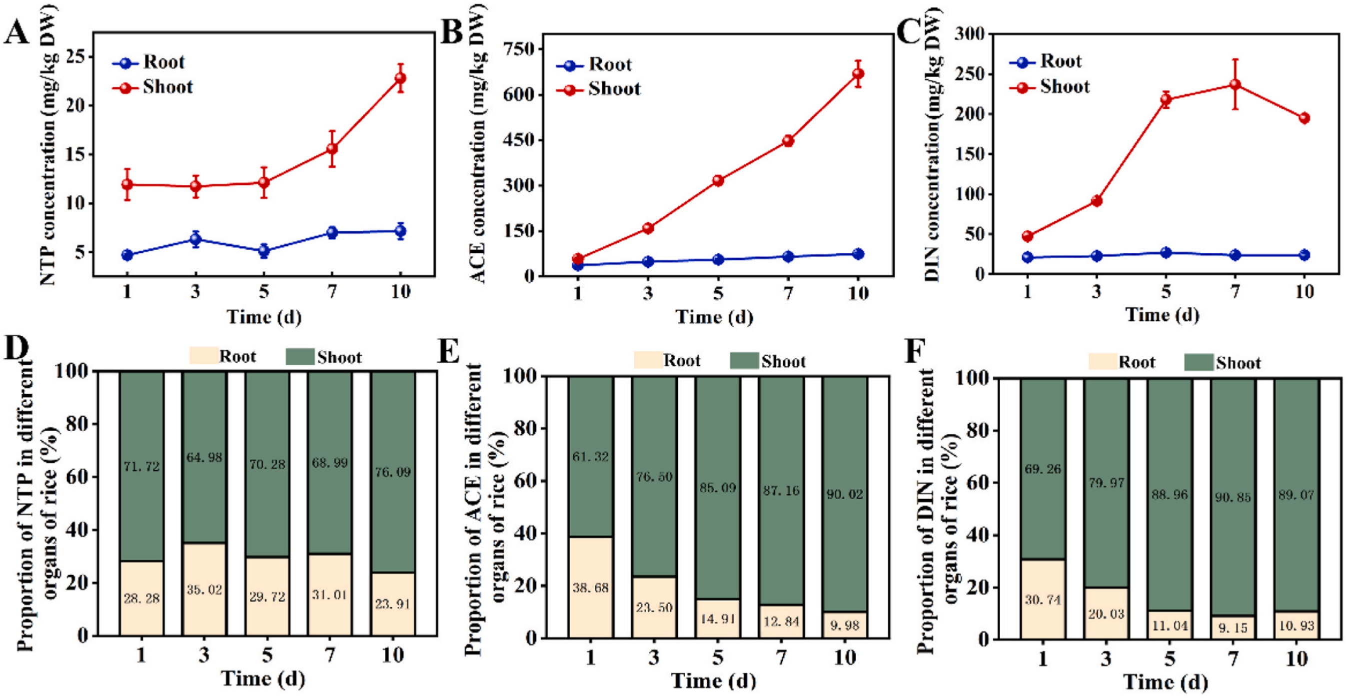

Fig. 1. Concentrations of NEOs in different tissues of rice seedlings. (A) (D) NTP. (B) (E) ACE, (C) (F) DIN. Experimental parameters: $[NEOs]_0 = 10$ mg/L.

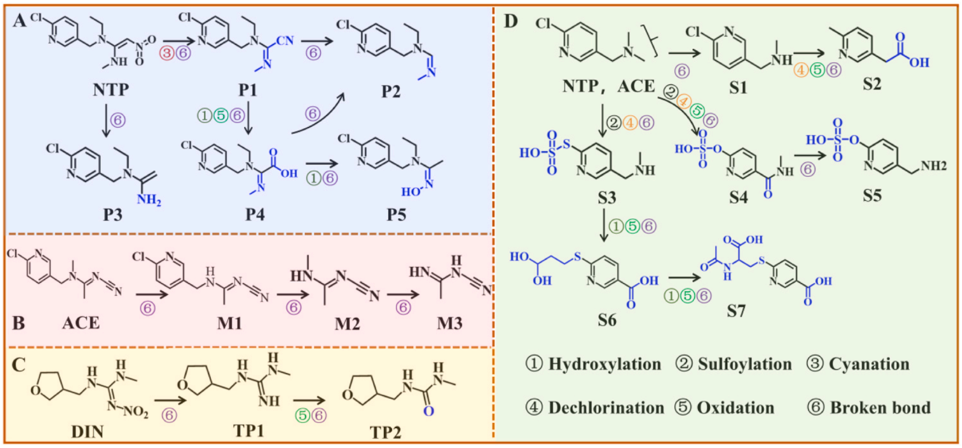

Fig. 2. Identification of degradation products and prediction of transformation pathways for NEOs in rice seedlings. (A) NTP. (B) ACE. (C) DIN. (D) NTP and ACE co-products.

substituents—they share several common metabolic pathways, with a total of 7 degradation intermediates identified (Fig. 2D). Intermediate S1 ($C_7H_9ClN_2$, m/z 157.04831) was formed through the removal of the nitro-ethylenediamine group from NTP and the acetamidine group from ACE. This intermediate subsequently underwent dechlorination, followed by C–N bond cleavage and oxidation to generate product S2 ($C_{10}H_{13}N$, m/z 148.11087). Under hydrolytic or oxidative conditions, further sulfonation occurred after chlorine removal, yielding intermediate S3 ($C_7H_{10}N_2O_3S_2$, m/z 235.02199) [26]. Oxidative cleavage of the C-N bond in S3, combined with hydrolysis and hydroxylation of the sulfonyl group, resulted in product S4 ($C_9H_{11}NO_4S$, m/z 230.04076). Subsequent hydroxylation and oxidation of S4 produced the final conjugated product S5 ($C_{11}H_{12}N_2O_5S$, m/z 285.05394). In addition, NTP and ACE could undergo an alternative pathway involving dechlorination followed by sulfonation and oxidation to form intermediate S6 ($C_7H_8N_2O_5S$, m/z 233.03027), which subsequently lost a carbonyl group and underwent demethylation to produce S7 ($C_6H_8N_2O_4S$, m/z 205.02759).

DIN has a unique tetrahydrofuran ring structure, which is connected via a nitromethyl group and a carbamate group. Two main intermediates of DIN, TP1 ($C_7H_{15}N_3O$, m/z 158.12952) and TP2 ($C_7H_{14}N_2O_2$, m/z 159.11284), were identified, as shown in Fig. 2C. DIN degradation was initiated by denitrification of the methyl group, yielding TP1, which subsequently underwent hydrolysis or oxidation to form the carbonyl-containing TP2 [27,28].

Overall, the proposed metabolic pathways reveal compound-specific transformation pathways of NEOs in rice seedlings. The elucidation of these pathways enhances understanding of the environmental fate of NEOs in agroecosystems and provides a scientific basis for developing targeted strategies for contaminant degradation and risk mitigation.

3.3. *Mineral content in rice under NEOs exposure*

Mineral elements are essential for maintaining molecular processes that support normal plant physiology [15]. However, the effects of insecticides on plant nutrient acquisition remain insufficiently understood. The eight mineral elements selected for analysis in this study were chosen based on their established essential roles in plant physiology: they serve as key enzyme cofactors (e.g., Cu/Zn/Fe in SODs, Mn in photosynthesis), osmotic regulators (K^+, Na^+), structural components (Ca in cell walls and signaling), and central nodes in energy metabolism (Mg in chlorophyll) [29,30]. Changes in the concentrations of major mineral elements, including Ca, Mn, Fe, Cu, Na, K, Mg, and Zn, in rice seedlings exposed to NEOs are shown in Fig. 3. Exposure to NTP, ACE, and DIN significantly altered the uptake of several mineral elements, particularly Ca, Mn, Na, K, Mg, and Zn. Compared with the control, the concentrations of Cu and Fe in rice seedlings remained relatively stable throughout the exposure period for all three NEOs. In contrast, Zn concentrations declined markedly during the first 3 days of exposure to ACE and DIN. Both Cu and Zn/Fe serve as essential cofactors for SODs in chloroplasts, where they catalyze the conversion of superoxide radicals to hydrogen peroxide and protect cells from oxidative damage [31]. The observed reduction in Zn concentrations suggests a disruption of enzyme activity and antioxidant defense capacity in rice seedlings under ACE and DIN stress.

Mn concentrations showed compound-specific responses. No significant change in Mn concentrations was observed under ACE exposure, whereas Mn accumulation increased significantly in the NTP- and DIN-treated groups. Mn plays a crucial role in photosynthesis, nitrogen assimilation, and ROS scavenging [32]. NTP and DIN exposure likely enhanced Mn uptake through the potential activation of Mn-related transport genes or modification of root exudate composition. Ca concentration decreased significantly in the presence of NTP and ACE. Ca participates in nitrogen metabolism and promotes the absorption of nitrate nitrogen [33]. The observed reduction in Ca concentrations suggests that NTP and ACE exposure may interfere with nitrogen assimilation processes in rice seedlings. No significant variation in Na levels was detected in the NTP-treated group, whereas ACE and DIN exposure greatly reduced Na concentration in rice seedlings at 1 d. Similarly, K concentrations were significantly lower in all NEOs-exposed groups during the first three days compared with the control, although a transient increase was observed on day 5 under DIN exposure. Overall, exposure to NEOs reduced the contents of Na and K simultaneously, indicating that the absorption and transport capacity of rice for Na^+ and K^+ decreased under NEO stress, thereby failing to ensure an adequate supply of the ions involved in key metabolic activities [34]. Mg plays a vital role in photosynthetic metabolism [35]. In the present study, Mg content only increased in the DIN exposure group during 1–3 d, indicating that DIN stress enhanced chlorophyll content and photosynthesis by promoting the uptake of Mg^{2+}. Similar reductions in mineral nutrient contents, including K, Mg, Ca, Fe, and Zn, have been reported in ginger leaves under abiotic stress conditions [36]. These findings collectively demonstrate that NEOs compromise normal seedling development by causing metabolic disturbances of mineral nutrients in rice.

3.4. Oxidative stress induced by NEOs

Oxidative stress induced by xenobiotic exposure promotes the

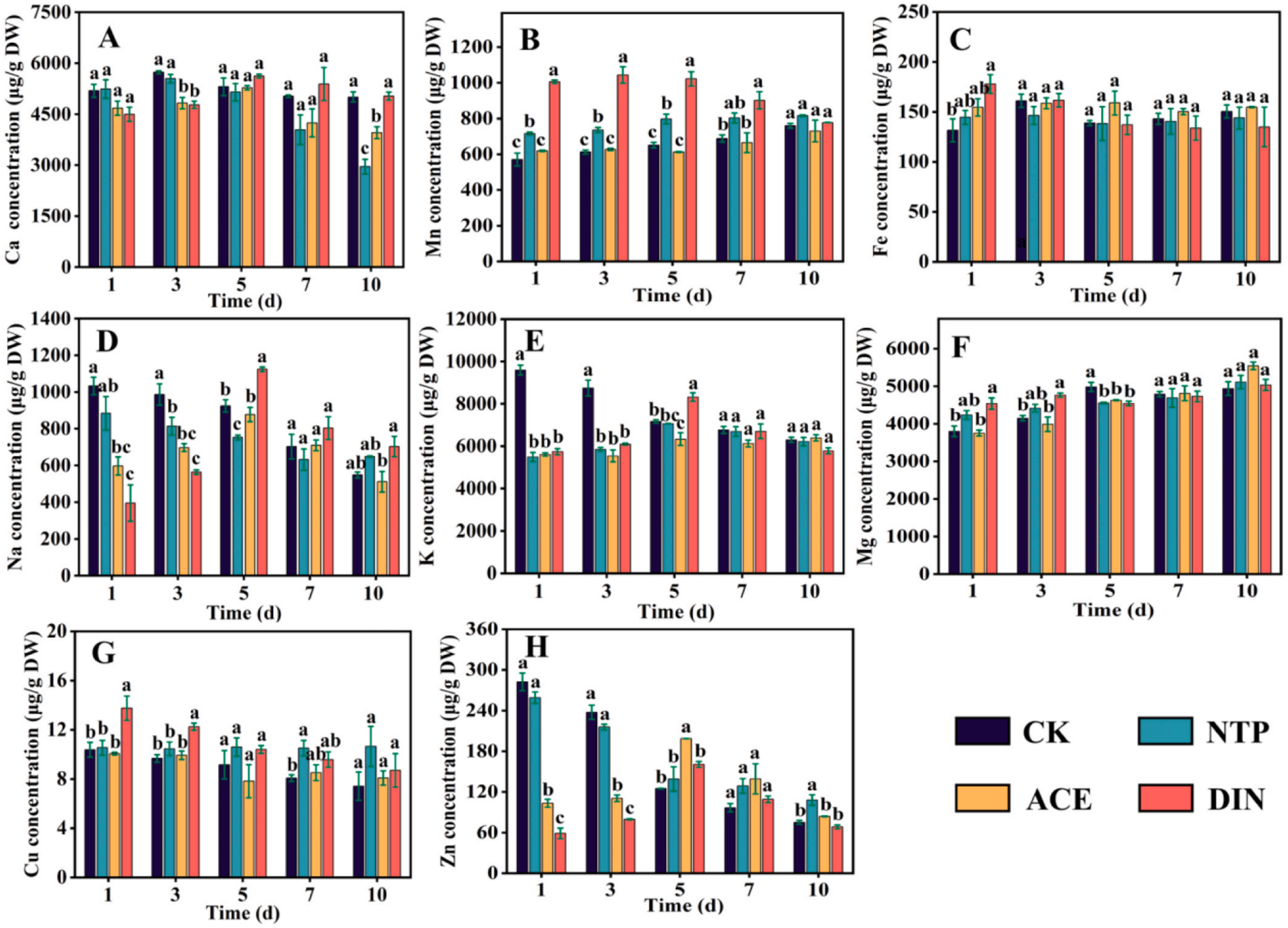

Fig. 3. Effects of NEOs on mineral element content in rice seedlings. (A) Ca. (B) Mn. (C) Fe. (D) Na. (E) K. (F) Mg. (G) Cu. (H) Zn. Experimental parameters: $[NEOs]_0 = 10$ mg/L. Data were analyzed by one-way ANOVA with Tukey's post hoc test. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

production of ROS in plants [37]. Under exposure to NTP, ACE, and DIN, both ROS and MDA levels followed similar temporal trends. Their concentrations increased in a time-dependent manner during the first 7 days of exposure and then declined slightly by day 10 (Figs. 4A and 4B). The pronounced accumulation of MDA indicated enhanced lipid peroxidation, reflecting membrane damage caused by excessive ROS generation under NEO stress [38]. Antioxidant enzymes play crucial role in alleviating oxidative damage in plants [39]. In the present study, CAT activity was initially suppressed following exposure to all three NEOs, followed by a slight recovery. However, CAT activity remained significantly lower than that of the control by the end of the exposure period (Fig. 4D). A similar response pattern was observed for SOD, whose activity decreased markedly under NEO stress (Fig. 4E). Reduced CAT and SOD activities limit the plant's capacity to scavenge excess ROS, thereby exacerbating oxidative stress [40]. In contrast, a remarkable increase in POD activity was observed following NEO exposure, indicating that NTP, ACE, and DIN treatments induced an antioxidant defense response in rice seedlings (Fig. 4C). Despite these enzyme-specific responses, no significant differences in oxidative stress intensity or antioxidant enzyme activities were observed among the three NEO treatments, indicating comparable oxidative stress effects at the tested concentrations. Consistent with these findings, previous studies have reported that exposure to high concentrations of fluroxypyr reduced the activities of multiple antioxidant enzymes, including CAT, SOD, and POD in rice [41]. Overall, NTP, ACE, and DIN exposure substantially increased intracellular ROS levels and suppressed key antioxidant enzymes, particularly CAT and SOD, resulting in enhanced lipid peroxidation as indicated by elevated MDA levels.

3.5. Metabolomics response to NEOs

After quality control, 83.7% of the characteristic peaks exhibited relative standard deviations (RSDs) below 30% (Fig. S3), which indicated satisfactory data stability and reliability for metabolomics analysis. Principal component analysis showed clear separation between control and NEO-treated groups, with PC1 and PC2 accounting for 25.3% and 20.1% of the total variance, respectively (Fig. S4). These results confirm that exposure to NTP, ACE, and DIN induced pronounced metabolic perturbations in rice seedlings. In addition, all NEOs-treated groups exhibited Q^2 values significantly greater than 0.5, demonstrating strong predictive performance of the OPLS-DA models and further validating data quality.

Non-targeted metabolomics analysis revealed 140, 136, and 79 significantly differentially expressed metabolites in the NTP, ACE, and DIN exposure groups, respectively, compared with the control group ($p < 0.05$). In the NTP-treated group, 34 metabolites were significantly upregulated and 106 were downregulated (Fig. 5A). Similarly, ACE exposure resulted in 52 upregulated and 84 downregulated metabolites (Fig. 5C), while DIN exposure led to 22 upregulated and 57 downregulated metabolites (Fig. 5E). The lower number of metabolites detected under DIN exposure likely reflects its distinct chemical structure (open-chain vs. heterocyclic), which may lead to faster degradation and the generation of highly polar products that are less efficiently extracted or ionized under the current analytical conditions. Overall, downregulated metabolites predominated under NEO stress. These metabolites primarily belonged to classes such as flavonoids, amino acids, lipids, pyridine and its derivatives, steroids and their derivatives, organic oxygen compounds, carboxylic acids and their derivatives, and other categories (Table S4 and Fig. S5). Flavonoids are important secondary metabolites of plants involved in antioxidant defense, stress tolerance, and signal transduction [42]. On the other hand, the accumulation of some amino acids (such as proline and glutamate) may be related to the plant's defense mechanism against oxidative stress [43]. These metabolites are crucial for rice defense against stress and for the maintenance of cellular structural integrity. Their pronounced downregulation is likely a consequence of NEO-induced oxidative damage, which disrupts intracellular homeostasis. Collectively, these findings indicate that the disruption of redox balance represents a key mechanism underlying NEO-induced phytotoxicity in rice.

Pathway enrichment analysis revealed a total of 21, 27, and 79

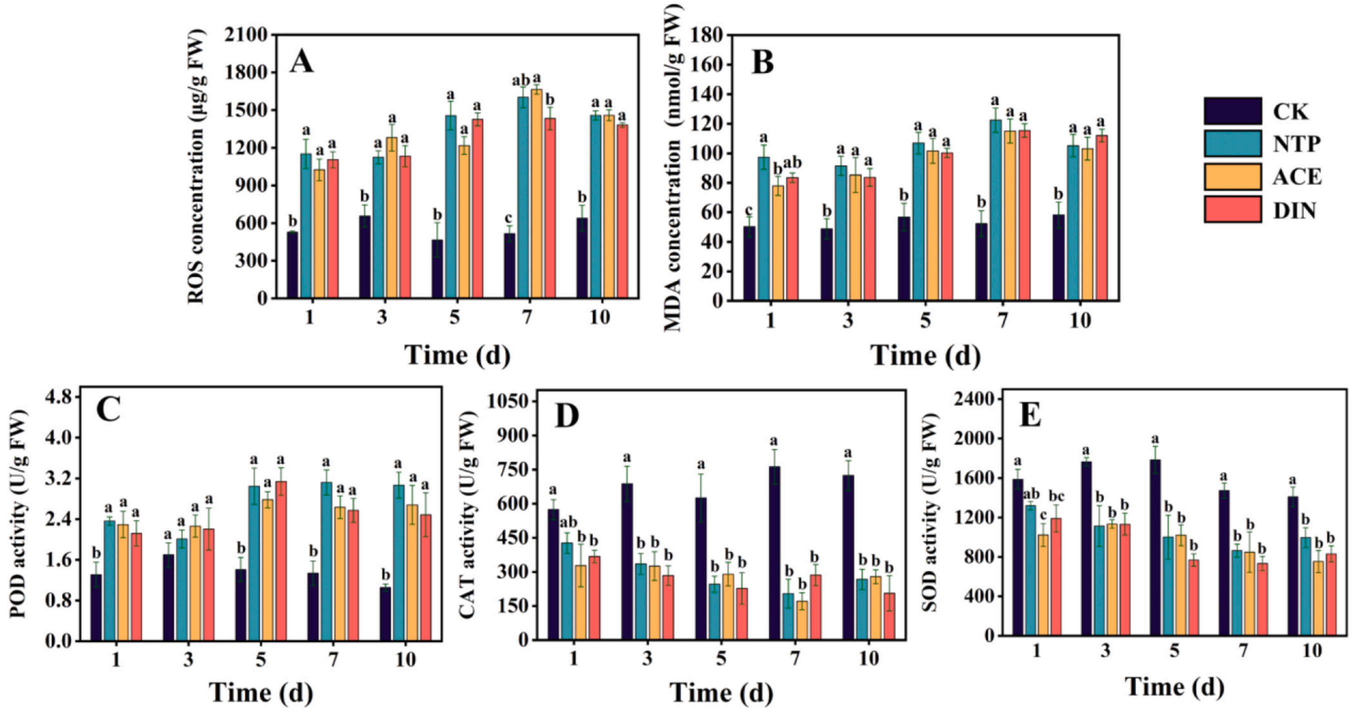

Fig. 4. Effects of NEO stress on oxidative damage and antioxidant enzyme activities in rice seedlings. (A) ROS. (B) MDA. (C) POD. (D) CAT. (E) SOD. Experimental parameters: $[\text{NEOs}]_0 = 10$ mg/L. Data were analyzed by one-way ANOVA with Tukey's post hoc test. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

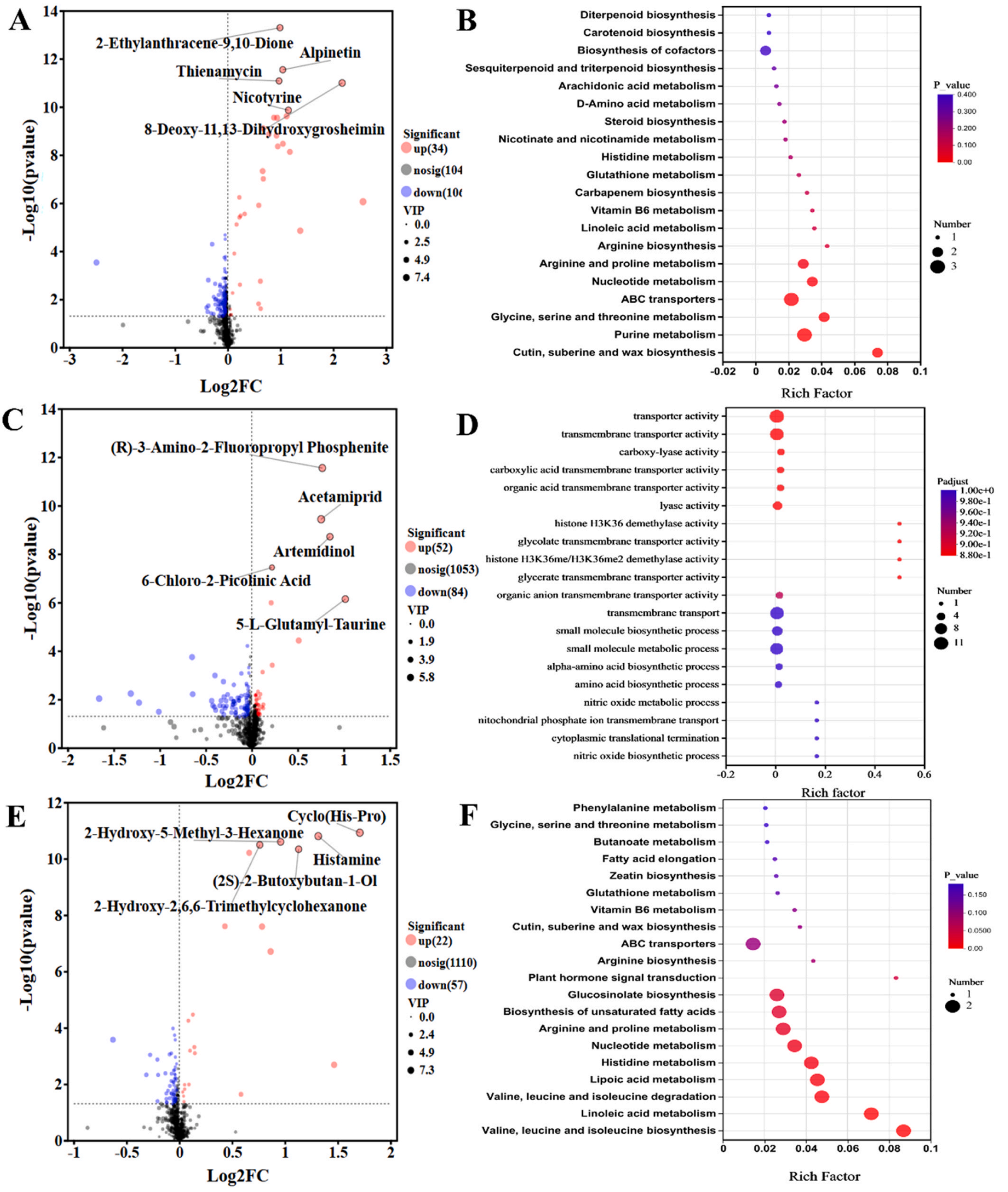

Fig. 5. Metabolome alteration in rice after exposure to NEOs. Differential metabolites expression (A: NTP, C: ACE, E: DIN) and metabolic pathways enrichment (B: NTP, D: ACE, F: DIN).

significantly affected metabolic pathways in the NTP, ACE, and DIN treatment groups, respectively. Among these, plant secondary, amino acid, and purine metabolism were the most prominently enriched pathways, each containing a high number of differentially expressed metabolites (Figs. 5B, 5D, and 5F). These results suggest that plants establish defense systems under NEO stress by significantly reprogramming key metabolic pathways to mitigate energy deficits, oxidative damage, and potential genotoxicity. Alterations in plant secondary metabolites are closely related to gene expression, hormone regulation, and environmental response, thereby affecting plant defense capacity, growth and development, and stress tolerance [44]. In the present study, secondary metabolism was characterized by upregulation of 22-hydroxydocosanoic acid and downregulation of 16-hydroxyhexadecanoic acid, 3-methyl-2-oxopentanoic acid, and leucine ketone. The accumulation of 22-hydroxydocosanoic acid, a characteristic product of fatty acid ω-oxidation, suggests reinforcement of the epidermal barrier to limit NEO penetration or reduce water loss [45]. In contrast, reduced levels of 16-hydroxyhexadecanoic acid may reflect NEO-induced disruption of lipid metabolism and membrane synthesis. The downregulation of 3-methyl-2-oxopentanoic acid and leucine ketone—intermediates of isoleucine and leucine metabolism—implied constrained flux toward acetyl-CoA and succinyl-CoA, thereby limiting energy production. Collectively, the predominant downregulation of metabolites in plant secondary metabolic pathways may reflect resource allocation priorities in rice seedlings under NEO stress. To enhance survival, plants likely redirect metabolic resources toward critical pathways such as osmoregulation and antioxidant defense. Furthermore, rice seedlings may actively suppress secondary metabolite synthesis through transcriptional regulation of related genes as an adaptive strategy to stressful conditions.

In amino acid metabolism, NEO exposure resulted in decreased levels of creatine, 5-aminolevulinate, and 2-oxobutanoic acid, accompanied by increased levels of L-ornithine, imidazole-4-acetaldehyde, histamine, and 5-L-glutamyl-taurine. Reduced creatine levels may compromise cellular energy homeostasis, while decreased 5-aminolevulinate—a precursor of chlorophyll biosynthesis—could impair photosynthetic capacity. The downregulation of 2-oxobutanoic acid, a crucial intermediate linking amino acid and lipid biosynthesis to the tricarboxylic acid (TCA) cycle, suggests disruption of carbon metabolism. This metabolic perturbation likely contributes to the observed accumulation of branched-chain amino acids and the subsequent impairment of signal transduction, consistent with previous reports [46]. Notably, the elevated levels of imidazole-4-acetaldehyde and histamine likely represent a stress adaptation mechanism, potentially through enhanced histidine metabolism and antioxidant defense systems. 5-L-glutamyl-taurine, a conjugated amino acid derived from glutamic acid and taurine, participates in multiple physiological processes, including nitrogen metabolism and cellular signaling [47]. Given the known roles of taurine in antioxidant defense and osmoregulation, increased 5-L-glutamyl-taurine levels may enhance rice tolerance to NEO stress by coordinating nitrogen utilization and oxidative stress responses.

Purine metabolism, a crucial pathway for nucleotide synthesis, serves as the foundation for DNA/RNA production while also participating in energy metabolism and signal transduction [48]. Under NEO stress, rice seedlings exhibited upregulation of key nucleotide metabolites, including cyclic guanosine monophosphate, agmatine, adenosine monophosphate, and oxalic acid. This metabolic shift likely indicates heightened cellular demands for energy metabolism and genetic information processing in response to stress conditions. Meanwhile, guanylate enters catabolism through uncharacterized guanosine monophosphate sulfatase and guanosine deaminase enzymes [49]. These distinct metabolic pathways are crucial for maintaining purine nucleotide homeostasis in plants. Agmatine has been implicated in DNA repair and antioxidant defense, and its upregulation likely represents an adaptive response to NEO-induced cellular damage. In contrast, the downregulation of hypoxanthine and inosine—key intermediates in purine salvage pathways [50]—suggests inhibition of nucleotide recycling and reduced efficiency of energy supply and genetic information processing. Overall, these metabolomic alterations demonstrate that NEO exposure induces extensive metabolic reprogramming in rice seedlings, affecting secondary metabolism, amino acid turnover, and purine biosynthesis. Such coordinated metabolic adjustments likely represent an adaptive strategy to cope with NEO-induced oxidative stress, energy imbalance, and cellular toxicity.

3.6. *Transcriptional response to NEOs*

To further investigate the physiological response of rice to NTP, ACE, and DIN stress, transcriptome profiling was performed using RNA sequencing to characterize global changes in gene expression. Venn diagrams, correlation heatmaps, and principal component analysis (PCA) plots revealed significant separation between experimental and control groups, confirming that all three NEOs substantially altered transcriptional profiles in rice (Fig. S6). Differential expression analysis revealed that 23, 41, and 53 genes were upregulated, whereas 1, 19, and 222 genes were downregulated in response to NTP, ACE, and DIN exposure, respectively (Fig. S7). These findings showed that the number of downregulated genes outnumbered those upregulated. Among these genes, six were randomly chosen for comparison and verification by RT-qPCR, as shown in Fig. S8. The results demonstrated that both RT-qPCR and transcriptome sequencing yielded highly consistent findings. Additionally, gene expression in rice was affected to varying degrees by exposure to the three NEOs, with the magnitude of response following the order of DIN > ACE > NTP. The observed discrepancy between the massive accumulation of ACE in shoots and its relatively modest transcriptomic perturbation, compared to the lower accumulation but stronger response induced by DIN, highlights a critical distinction between bioaccumulation and biological activity. This apparent paradox likely stems from differences in subcellular distribution and bioavailability. The high tissue concentration of ACE may largely represent sequestered pools in apoplastic spaces or vacuoles, reducing its effective concentration at sites of molecular action. Conversely, DIN may more readily reach cytosolic targets or be perceived as a more potent stress signal, eliciting a stronger transcriptomic response.

GO annotation analysis was conducted to assess the functional significance of DEGs. Most DEGs were associated with cellular processes, metabolic processes, cellular anatomical entities, binding, and catalytic activities (Fig. S9). Enrichment analysis further showed that DEGs in rice seedlings exposed to the three NEOs were significantly enriched in biological processes, cellular components, and molecular functions, indicating broad impacts on cellular organization and metabolism (Fig. S10).

Compound-specific transcriptional responses were observed among the three NEO treatments. In NTP-exposed seedlings, significant changes were detected in genes encoding allantoinase (LOC_Os04g0680400, 3.11-fold) and isocitrate lyase (LOC_Os07g0529000, 20.31-fold). Allantoin-related metabolites are known to enhance plant stress tolerance through activation of abscisic acid–related signaling pathways, suggesting a role for NTP-induced modulation of stress hormone metabolism [51]. ACE exposure induced marked upregulation of genes associated with transmembrane transport, carboxy-lyase activity, and key enzymatic nodes. Studies have demonstrated that impairment of plasma membrane transport systems can lead to the abnormal accumulation or misdistribution of metabolites, thereby disrupting normal cellular physiological activities [52]. In addition, DIN-treated seedlings primarily activated lipid metabolism pathways, signal transduction cascades, and root development-related gene networks as defensive mechanisms.

To elucidate the functional implications of differential gene expression, KEGG pathway analysis was performed to link DEGs with metabolic pathways in rice (Figs. 6A, 6C, and 6E). Under NTP exposure, DEGs were mainly enriched in carbohydrate metabolism, signal transduction/transport, and catabolic metabolism. In the ACE-treated group, energy, carbohydrate, and amino acid metabolism were the predominantly enriched pathways. For DIN exposure, DEGs were primarily associated with biosynthesis of secondary metabolites, lipid metabolism, and metabolism of amino acid derivatives. KEGG enrichment analysis showed that DEGs under NTP, ACE, and DIN stress were significantly enriched in 1, 3, and 15 metabolic pathways, respectively (Figs. 6B, 6D, and 6F). The most prominent pathways included cutin, suberin, and wax biosynthesis under NTP exposure, glycolysis and gluconeogenesis under

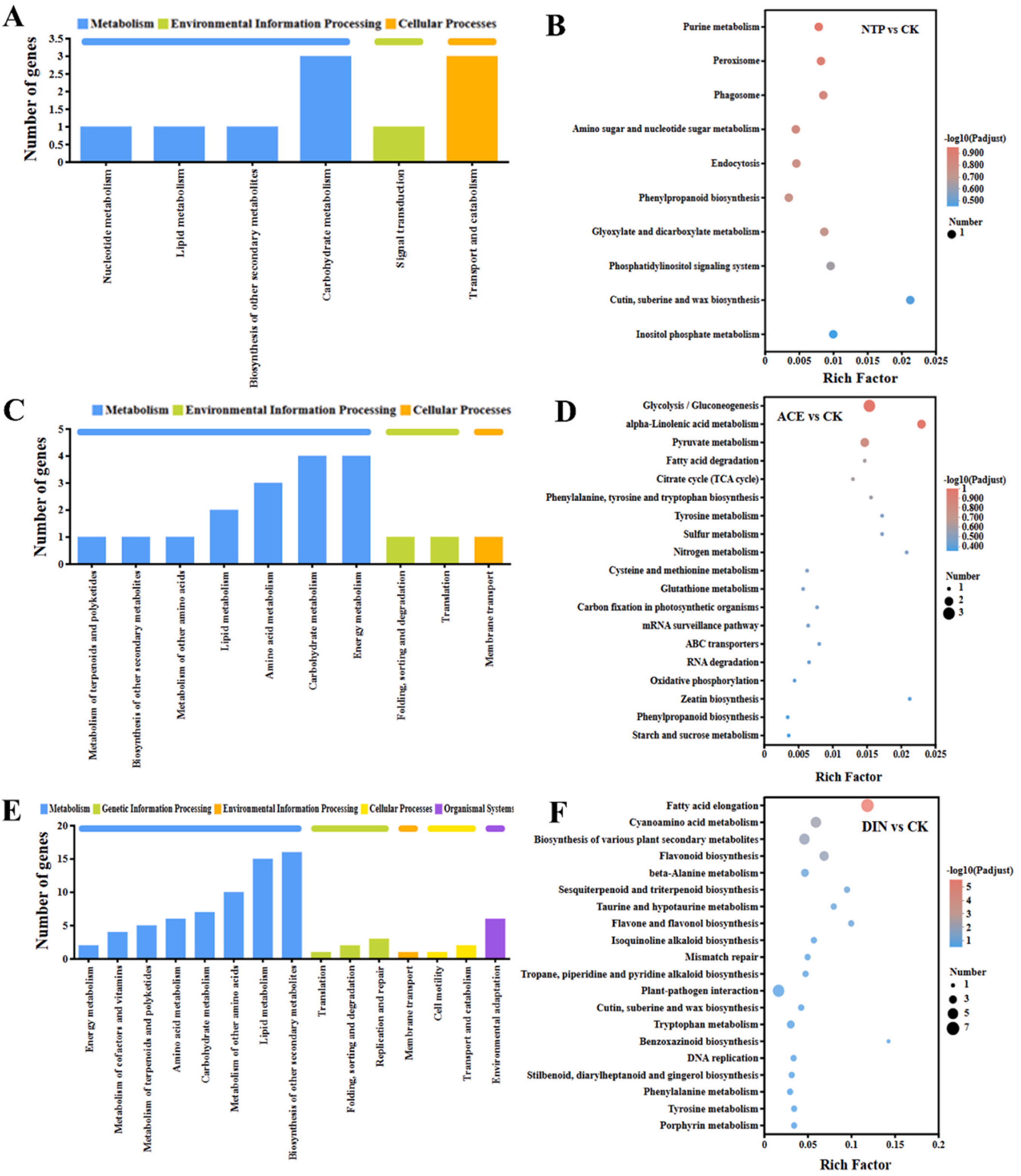

Fig. 6. KEGG annotation and enrichment analysis of DEGs in rice seedlings under different NEO stress. (A) (B) NTP. (C) (D) ACE. (E) (F) DIN.

ACE exposure, and fatty acid elongation under DIN exposure.

Carbohydrate metabolism plays a central role in cellular energy supply, with glycolysis serving as the core pathway that converts glucose into pyruvate and generates adenosine triphosphate [19]. Several genes involved in the TCA cycle and associated metabolic processes were deferentially expressed following NEO exposure, including genes encoding glycoside hydrolase (LOC_Os12g0178000, 0.16-fold in DIN), isocitrate lyase (LOC_Os07g0529000, 20.31-fold in NTP), phosphatidylinositol-4-phosphate 5-kinase (LOC_Os03g0356582, 3.96-fold in NTP), pyruvate decarboxylase (LOC_Os07g0693100, 4.28-fold in NTP; LOC_Os01g0160100, 2.48-fold in ACE), alcohol dehydrogenase (LOC_Os11g0210500, 0.14-fold in ACE), and phosphoenolpyruvate carboxykinase (LOC_Os10g0204400, 0.20-fold in ACE). Glycolysis provides precursor substrates for fatty acid biosynthesis, and downregulation of pyruvate metabolism-related genes suggests that NEO exposure may suppress energy production and carbon flux allocation in rice seedlings [53].

Under aerobic conditions, pyruvate is converted to acetyl coenzyme A by the pyruvate dehydrogenase complex, enabling entry into the TCA cycle, where substantial energy is generated through oxidative metabolism [54]. The significant downregulation of phosphoenolpyruvate carboxykinase (LOC_Os10g0204400, 0.20-fold in ACE) suggested that NEOs may inhibit the conversion of oxaloacetate to phosphoenolpyruvate, thereby reducing metabolic flux and energy supply through the TCA cycle. In contrast, pyruvate decarboxylase (LOC_Os07g0693100, 4.28-fold in NTP; LOC_Os01g0160100, 2.48-fold in ACE) was significantly upregulated, promoting the conversion of pyruvate to acetaldehyde and carbon dioxide. Meanwhile, alcohol dehydrogenase (LOC_Os11g0210500, 0.14-fold in ACE) was downregulated, which may restrict acetaldehyde conversion to ethanol. This coordinated regulation likely serves to stabilize acetaldehyde levels and prevent cytotoxic effects associated with its excessive accumulation.

Lipid metabolism is essential for cellular energy supply and maintenance of membrane integrity. Several genes involved in lipid metabolism, including those encoding fatty acyl coenzyme A (acyl-CoA) reductase (LOC_Os09g0567500, 5.14-fold in NTP), glycerophosphodiester phosphodiesterase (LOC_Os03g0603600, 0.22-fold in DIN), phosphatidylserine synthase (LOC_Os05g0554400, 0.49-fold in DIN), and gamma-glutamyltransferase (LOC_Os01g0151500, 0.22-fold in DIN), were differentially expressed following NEO exposure. Notably, fatty acyl-CoA reductase (LOC_Os09g0567500, 5.14-fold in NTP) was upregulated in the cutin, suberin, and wax biosynthetic pathways, suggesting enhanced epidermal wax accumulation. This response may strengthen the physical barrier of rice seedlings, thereby reducing water loss and limiting the penetration of external stressors. Previous studies have shown that fatty acid synthase is a key enzyme in the initiation of lipid synthesis, and its activity determines the rate of fatty acid synthesis in cells [55].

In the fatty acid elongation pathway, several DEGs in rice seedlings were significantly altered, and all were related to fatty acid synthesis in the endoplasmic reticulum. A total of six DEGs (LOC_Os11g0566800, 0.15-fold in ACE; LOC_Os07g0526400, 0.09-fold in DIN; LOC_Os02g0731900, 0.18-fold in DIN; LOC_Os06g0262800, 0.19-fold in DIN; LOC_Os03g0245700, 0.13-fold in DIN; LOC_Os03g0162800, 0.12-fold in DIN) were significantly downregulated during the conversion of long-chain acyl-CoA and malonyl-CoA to long-chain 3-oxoacyl intermediates. These results suggest that NEO exposure inhibits the formation of long-chain 3-oxoacyl derivatives. In addition, protein tyrosine phosphatase (LOC_Os02g0771400, 0.20-fold in DIN) was significantly downregulated during the conversion of long-chain 3-hydroxyacyl intermediates to long-chain trans-2,3-dehydroacyl derivatives, which may lead to reduced dehydrogenase activity, decreased production of long-chain trans-2,3-dehydroacyl intermediates, and impaired synthesis of unsaturated fatty acids. These changes may represent an adaptive response of rice seedling cells to NEO stress, maintaining cell membrane stability and function by adjusting lipid metabolism [56]. In conclusion, these findings further elucidate the molecular mechanisms underlying the response of rice seedlings to NEO stress.

Integrated transcriptomic and metabolomic analyses revealed that exposure to three neonicotinoid insecticides (NEOs) triggers distinct yet coordinated metabolic reprogramming in rice seedlings, with each compound eliciting a unique signature of gene–metabolite associations. Under ACE stress, a defensive metabolic response was evident, characterized by the upregulation of pyruvate decarboxylase (LOC_Os01g0160100, 2.48-fold), which strongly correlated with the secondary metabolite artemidinol (1.80-fold), alongside the induction of pathogenesis-related protein PR10 (LOC_Os12g0555300, 16.74-fold) and its correlation with the osmoprotective conjugate 5-L-glutamyl-taurine (2.02-fold). Additionally, a phospholipid-transporting ATPase (LOC_Os03g0326200, 2.54-fold) was positively associated with the cutin monomer 22-hydroxydocosanoic acid (1.08-fold), suggesting reinforcement of the physical barrier. In contrast, DIN exposure primarily suppressed growth-related and lipid biosynthetic pathways, as indicated by the downregulation of 3-ketoacyl-CoA synthase 5 (LOC_Os06g0262800, 0.19-fold), which positively correlated with reduced palmitic acid (0.98-fold), and the coordinated decrease in divinyl chlorophyllide a 8-vinyl-reductase (LOC_Os03g0351200, 0.36-fold) alongside creatine depletion (0.65-fold), reflecting compromised lipid biosynthesis and energy status. NTP exposure, meanwhile, induced lipid remodeling centered on the hydrophobic protein LTI6A (LOC_Os07g0635900, 2.79-fold), which showed a positive correlation with the cutin monomer 22-hydroxydocosanoic acid (1.07-fold) and negative correlations with medium-chain fatty acids such as 16-hydroxyhexadecanoic acid (0.97-fold), suggesting a redirection of lipid resources toward cuticle reinforcement. Collectively, these findings demonstrate that NEO stress elicits compound-specific transcriptional and metabolic adjustments—ranging from the activation of secondary metabolism and barrier formation (ACE) to the suppression of lipid and energy metabolism (DIN) and targeted lipid remodeling (NTP)—highlighting the complex adaptive networks that underpin plant resilience to xenobiotic exposure.

3.7. Limitations

(1) The single-concentration design of this study enabled the identification of the spectrum of NEO-induced metabolic disruptions. However, multi-concentration experiments are needed to fully characterize the dose-dependent nature of these effects. (2) The relatively high exposure concentration and short-term duration were intentionally chosen to elucidate the mechanistic basis of NEO uptake and toxicity under controlled conditions, but they do not fully represent long-term, environmentally relevant scenarios. Investigating chronic phytotoxicity at environmentally relevant concentrations across diverse agricultural ecosystems is essential to bridge the gap between laboratory findings and field applications.

4. Conclusion

This study systematically investigated the uptake, translocation, biotransformation, and phytotoxic effects of three representative neonicotinoid insecticides (NTP, ACE, DIN) in rice seedlings. Compound-specific accumulation patterns were observed, indicating efficient root-to-shoot translocation. A total of 17 transformation products were identified, involving hydroxylation, sulfonation, cyanation, dechlorination, oxidation, and bond-cleavage reactions. Integrated multi-omics analyses revealed that NEO exposure significantly disrupted flavonoid, amino acid, and lipid metabolism, with transcriptomic enrichment in pathways associated with secondary metabolite biosynthesis and energy metabolism. These findings highlight that rice can act as a significant sink and potential vector for NEO entry into food chains. Future research should prioritize long-term field validation and assessment of trophic transfer to fully characterize the ecological implications of NEOs in agroecosystems.

Environmental Implication

Neonicotinoid insecticides (NEOs) are among the most extensively used insecticides in modern agriculture, and they pose significant risks to ecosystems and human health. This study is the first to demonstrate the uptake, translocation, and biotransformation of three representative NEOs in rice, including nitenpyram, acetamiprid, and dinotefuran. Our results highlight that NEO exposure disrupted mineral homeostasis and induced oxidative stress in rice. Moreover, NEOs altered the expression of genes involved in anabolism and signal transduction, and also triggered systemic metabolic reprogramming in rice. These findings provide critical mechanistic insight into the behavior and biological effects of

NEOs in rice.

CRediT authorship contribution statement

Shanhong Lan: Software, Resources. **Kun Wei:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lichang Zhao:** Validation, Methodology, Investigation, Data curation. **Chang He:** Investigation, Formal analysis. **Zhen Yu:** Validation, Supervision, Conceptualization. **Shaoyu Tang:** Visualization, Methodology, Funding acquisition, Conceptualization. **Yuanyuan Yu:** Validation, Funding acquisition.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.142191.

Data availability

Data will be made available on request.

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