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The Effects of Nitrogen and Phosphorus Deficiency on the Main Physiology of Ilex chinensis and Transcriptomic Analysis

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Abstract

The elements nitrogen (N) and phosphorus (P) are essential nutrients required for normal growth and photosynthesis in plants. However, nitrogen and phosphorus deficiencies can have a significant impact on plant physiology and metabolism. In this study, the two-year-old *Ilex chinensis* seedlings were used as the research object, the growth environment of low nitrogen (LN) and low phosphorus (LP) was simulated, and two treatment gradients of mild and severe stress were set up, and the changes of the growth and development of wintergreen and the main physiological indexes were studied under normal growth conditions as the control. Differentially expressed genes (DEGs) were identified by transcriptomic analysis at 10 weeks of severe stress. The results showed that nitrogen and phosphorus deficiency inhibited the growth of shoots and promoted root development of *I. chinensis*, and LN_2 and LP_2 treatments had the most serious effects. The physiological indexes showed that the contents of nitrate reductase (NR), glutamine synthetase (GS), superoxide dismutase (SOD) and peroxidase (POD) and malondialdehyde (MDA) in the LN_2 group were significantly increased by 112.36%, 290.19%, 67.56%, 151.79% and 248.04%, respectively, compared with the CK group after 10 weeks of treatment. The activities of acid phosphatase (ACP), SOD and POD, anthocyanins and MDA increased by 77.1%, 65.46%, 97.81%, 144.43% and 134.25%. Transcriptome analysis revealed that the key differentially expressed genes under the nitrogen and phosphorus deficit of wintergreen were mainly involved in the regulation of plant growth, root development, nitrogen and phosphorus uptake and other biological processes. These findings provide insights into the adaptation mechanisms of *I. chinensis* under nitrogen and phosphorus deficits and highlight potential target genes for improving nutrient use efficiency. This study contributes to a better understanding of the physiological and molecular responses of *I. chinensis* to nitrogen and phosphorus deficiency stress, and provides valuable information for optimizing its cultivation under nutrient-constrained conditions.

1 Introduction

Nitrogen (N) and phosphorus (P) are key macroelements for plant growth and development, and are important components of biological macromolecules such as nucleic acids and proteins in plants. They are widely involved in physiological processes such as photosynthesis, respiration, and signal transduction in plants. The demand for nitrogen and phosphorus in plants runs through their entire life cycle[1, 2]. Lack of nitrogen and phosphorus can lead to significant changes in plant morphology, growth, and development, ultimately resulting in reduced plant yield, decreased quality, poor ornamental value, and decreased economic value.

Plants subjected to nitrogen and phosphorus deficiency stress will initiate a series of complex molecular response mechanisms to enhance the absorption and utilization of limited nutrients. In nitrogen deficient environments, plants enhance nitrogen uptake and transport by upregulating the expression of nitrate transporter genes (*NRT1* and *NRT2* family genes), which are typically tissue-specific. The root system is often the primary site in response to low nitrogen stress [3, 4]. In addition, up-regulation of *GS1*

[5], *BnaGLNs* and *BnaNIAs*[6]gene expression promotes nitrogen assimilation and utilization. Under low phosphorus conditions, plants induce up-regulation of the purple acid phosphatase gene *PpAPs* to promote the synthesis and secretion of acid phosphatase (ACP) in roots, enhancing the activation and absorption capacity of insoluble phosphorus in soil [7]. Meanwhile, up-regulation of phosphorus transport related genes (*PHT1*, *PHT1.10*, *SPX6*, and *SPX-MFS2*) contributes to the effective transport and redistribution of phosphorus in plants [8–10]. These molecular response mechanisms reveal how plants optimize nutrient absorption and utilization through gene expression regulation under nutritional stress, providing important molecular targets for cultivating crop varieties with high nutrient utilization efficiency.

Under nitrogen and phosphorus deficiency, plant root growth is restricted and photosynthesis is hindered. Therefore, plants optimize root growth and maintain photosynthetic efficiency by regulating the expression of a series of genes. Under low nitrogen and low phosphorus conditions, plants regulate root development by modulating plant hormone signals and interacting with related genes. For example, under low phosphorus treatment, *GmGDPD2* regulates the levels of root hormones such as gibberellin, auxin, and ethylene by sensing the upstream signal *GmMyb73*, inhibiting main root elongation, inducing lateral root and root hair formation, thereby affecting phosphorus absorption and utilization [11]; The expression of *GmEIL4* in soybean roots is significantly upregulated, which can promote the growth and development of hairy roots, increase root hair density, root length, and root surface area, improve phosphorus absorption and utilization ability, and *GmEIL4* and *GmEBF1* jointly participate in regulating the ethylene metabolism pathway of soybean, thereby affecting the response of roots to low phosphorus stress [12]. Under low nitrogen stress, the expression of auxin synthesis gene *YUCCA* and auxin inward transporter *AUX1* related genes in peanuts is downregulated, leading to a decrease in auxin content and limiting the occurrence and elongation of lateral roots [13]; The *BnIPTs* gene in rapeseed is downregulated in the roots, leading to a decrease in cytokinin concentration in the roots, which promotes root elongation and increases the number of root tips, enhancing their ability to absorb nutrients from the soil and increasing their resistance to low nitrogen stress [14]. In addition, due to adversity stress, plants cope with the increase in reactive oxygen species content in their bodies by upregulating the expression of antioxidant enzyme related genes (*SOD9* and *CAT2*), thereby increasing the activity of antioxidant enzymes in plants and maintaining the balance of intracellular reactive oxygen species [15]. However, the expression levels of chlorophyll catabolism related genes (*MdNYC1*, *MdPAO*, and *MdSGR1*) significantly increased [16], while the expression levels of PSII related genes (*PsbO*, *PsbP*, *PsbQ*, and *PsbY*) were downregulated [17, 18], resulting in a decrease in chlorophyll content and photosynthetic efficiency. These molecular regulatory mechanisms reveal how plants balance growth needs and resource constraints through precise regulation of gene expression under nutritional stress, providing important molecular targets for cultivating stress tolerant crop varieties.

Ilex chinensis is widely used in urban greening and disease treatment due to its excellent ornamental value and significant medicinal value [19–21]. However, the significant variations in climatic conditions and soil characteristics across different regions may lead to deficiencies or imbalances of N and P in the

soil. For example, some areas lack essential N and P elements due to climate drought or soil barrenness, while others are affected by industrial pollution, frequent acid rain, and soil pH decline, which in turn reduce the availability of N and P. All of these factors have adverse effects on the growth and survival rate of *I. chinensis*. Therefore, investigating the optimal concentrations of N and P for the growth of *I. chinensis*, clarifying the roles of N and P in its growth and development, and understanding the impacts of N and P deficiency on plant growth are of great significance for the cultivation management and sustainable development of this species.

The aim of this study is to evaluate the effects of different nitrogen and phosphorus concentrations on the growth status and physiological indicators of *I. chinensis* by simulating nitrogen and phosphorus deficit conditions and comparing them with normal growth conditions. The aim is to clarify the phenotypic changes of *I. chinensis* under nitrogen and phosphorus deficit conditions. The research results will provide theoretical support for screening suitable nitrogen and phosphorus concentrations for *I. chinensis* growth. In addition, this study will explore the gene response mechanism of *I. chinensis* under nitrogen and phosphorus deficiency through transcriptome analysis, and identify key response genes. The research results will provide scientific basis for creating transgenic *I. chinensis* varieties with high nitrogen and phosphorus utilization efficiency, and provide theoretical references for efficient plant cultivation, water and fertilizer management, and development and utilization.

2 Materials and Methods

2.1 Plant Materials and Growth Conditions

The plant materials for this experiment were wild species of *Ilex chinensis* Sims, collected from Tianmu Mountain in Hangzhou, Zhejiang Province, eastern China. The collected seeds were stored in low-temperature sand, and seedlings of *I. chinensis* were obtained through seedling cultivation. Two-year-old seedlings with a uniform height of 1.0-1.10 m were selected for the study. Two weeks before the start of the experiment, the seedlings were transplanted into pots (height 30 cm, diameter 20 cm) filled with river sand, with one seedling per pot. During the experimental period, the seedlings were maintained in a greenhouse with a temperature of (25 ± 2)°C during the day and (20 ± 2)°C at night, a relative humidity of (70 ± 5)%, and natural light intensity.

The plant species were identified by plant classification experts Yan Daoliang and Lou Luhuan. Voucher specimens have been deposited at the Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE), with the accession number 4111.

2.1.1 Compliance with Guidelines

All experimental research on plants, including the collection of plant materials, was conducted in compliance with institutional, national, and international guidelines. Field studies were conducted in

accordance with local legislation. The necessary permissions and/or licenses were obtained for the collection of wild plant materials.

2.1.2 Compliance with the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)

This study adheres to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The plant species used in this study are not listed as endangered under CITES, and all collection and use of plant materials were conducted in accordance with the guidelines provided by CITES.

2.2 Experimental Design

The experiment utilized a modified Hoagland nutrient solution with the following composition (mg/L): $Ca(NO_3)_2{\cdot}4H_2O$ 616.64, KNO_3 376.34, KH_2PO_4 101.49 and $MgSO_4$ 60. Iron was supplied as Fe-EDTA solution (2.5 mL/L), and micronutrient solution (5 mL/L) was added, containing (mg/L): KI 0.83, H_3BO_3 6.2, $MnSO_4{\cdot}4H_2O$ 22.3, $ZnSO_4{\cdot}7H_2O$ 8.6, $Na_2MoO_4{\cdot}2H_2O$ 0.25, $CuSO_4{\cdot}5H_2O$ 0.025 and $CoCl_2{\cdot}6H_2O$ 0.025. Five treatment groups were established: (1) normal nutrient supply (CK, nitrogen (N) concentration 125 mg/L, phosphorus (P) concentration 0.75 mmol/L), (2) mild low-N (LN_1, N concentration 25 mg/L, P concentration 0.75 mmol/L), (3) severe low-N (LN_2, N concentration 8.33 mg/L, P concentration 0.75 mmol/L), (4) mild low-P (LP_1, N concentration 125 mg/L, P concentration 0.15 mmol/L), and (5) severe low-P (LP_2, N concentration 125 mg/L, P concentration 0.05 mmol/L). Other essential elements were maintained at standard levels to meet plant growth requirements. Each treatment was replicated three times, with 10 plants per replicate. The corresponding nutrient solution was applied every 7 days until leaching from the bottom of the pots, for a total of 10 weeks. Sampling was conducted at week 0 (T_0), week 5 (T_1), and week 10 (T_2) to measure plant growth indicators and physiological parameters.

2.3 Measurements of Growth and Physiological Parameters

2.3.1 Total Biomass and Root-Shoot Ratio

Three plants were randomly selected from each treatment group for de-potting. The above-ground and below-ground parts were separated and thoroughly washed with deionized water to remove adhering soil and impurities. Subsequently, the samples were placed in a constant-temperature oven at 80°C until a constant weight was achieved. The dry weights of the above-ground and below-ground parts were measured using an electronic balance with a precision of 0.0001 g (ME104E, Mettler Toledo, Greifensee, Switzerland). The root-shoot ratio (RSR) was then calculated.

RSR = Root Dry Weight / Shoot Dry Weight

2.3.2 Determination of Plant Root System Parameters

Three plants were randomly selected from each treatment group for de-potting. The roots were carefully washed to remove any adhering soil and impurities. The total root length and root branch number were then measured using the LA-S plant image analysis system (LA-S, Hangzhou Wanshen Detection Technology Co., Ltd., Hangzhou, China).

2.3.3 Determination of leaf antioxidant enzymatic system index

Enzyme extracts were prepared using phosphate buffer (pH 7.8), and enzyme activities were measured using standard colorimetric methods. The content of malondialdehyde (MDA) was determined using the thiobarbituric acid reaction method [22]. The activity of superoxide dismutase (SOD) was assessed by its ability to inhibit the reduction of nitroblue tetrazolium (NBT) [23]. The activity of peroxidase (POD) was measured by its capacity to oxidize guaiacol [24].

2.3.4 Determination of enzyme activity and anthocyanin content related to nitrogen and phosphorus metabolism in leaves

The activities of acid phosphatase (ACP), nitrate reductase (NR), and glutamine synthetase (GS) in leaves, as well as anthocyanin content, were measured using commercial kits from Suzhou Mengxi Biotechnology Co., Ltd. (Suzhou, China).

2.4 Transcriptomic Analysis

Leaves from *I. chinensis* plants under N and P deficiency at time point T_2 were collected from three treatment groups: CK, LN_2, and LP_2. The leaves were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis, with three biological replicates per sample. Total RNA was extracted from *I. chinensis* leaves by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and its purity and quantity were checked via a bioanalyzer (Agilent, Santa Clara, CA, USA). Due to the low alignment rate of *I. chinensis* to existing genomes, the analysis of transcriptome was conducted without the reference genome. NovaseqTM 6000 (Illumina, San Diego, CA, USA) was used to sequence samples in PE150 mode, and Trinity was used to perform gene de novo assembly on sequencing data to obtain unigenes and transcripts (gene isoforms). Using DIAMOND for functional annotation of unigenes, and six databases (NCBI_NR, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Pfam, SwissProt, and eggNOG) were used to perform the annotation, with an E-value < 0.00001.

Differential expression analysis was conducted using the criteria of fold change (FC) ≥ 2 or FC ≤ 0.5 (i.e., |log2FC| ≥ 1) and false discovery rate (FDR) < 0.05 to identify differentially expressed genes (DEGs). For multiple group comparisons, genes with FDR < 0.05 were selected to identify statistically significant differences across groups. To further classify the functions of the DEGs, GO functional annotation and KEGG pathway enrichment analysis were conducted.

2.5 Statistical Analysis

All data were subjected to statistical analysis using SPSS 22.0 software. One-way analysis of variance (ANOVA) was employed to compare differences among different treatment groups, with the significance level set at $P < 0.05$.

3 Results

3.1 Effects of low-nitrogen (LN) and low phosphorus (LP) on growth traits of *Ilex chinensis*

As shown in Fig. 1, at time point T_2, significant changes in leaf area and color were observed among the different treatment groups. Compared with the CK, under low-nitrogen (LN) stress, the color of *I. chinensis* leaves gradually lightened from dark green to light green with increasing stress intensity. The leaves also became thinner and exhibited reduced leaf area. Under LP_1, the leaf color of *I. chinensis* only slightly lightened, with no significant difference in leaf area. However, under LP_2, the leaves showed obvious chlorosis and yellowing, along with the appearance of dry, yellow spots.

As shown in Fig. 2, nitrogen and phosphorus deficiency significantly inhibited the growth and development of *I. chinensis*. Under CK conditions, the total biomass of *I. chinensis* increased by 11.05% and 14.97% at T_1 and T_2, respectively, compared with T_0. However, under LN and LP stress, the increase in total biomass was lower than that of CK and showed a continuous downward trend with prolonged stress duration. At T_2, compared with T0, the total biomass of the LN_2 and LP_2 treatments only increased by 10.8% and 12.14%, respectively (Fig. 2A). Moreover, N and P deficiency significantly increased the root-shoot ratio (RSR) of *I. chinensis* (Fig. 2B). At T_2, the RSR of the LN_2 and LP_2 treatments increased by 27.73% and 23.25%, respectively, compared with CK. Nevertheless, no significant differences were observed between the LN and LP treatment groups at T_1 and T_2.

Figures 2C and 2D indicate that N and P deficiency significantly affected the root growth of *I. chinensis*. At T_0, there were no significant differences in primary root length and root branch number among the different treatment groups of *I. chinensis*. At T_1 and T_2, no significant differences were observed in these indices within each LN and LP treatment group. However, compared with CK, the primary root length of the LN treatment groups LN_1 and LN_2 significantly increased by 14.77% and 7.44% at T_1, and by 16.28% and 9.22% at T_2, respectively. The root branch number significantly increased by 16.66% and 20.03% at T_1, and by 10.33% and 12.97% at T_2, respectively. For the LP treatment groups LP_1 and LP_2, the primary root length significantly increased by 13.52% and 8.29% at T_1, and by 15.53% and 8.9% at T_2, respectively. The root branch number increased by 17.45% and 18.45% at T_1, and by 11.62% and 12.88% at T_2, respectively. Compared with T_0, at T_1, the primary root length of LN_1 and LN_2 increased by 21.58% and 23.81%, respectively, and the root branch number significantly increased by 22.16% and 15.71%, respectively. For LP_1 and LP_2, the primary root length increased by 20.35% and 22.55%, respectively, and the root branch number significantly increased by 22.99% and 16.89%, respectively.

3.2 Effects of LN and LP on Antioxidant Enzyme Activities in *I. chinensis*

As shown in Fig. 3, at T_2, the activities of superoxide dismutase (SOD) and peroxidase (POD), as well as the content of malondialdehyde (MDA), increased most significantly in the LN_2 and LP_2 groups. Specifically, in the LN_2 group, the activities of SOD and POD and the content of MDA were 1072.95 $U \cdot g^{-1}$, 153.8 U/g·min, and 23.98 μmol/g, respectively, which were significantly higher than those in the CK group by 67.56%, 151.79%, and 248.04%, respectively. In the LP_2 group, the activities of SOD and POD and the content of MDA were 1058.48 U/g, 120.8 U/g·min, and 16.14 $\mu mol \cdot g^{-1}$, respectively, which were significantly higher than those in the CK group by 65.46%, 97.81%, and 134.25%, respectively. At each treatment period, the activities of SOD and POD and the content of MDA in the LN_2 group were significantly higher than those in the LN_1 group. Under low-phosphorus stress, only the trends of SOD activity and MDA content were similar to those under low-nitrogen stress. At T_1, there were no significant differences in POD activity between the LP_1 and LP_2 groups, but at T_2, the POD activity in the LP_2 group was significantly lower than that in the LP_1 group.

3.3 Effects of LN Treatment on the Activities of Nitrate Reductase (NR) and Glutamine Synthetase (GS) in I. chinensis Leaves

Under LN stress, the activities of NR and GS in the leaves of all treatment groups increased (Fig. 4), but the extent of the increase varied among groups. As the stress duration extended, the NR activity in the LN_1 and LN_2 groups was significantly higher than that in the CK group, reaching a peak at T_2, with increases of 130.15% and 290.19% compared to CK, respectively. Additionally, the GS activity in LN_2 was significantly higher than that in LN_1 and much higher than CK throughout all treatment periods. Specifically, at T_1 and T_2, the GS activity in LN_2 was 60.89% and 112.36% higher than CK, respectively, and 23.78% and 56.02% higher than LN_1, respectively.

3.3 Effects of LP Treatment on Anthocyanin Content and Acid Phosphatase (ACP) Activity in I. chinensis Leaves

Under LP treatment, both anthocyanin content and ACP activity exhibited a continuous upward trend, with the LP_2 group consistently showing higher levels than the LP_1 group across all treatment periods (Fig. 5). For instance, at T_1, the anthocyanin content and ACP activity in the LP_2 group were 37.07% and 22.33% higher than those in the LP_1 group, respectively. By T_2, the difference in anthocyanin content further increased, while the difference in ACP activity decreased, with the LP_2 group showing 52.08% higher anthocyanin content and 9.68% higher ACP activity compared to the LP_1 group.

3.4 Transcriptome Sequencing Analysis

3.4.1 Assessment and Assembly of Transcriptome Sequencing Data

Illumina sequencing technology was employed to compare and analyze the transcriptome data of CK, LN_2, and LP_2 samples. A total of 55.34 Gb of valid reads were obtained, comprising 376,227,290 reads. The effective data volume for each sample ranged from 5.05 Gb to 7.38 Gb. The proportion of bases with a quality value of ≥ 20 (Q20) was over 98.09%, and the average GC content was 45.22% (Table 1). Pearson correlation analysis of the samples (Fig. 6) revealed high similarity among biological replicates and minor differences in gene expression levels within each treatment group, while distinct differences were observed between different treatment groups. Therefore, the sequencing quality was satisfactory, and the data were suitable for subsequent comparative and analytical studies.

Table 1
Sample sequencing data

Sample	Raw_Reads	Raw_Bases	Valid_Reads	Valid_Bases	Valid%	Q20%	GC%
CK_1	43563974	6.53G	41599030	6.11G	95.49	98.31	45.17
CK_2	42925382	6.44G	40531846	5.94G	94.42	98.38	45.76
CK_3	42502884	6.38G	41094998	6.06G	96.69	98.34	44.97
LN2_1	41826864	6.27G	40459498	5.96G	96.73	98.29	44.79
LN2_2	41153310	6.17G	39746028	5.86G	96.58	98.32	44.55
LN2_3	52467644	7.87G	50180432	7.38G	95.64	98.32	44.85
LP2_1	48985036	7.35G	46849450	6.89G	95.64	98.37	45.64
LP2_2	43207630	6.48G	41426462	6.09G	95.88	98.31	45.59
LP2_3	35648318	5.35G	34339546	5.05G	96.33	98.09	45.74

3.4.2 Differential Gene Expression Analysis

In this study, two comparisons were established: LN_2 vs. CK and LP_2 vs. CK. A total of 2480 differentially expressed genes (DEGs) were identified in the LN_2 vs. CK comparison, and 960 DEGs were identified in the LP_2 vs. CK comparison. Compared with the CK group, 845 DEGs were up-regulated and 1635 DEGs were down-regulated in the LN_2 treatment (LN_2 vs. CK). In the LP_2 treatment (LP_2 vs. CK), 401 DEGs were up-regulated and 559 DEGs were down-regulated. Under LN and LP, both comparison groups exhibited fewer up-regulated DEGs than down-regulated DEGs. Additionally, the total number of DEGs identified in the LN treatment group was higher than that in the LP treatment group (Fig. 7A). This suggests that the response mechanisms of *I. chinensis* to LN conditions are more complex. Furthermore, a Venn diagram was constructed to compare the shared DEGs among the treatment groups (Fig. 7B). A total of 445

common DEGs were identified in both LN and LP treatment groups. These shared DEGs likely play important roles in the resistance of *I. chinensis* to N and P deficiency stress.

3.4.3 GO and KEGG Enrichment Analysis of DEGs

The GO enrichment analysis (Fig. 8A) elucidated the functional profiles of differentially expressed genes (DEGs) in *I. chinensis* under LN and LP stress conditions. Under LN stress, DEGs in the LN_2 group were predominantly enriched in terms associated with chloroplast thylakoid membrane, ribosomal structure, thylakoids, photosynthesis, transition metal ion binding, and cellular transition metal ion homeostasis. In contrast, under LP stress, DEGs in the LP_2 group were primarily enriched in terms related to cell wall, heat response, ARF guanine nucleotide exchange factor, extrinsic component of the plasma membrane, plant-type hypersensitive response, and signal transduction.

KEGG enrichment analysis (Fig. 8B) further elucidates the metabolic regulatory pathways in *I. chinensis* under LN and LP stress. The results indicate that *I. chinensis* employs both common and unique metabolic regulatory pathways in response to LN and LP stress. In the LN treatment group, the KEGG metabolic pathways are primarily enriched in photosynthesis, ribosomes, cyanate metabolism, phenylpropanoid biosynthesis, flavonoid biosynthesis, photosynthesis - antenna proteins, nitrogen metabolism, and tyrosine metabolism. In the LP treatment group, the KEGG metabolic pathways are mainly enriched in flavonoid biosynthesis, arachidonic acid metabolism, plant-pathogen interaction, isoquinoline alkaloid biosynthesis, cutin and suberin biosynthesis, and tyrosine metabolism. The metabolic pathways commonly enriched by both LN and LP treatment groups include cyanate metabolism, phenylpropanoid biosynthesis, and flavonoid biosynthesis.

These results indicate that *I. chinensis* adapts to LN and LP stress by modulating the expression of different genes. Under LN conditions, the DEGs in *I. chinensis* are primarily involved in processes related to photosynthesis and protein synthesis. In contrast, under LP conditions, the DEGs are more extensively involved in biosynthetic processes and cellular development-related processes.

3.4.4 Expression Patterns of DEGs in Key KEGG Pathways

Based on the KEGG-related metabolic pathway enrichment results in response to nitrogen and phosphorus deficiency in *I. chinensis*, the significantly enriched key KEGG pathways in each treatment group were selected for further analysis. A heatmap analysis was performed to illustrate the expression patterns of the differentially expressed genes in the key KEGG pathways in the low-nitrogen treatment group (Fig. 9). The results showed that most of the differentially expressed genes involved in the key KEGG pathways exhibited down-regulated expression, with only a small proportion of genes showing up-regulated expression.

Specifically, genes involved in photosynthesis included TRINITY_DN17859_c0_g1 (*PSBO*), TRINITY_DN4395_c0_g1 (*PSBO*), TRINITY_DN9156_c0_g1 (*XYLA*), TRINITY_DN15339_c0_g1 (*PETE*), TRINITY_DN2383_c0_g1 (*PSBW*), TRINITY_DN6719_c0_g1 (*PSBW*), TRINITY_DN2042_c2_g1 (*SEND33*), TRINITY_DN14464_c0_g1 (*fdxH1*), TRINITY_DN4423_c0_g1 (*PSAK*), TRINITY_DN383_c1_g1 (*ATPC*), and

TRINITY_DN51_c0_g1 (*PSAO*), with the exception of TRINITY_DN9156_c0_g1 (*XYLA*), which was up-regulated, while the others were down-regulated.

Genes involved in ribosome metabolism included TRINITY_DN5698_c0_g1 (*RCA*), TRINITY_DN320_c1_g1 (*SOG1*), TRINITY_DN4378_c0_g1 (*APS1*), TRINITY_DN37697_c0_g1 (*mrpl19*), TRINITY_DN16074_c0_g1 (*RPP1A*), TRINITY_DN2782_c0_g1 (*UBI3*), TRINITY_DN7984_c0_g1 (*ALTA5*), TRINITY_DN412_c0_g1 (*UBQ8*), TRINITY_DN2973_c0_g1 (*RPS28*), TRINITY_DN9933_c0_g1 (*RPL18B*), TRINITY_DN6931_c0_g2 (*ycf3*), TRINITY_DN4920_c0_g1 (*RAN1A*), and TRINITY_DN3843_c0_g1 (*RPL24*), with the exception of TRINITY_DN320_c1_g1 (*SOG1*), TRINITY_DN9933_c0_g1 (*RPL18B*), and TRINITY_DN3843_c0_g1 (RPL24), which were up-regulated, while the others were down-regulated.

Genes involved in cyanamide metabolism included TRINITY_DN11758_c0_g1 (*CAS2*), TRINITY_DN26728_c0_g1 (*SABP2*), TRINITY_DN5704_c0_g1 (*MES10*), TRINITY_DN29012_c0_g2 (*AtMg00310*), TRINITY_DN32055_c0_g1 (*AtMg01250*), TRINITY_DN8246_c0_g1 (*At1g65750*), and TRINITY_DN4045_c0_g1 (*BGLU12*), with the exception of TRINITY_DN11758_c0_g1 (*CAS2*) and TRINITY_DN4045_c0_g1 (*BGLU12*), which were down-regulated, while the others were up-regulated.

Genes involved in phenylpropanoid biosynthesis included TRINITY_DN9992_c0_g1 (*F6'H1*), TRINITY_DN8210_c0_g1 (*4CL1*), TRINITY_DN9046_c0_g1 (*PER42*), TRINITY_DN3267_c0_g2 (*PAL*), TRINITY_DN24156_c1_g1 (*10HGO*), TRINITY_DN4868_c0_g1 (*PNC1*), and TRINITY_DN9532_c0_g1 (*PER43*), with the exception of TRINITY_DN4868_c0_g1 (*PNC1*), which was up-regulated, while the others were down-regulated.

Genes involved in flavonoid biosynthesis included TRINITY_DN5453_c0_g1 (*ANS*), TRINITY_DN5906_c0_g1 (*DLO2*), TRINITY_DN28994_c0_g1 (*DFR*), TRINITY_DN5516_c1_g1 (*NCS1*), TRINITY_DN938_c0_g1 (*SRG1*), TRINITY_DN10020_c0_g2 (*HST*), TRINITY_DN37560_c0_g1 (*CHAT*), TRINITY_DN13010_c0_g1 (*ACT*), TRINITY_DN1253_c0_g2 (*mdmC*), TRINITY_DN10247_c0_g1 (*LAR*), and TRINITY_DN8461_c0_g1 (*BP80*), with the exception of TRINITY_DN5516_c1_g1 (*NCS1*), which was up-regulated, while the others were down-regulated.

The gene TRINITY_DN9112_c0_g1 (RLP30), involved in plant stress resistance, was significantly up-regulated. Genes involved in nitrogen metabolism included TRINITY_DN26004_c0_g1 (*ACA1*), TRINITY_DN747_c0_g1 (*GS1-2*), TRINITY_DN2414_c0_g1 (*GLT1*), TRINITY_DN1838_c0_g1 (*CYN*), TRINITY_DN2438_c0_g1 (*NIA2*), and TRINITY_DN3357_c0_g1 (*BCA5*), with the exception of TRINITY_DN2438_c0_g1 (*NIA2*), which was up-regulated, while the others were down-regulated. Genes involved in tyrosine metabolism included TRINITY_DN3259_c0_g1 (*ADH1*), TRINITY_DN4933_c2_g1 (*ASP1*), TRINITY_DN8458_c0_g1 (*At1g62810*), and TRINITY_DN4153_c0_g3 (*co-2*), with the exception of TRINITY_DN8458_c0_g1(*At1g62810*), which was up-regulated, while the others were down-regulated. all of which were down-regulated.

A heatmap analysis was conducted to illustrate the expression patterns of the differentially expressed genes in the key KEGG pathways in the low-phosphorus treatment group (Fig. 9). The results indicated

that the majority of the differentially expressed genes involved in the key KEGG pathways were down-regulated, while a portion of the genes exhibited up-regulated expression.

Specifically, genes involved in flavonoid biosynthesis included TRINITY_DN38553_c0_g1 (*At5g05600*), TRINITY_DN44_c1_g1 (*CODM*), TRINITY_DN28994_c0_g1 (*DFR*), TRINITY_DN938_c0_g1 (*SRG1*), TRINITY_DN44_c3_g1 (*FHT*), and TRINITY_DN10247_c0_g1 (*LAR*). Except for TRINITY_DN38553_c0_g1 (*At5g05600*), TRINITY_DN44_c1_g1 (*CODM*), and TRINITY_DN44_c3_g1 (*FHT*), all others were down-regulated.

Genes involved in the biosynthesis of stilbenes, diarylheptanoids, and gingerols included TRINITY_DN13010_c0_g1 (*ACT*) and TRINITY_DN37560_c0_g1 (*CHAT*), both of which were down-regulated. Genes involved in arachidonic acid metabolism included TRINITY_DN23164_c0_g1 (*EPHX2*), TRINITY_DN8168_c0_g1 (*OsI_027940*), and TRINITY_DN5361_c0_g1 (*yfhM*), all of which were down-regulated.

Genes involved in plant-pathogen interaction included TRINITY_DN10034_c0_g2 (*At1g59620*), TRINITY_DN36138_c0_g1 (*EFR*), TRINITY_DN22339_c1_g1 (*R1A-10*), TRINITY_DN12377_c0_g2 (*R1A-3*), TRINITY_DN16250_c0_g1 (*At4g27190*), TRINITY_DN29459_c0_g3 (*RPM1*), TRINITY_DN37700_c0_g1 (*MKK6*), TRINITY_DN5041_c0_g3 (*RPP8L4*), TRINITY_DN3065_c0_g1 (*WRKY53*), TRINITY_DN33342_c0_g1 (*CRF3*), and TRINITY_DN8177_c0_g1 (*CG5412*). Except for TRINITY_DN36138_c0_g1 (*EFR*), TRINITY_DN16250_c0_g1 (*At4g27190*), TRINITY_DN29459_c0_g3 (*RPM1*), TRINITY_DN37700_c0_g1 (*MKK6*), TRINITY_DN5041_c0_g3 (*RPP8L4*), and TRINITY_DN33342_c0_g1 (*CRF3*), all others were down-regulated.

Genes involved in the biosynthesis of cutin, suberin, and wax included TRINITY_DN6750_c1_g1 (*PXG4*), TRINITY_DN6366_c0_g1 (*CER1*), and TRINITY_DN9279_c0_g1 (*CYP704C1*). Except for TRINITY_DN6750_c1_g1 (*PXG4*), all others were down-regulated. Genes involved in endocytosis included TRINITY_DN30224_c0_g2 (*AtMg00810*), TRINITY_DN8151_c0_g1 (*MED37D*), TRINITY_DN446_c1_g1 (*HSP70*), TRINITY_DN13409_c0_g1 (*GN*), TRINITY_DN17085_c0_g1 (*GN*), TRINITY_DN18740_c1_g1 (*GN*), TRINITY_DN4537_c0_g1 (*HSC-2*), TRINITY_DN9093_c0_g1 (*VPS2.3*), and TRINITY_DN32516_c0_g1 (*ARF1*). Except for TRINITY_DN30224_c0_g2 (*AtMg00810*), TRINITY_DN13409_c0_g1 (*GN*), and TRINITY_DN9093_c0_g1 (*VPS2.3*), all others were down-regulated.

Genes involved in sulfur metabolism included TRINITY_DN573_c1_g1 (*APR3*) and TRINITY_DN125_c0_g2 (*APS1*), both of which were up-regulated. Genes involved in cyanamide metabolism included TRINITY_DN3601_c0_g1 (*BGLU12*), TRINITY_DN5704_c0_g2 (*SABP2*), and TRINITY_DN8246_c0_g1 (*At1g65750*). Except for TRINITY_DN3601_c0_g1 (*BGLU12*), all others were up-regulated. The gene involved in tyrosine metabolism was TRINITY_DN4153_c0_g3 (*co-2*), which was down-regulated.

Genes involved in RNA degradation included TRINITY_DN10720_c0_g1 (*PAB7*), TRINITY_DN9881_c0_g1 (*PAB2*), TRINITY_DN9881_c0_g2 (*PAB6*), TRINITY_DN4343_c0_g1 (*PUP1*), TRINITY_DN22471_c0_g1

(*NAC021*), and TRINITY_DN7967_c0_g1 (*NAC029*). Except for TRINITY_DN4343_c0_g1 (*PUP1*), all others were down-regulated. Genes involved in phenylpropanoid biosynthesis included TRINITY_DN5846_c1_g1 (*TKPR1*), TRINITY_DN6578_c1_g1 (*CAD1*), and TRINITY_DN6456_c0_g1 (*gluA*). Except for TRINITY_DN5846_c1_g1 (*TKPR1*), all others were down-regulated.

4 Discussion

Nitrogen (N) and phosphorus (P) are essential macronutrients for plant growth and development and are common limiting factors in nutrient availability. The concentrations of nitrogen and phosphorus in the soil significantly influence various vital activities of plants, including the synthesis of proteins, phospholipids, nucleic acids, and overall growth and development. However, in practical agricultural and ecological settings, the availability of nitrogen and phosphorus in the soil is often affected by a variety of factors, such as human activities, soil type, natural conditions, and soil microbial communities. These factors can lead to low levels of available N and P in the soil, subjecting plants to P and N deficiency stresses, which are detrimental to plant growth and development.

To effectively cope with phosphorus and nitrogen deficiency stresses, plants employ a range of complex mechanisms to maximize their resilience. These mechanisms include phenotypic changes in plant morphology and alterations in physiological and biochemical indicators, all of which help to mitigate the adverse effects of nitrogen and phosphorus deficiency on plant growth and development. Building on this understanding, the present study primarily investigates the morphological, physiological, and molecular responses of *Ilex chinensis* to nitrogen and phosphorus deficiency and provides theoretical insights into the tolerance mechanisms of *I. chinensis* under nitrogen and phosphorus deficiency conditions.

4.1 Morphological Adaptations of *I. chinensis* Facilitate Acclimation to LN and LP

The phenotypic characteristics of the plant and the growth, morphology, and physiological traits of the root system are directly influenced by the nitrogen and phosphorus concentrations in the soil. In this study, compared with the CK group, the low-nitrogen (LN) treatment group exhibited significant phenotypic changes at T_2: the leaves of *I. chinensis* became thinner, and the color changed from dark green to light green, with yellowing signs observed in the LN_2 group. In contrast, in the low-phosphorus (LP) treatment group, the leaves in both LP_1 and LP_2 groups lacked luster. The leaf color in the LP_1 group was not significantly different from that in the CK group, while the LP_2 group showed obvious chlorosis and yellowing. These results are consistent with previous studies in *Solanum lycopersicum* [25], *Arabidopsis thaliana* [26], and *Malus pumila* [27], indicating that LP and LN stresses significantly affect the leaf development of *I. chinensis*.

Other studies have shown that under LN and LP stresses, the total plant biomass decreases, while the root-shoot ratio significantly increases, along with an increase in the length of the primary root and the number of root branches [28–30]. This conclusion is consistent with the results of the present study, where LN and LP significantly inhibited the growth of *I. chinensis*, manifested as reduced biomass but an increased root-shoot ratio. This suggests that *I. chinensis* allocates more resources to the root system to enhance the plant's absorption and utilization efficiency of N and P from the soil, thereby maintaining plant growth under N and Pdeficiency conditions.

4.2 Resistance of I. chinensis to LN and LP Stresses is Enhanced through the Antioxidant Enzyme System

The increase in malondialdehyde (MDA) content is indicative of lipid peroxidation of cell membranes and the accumulation of reactive oxygen species (ROS), reflecting the degree of membrane damage. Numerous studies have demonstrated that under low-nitrogen and LP stresses, the MDA content in plant leaves significantly increases [30–33]. The results of this study show that in *I. chinensis* leaves subjected to LN and LP treatments, the MDA content continuously rose with extended treatment duration, and this increase was more pronounced under severe stress conditions. This finding is consistent with previous research and indicates that the cell membranes of *I. chinensis* leaves are severely damaged under N and P deficiency.

To cope with ROS accumulation and membrane oxidative damage, plants enhance the activities of antioxidant enzymes (such as SOD, POD, and CAT) to scavenge excess ROS within the plant, thereby strengthening their ability to withstand adverse conditions [17, 33, 34]. In this study, under low-nitrogen stress, the activities of SOD and POD in *I. chinensis* leaves were significantly elevated and positively correlated with the duration of stress, with greater increases observed in the LN_2 group compared to the LN_1 group. Under low-phosphorus stress, the changes in SOD activity were similar to those under low-nitrogen stress. However, while POD activity was higher than that of the CK group at T_1, no significant differences were observed between the LP_1 and LP_2 groups. At T_2, POD activity significantly increased in both LP_1 and LP_2 groups, with LP_1 showing significantly higher POD activity than LP_2. This phenomenon may be attributed to the more severe phosphorus deficiency under severe stress conditions, which could inhibit the enhancement of enzyme activity.

These conclusions indicate that *I. chinensis* can mitigate damage under low-nitrogen and low-phosphorus stresses by stimulating the synthesis and activity of antioxidant enzymes, activating the antioxidant system, and scavenging excess free radicals within the plant.

4.3 Accumulation of Nitrate reductase (NR) and glutamine synthetase (GS) Activities Enhances LN Stress Resistance in I. chinensis

NR and GS are key enzymes in plant nitrogen metabolism and nitrogen absorption, and their activities are closely related to changes in nitrogen content. Their activities not only directly reflect the plant's

ability to utilize and assimilate inorganic nitrogen but also indirectly affect the plant's ability to absorb and accumulate nitrate [35, 36]. The trends in NR and GS activities under low-nitrogen stress have been confirmed in many plants, such as *Populus simonii* [37], *Triticum aestivum* [38], *Camellia sinensis*[3], and *Spinacia oleracea* [39].

The results of this study are consistent with previous research. Under low-nitrogen stress, the activities of NR and GS in *I. chinensis* leaves increased continuously with prolonged stress duration, indicating enhanced nitrogen metabolism and assimilation capabilities within the plant, which in turn improved the utilization of limited nitrogen resources. Additionally, GS activity was higher in the LN_2 group than in the LN_1 group at both T_1 and T_2, whereas NR activity showed no significant difference between the two treatment groups at T_1 but was significantly higher in the LN_2 group than in the LN_1 group at T_2. This suggests that GS activity in *I. chinensis* may be more sensitive to nitrogen deficiency than NR activity. Under short-term severe stress, *I. chinensis* may first increase GS activity to improve the utilization of limited nitrogen sources, thereby stabilizing the nitrogen content within the plant to cope with stress.

4.4 Accumulation of Acid phosphatase (ACP) Activity and Anthocyanin Content Enhances LP Stress Resistance in I. chinensis

ACP is activated in response to phosphorus starvation signals in plants, promoting the decomposition of organic, insoluble phosphorus in the environment and thereby enhancing the plant's phosphorus utilization efficiency. This mechanism plays a significant role in plant phosphorus metabolism and is one of the important strategies for plants to cope with low-phosphorus stress [40–43]. In this study, under low-phosphorus stress, the activity of ACP in *I. chinensis* leaves continuously increased with prolonged stress duration, with the most significant increase observed at T_2. Moreover, the ACP activity in the LP_2 group was significantly higher than that in the LP1 group at all treatment periods, which is consistent with previous studies in *Gossypium hirsutum* [44] and *Zea mays* [45]. These findings indicate that *I. chinensis* enhances its absorption and utilization efficiency of phosphorus from the environment under low-phosphorus conditions, thereby improving its adaptability to phosphorus-deficient environments and alleviating the adverse effects caused by phosphorus deficiency.

Anthocyanins are one of the important secondary metabolites in plants and are widely distributed in various plant organs, including roots, stems, leaves, flowers, and fruits. Under biotic and abiotic stresses, the synthesis and accumulation of anthocyanins are crucial for plant stress resistance, as they can enhance the plant's ability to scavenge ROS and mitigate damage to nucleic acids and chloroplasts caused by phosphorus deficiency [46]. In this study, the anthocyanin content in *I. chinensis* subjected to low-phosphorus stress continuously increased with prolonged stress duration. This finding is consistent with previous studies, which have shown that low-phosphorus stress promotes the synthesis and accumulation of anthocyanins in plants [17, 47–50].

In this study, the trends in anthocyanin content and ACP activity were consistent. This coordinated change may be related to the mechanisms by which plants adapt to environmental changes under stress

conditions through the regulation of metabolic pathways.

4.5 Molecular Response Mechanisms of *I. chinensis* to LN and LP Stresses Revealed by Transcriptomic Analysis

Previous studies have revealed the critical roles of multiple genes in plant growth and stress responses. Specifically, genes such as TRINITY_DN9156_c0_g1 (*XYLA*), TRINITY_DN320_c1_g1 (*SOG1*), TRINITY_DN3843_c0_g1 (*RPL24*), TRINITY_DN5516_c1_g1 (*NCS1*), and TRINITY_DN2438_c0_g1 (*NIA2*) are involved in the regulation of plant growth and nitrogen metabolism [51, 52]. Additionally, TRINITY_DN9112_c0_g1 (*RLP30*) and TRINITY_DN29459_c0_g3 (*RPM1*) participate in plant immune responses and modulate tolerance to abiotic stresses [53–55]. Furthermore, genes including TRINITY_DN38553_c0_g1 (*At5g05600*), TRINITY_DN44_c3_g1 (*FHT*), TRINITY_DN37700_c0_g1 (*MKK6*), TRINITY_DN573_c1_g1 (*APR3*), TRINITY_DN125_c0_g2 (*APS1*), and TRINITY_DN6750_c1_g1 (*PXG4*) are implicated in the regulation of phosphorus uptake and utilization, enhancing plant adaptability to phosphorus-deficient environments [56–58]. Additionally, TRINITY_DN33342_c0_g1 (*CRF3*) and TRINITY_DN4343_c0_g1 (*PUP1*) influence root development, thereby improving phosphorus uptake and utilization efficiency [59–61]. These genes may play significant roles in plant adaptation to nitrogen and phosphorus deficiency stresses.

In this study, based on the quantitative expression results, we found that the differentially expressed genes in *I. chinensis* under low-nitrogen and low-phosphorus treatments were predominantly down-regulated. However, under nitrogen deficiency stress, genes related to plant growth and nitrogen metabolism regulation, including TRINITY_DN9156_c0_g1 (*XYLA*), TRINITY_DN320_c1_g1 (*SOG1*), TRINITY_DN3843_c0_g1 (*RPL24*), and TRINITY_DN2438_c0_g1 (*NIA2*), as well as the stress-responsive gene TRINITY_DN9112_c0_g1 (*RLP30*), were significantly up-regulated. Under phosphorus deficiency stress, genes associated with phosphorus uptake and utilization, such as TRINITY_DN38553_c0_g1 (*At5g05600*), TRINITY_DN44_c3_g1 (*FHT*), TRINITY_DN37700_c0_g1 (*MKK6*), TRINITY_DN573_c1_g1 (*APR3*), TRINITY_DN125_c0_g2 (*APS1*), TRINITY_DN6750_c1_g1 (*PXG4*), TRINITY_DN29459_c0_g3 (*RPM1*), TRINITY_DN33342_c0_g1 (*CRF3*), and TRINITY_DN4343_c0_g1 (*PUP1*), were also up-regulated. These findings indicate that these genes may play crucial roles in the adaptation of *I. chinensis* to nitrogen and phosphorus deficiency stresses.

Combining the results of GO functional enrichment analysis and KEGG metabolic pathway enrichment analysis, we further elucidated the molecular mechanisms of these genes in the adaptation of *I. chinensis* to nitrogen and phosphorus deficiency stresses. Under nitrogen-deficient conditions, the up-regulated genes were primarily enriched in pathways related to nitrogen metabolism, photosynthesis, and antioxidant responses. This is consistent with the strategies employed by plants such as *Camellia sinensis* [3], *Brassica napus* [6], and *Malus pumila* [16], which enhance nitrogen metabolism and antioxidant defenses to maintain growth under nitrogen stress. Under phosphorus-deficient conditions, the up-regulated genes were mainly enriched in pathways related to phosphorus metabolism, plant-pathogen interactions, and root development. This aligns with the strategies of plants like *Pinus*

massoniana [62], *Zea mays* [15], and *Gossypium hirsutum* [63], which optimize phosphorus absorption and utilization as well as root structure to adapt to phosphorus stress. These results indicate that *I. chinensis* adapts to nitrogen and phosphorus deficiency stresses by regulating the expression of specific genes and metabolic pathways.

5 Conclusions

In this study, the physiological and molecular response mechanisms of *Ilex chinensis* to low-nitrogen and low-phosphorus conditions were analyzed through physiological indices and transcriptomic analysis (Fig. 10). The increased activities of SOD and POD enhanced tolerance to nitrogen and phosphorus deficiency. The expression of genes responsive to nitrogen and phosphorus deficiency was regulated through pathways involved in plant growth, plant immune response, phosphorus uptake, and nitrogen metabolism, thereby improving tolerance to nitrogen and phosphorus deficiency. This study elucidates the differences in growth traits, antioxidant defense systems, and nitrogen and phosphorus metabolism of *I. chinensis* under nitrogen and phosphorus deficiency stress, providing important physiological and molecular data for understanding the mechanisms by which plants enhance their tolerance to nitrogen and phosphorus deficiency.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Competing interests: The authors declare that they have no conflict of interest.

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Figures

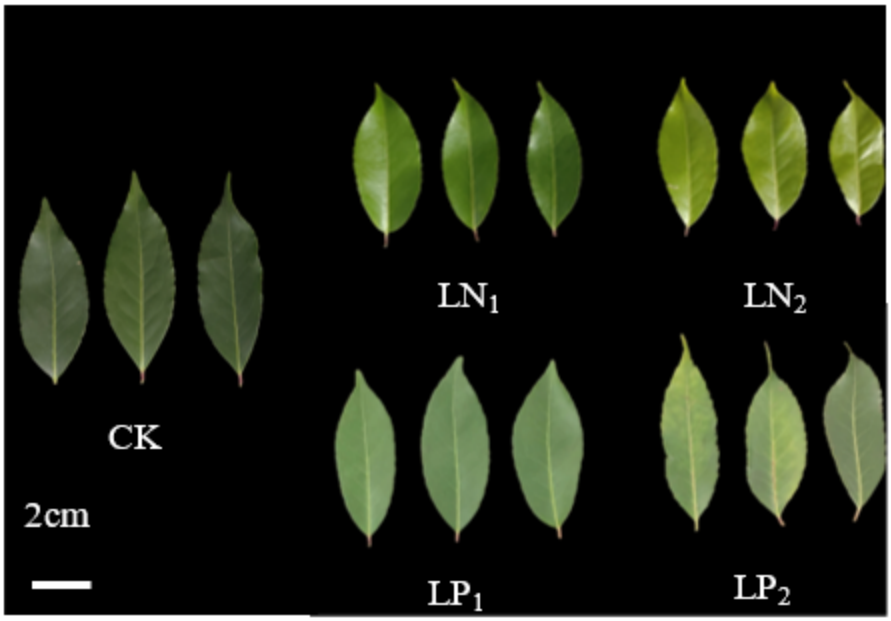


Figure 1

Phenotypic changes of *I. chinensis* leaves after T_2 treatment with LN and LP.

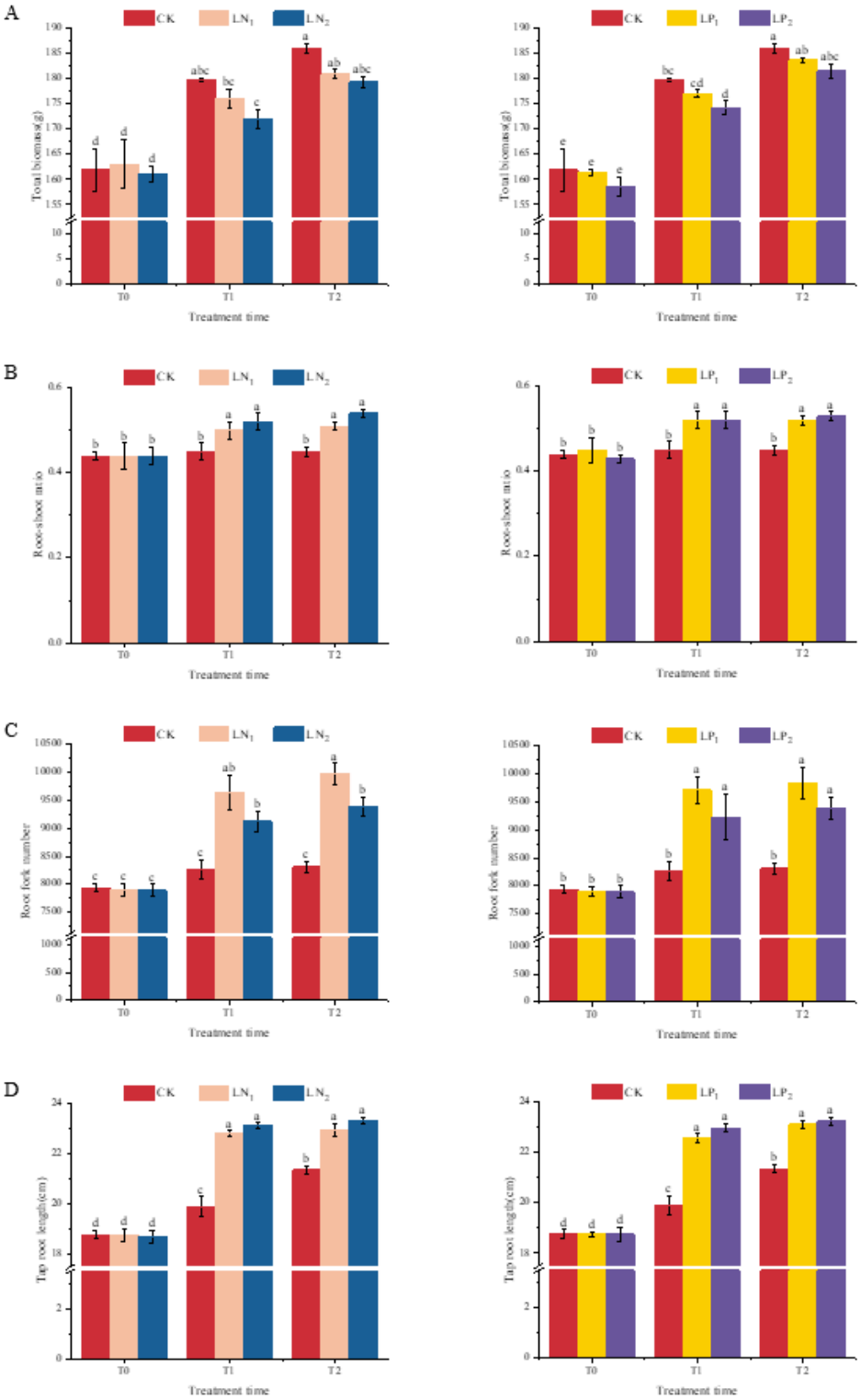


Figure 2

The effects of LN (left) and LP (right) treatments on the main growth indicators of *I. chinensis*

Notes: T_0=week 0, T_1=week 5, T_2=week 10; Different letters represent significant differences between the treatment groups (P<0.05).

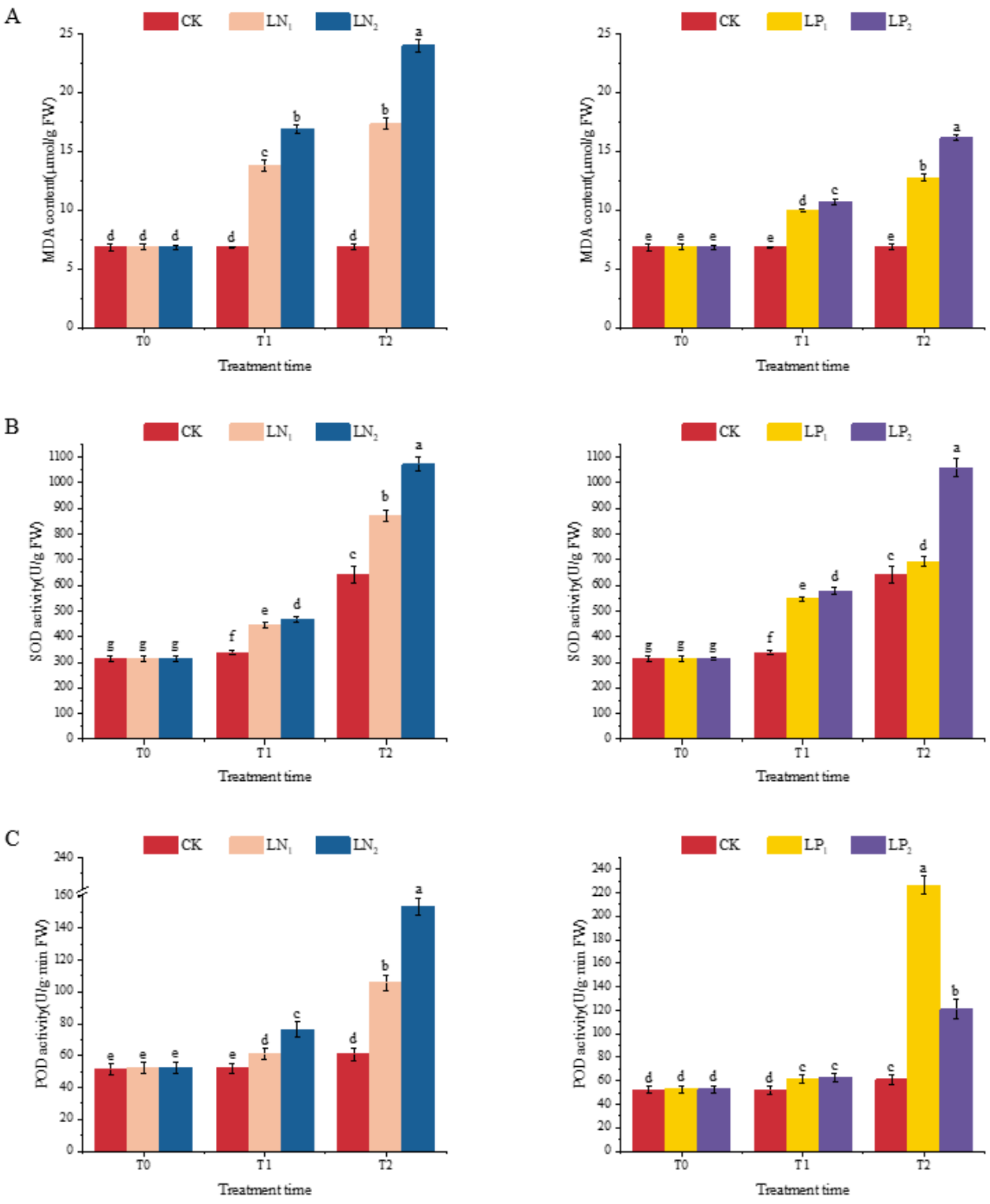


Figure 3

Determination of antioxidant enzyme system indexes of *I. chinensis* leaves under LN (left) and LP (right) treatments

Notes: T_0=week 0, T_1=week 5, T_2=week 10; Different letters represent significant differences between the treatment groups (P<0.05).

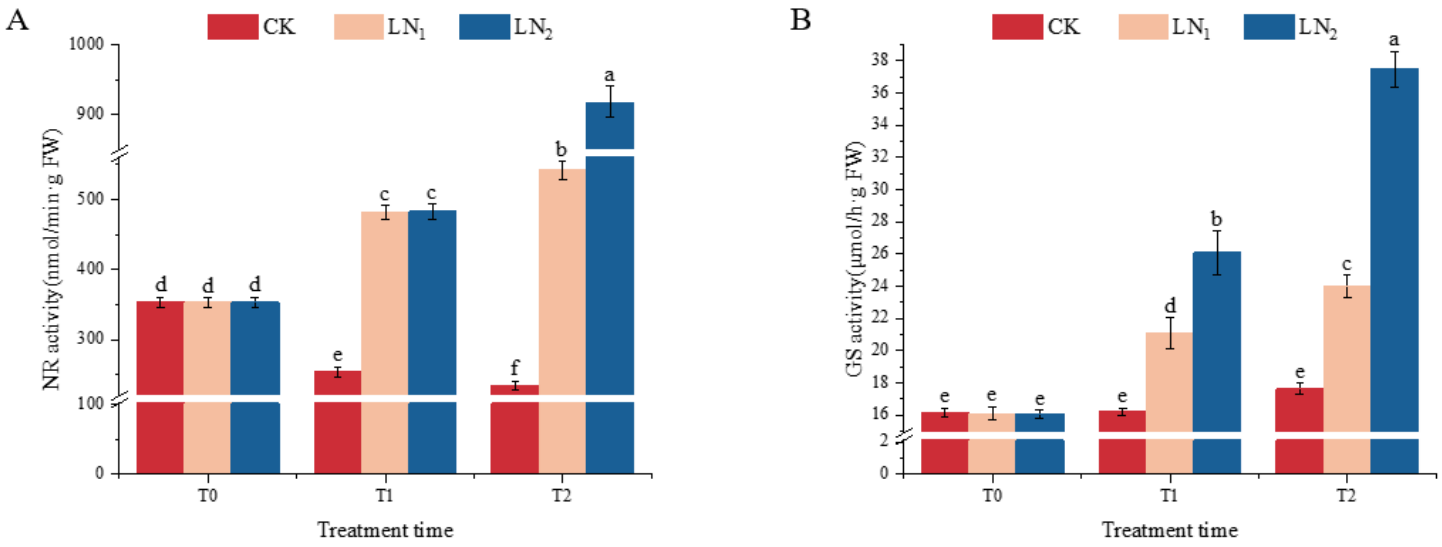


Figure 4

The effect of low nitrogen treatment on the activities of NR (A) and GS (B) in *I. chinensis* leaves

Notes: T_0=week 0, T_1=week 5, T_2=week 10; Different letters represent significant differences between the treatment groups (P<0.05).

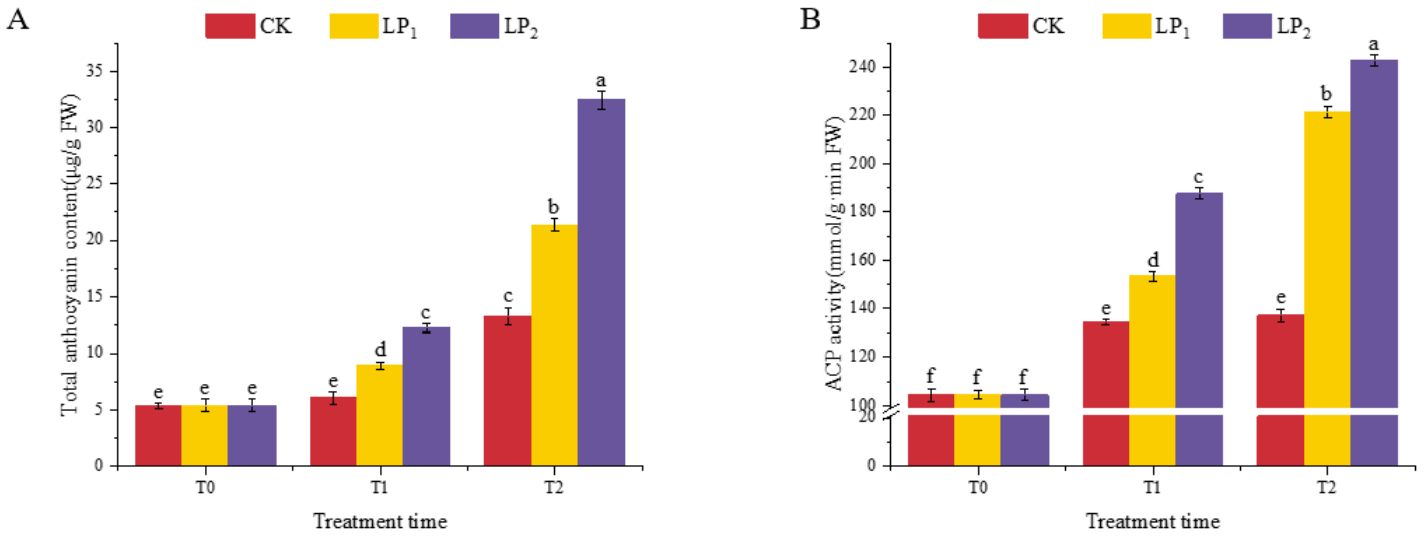


Figure 5

The effect of low phosphorus treatment on anthocyanin content (A) and ACP (B) activity in *I. chinensis* leaves

Notes: T_0=week 0, T_1=week 5, T_2=week 10; Different letters represent significant differences between the treatment groups (P<0.05).

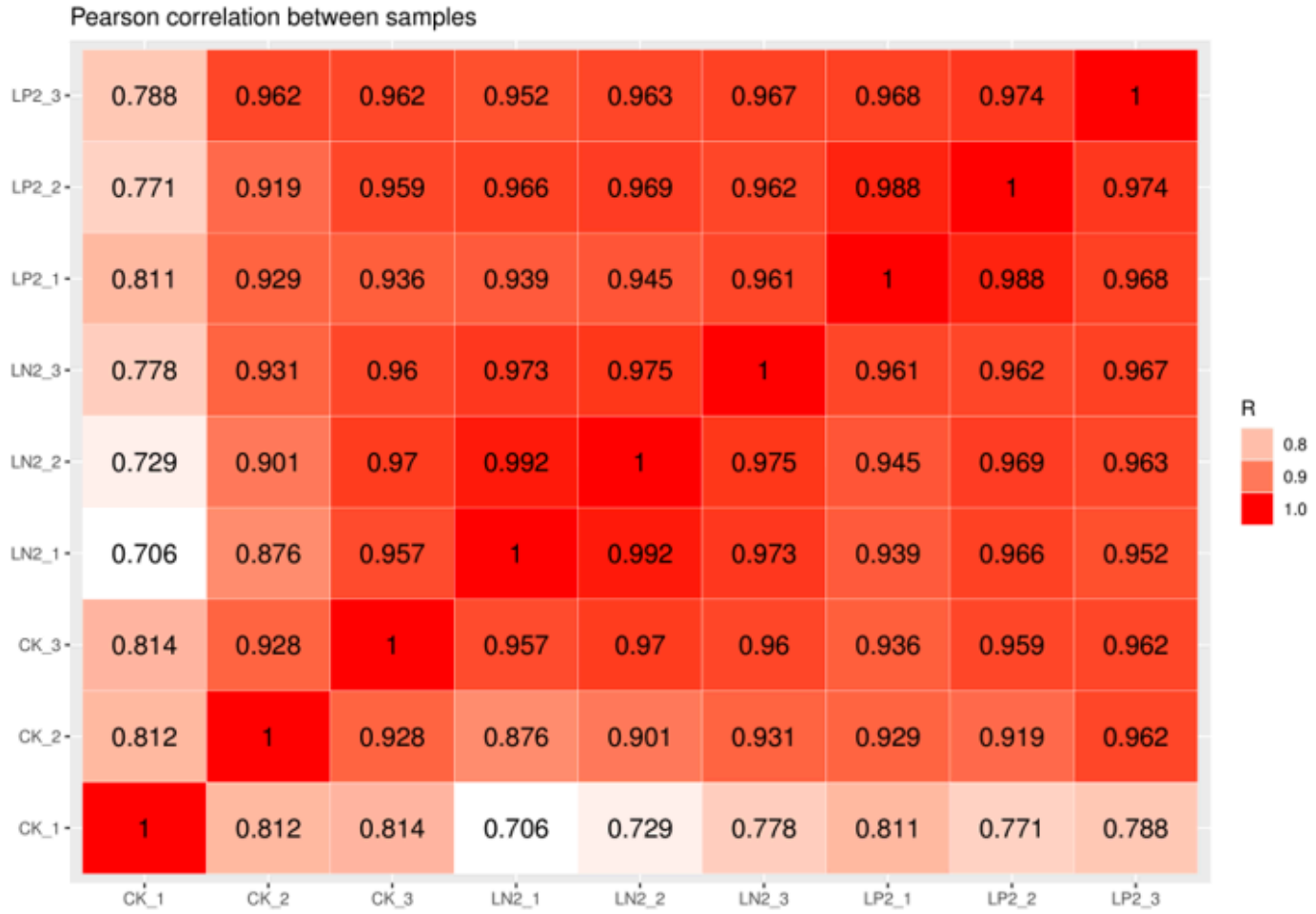


Figure 6

Pearson correlation coefficients among all samples

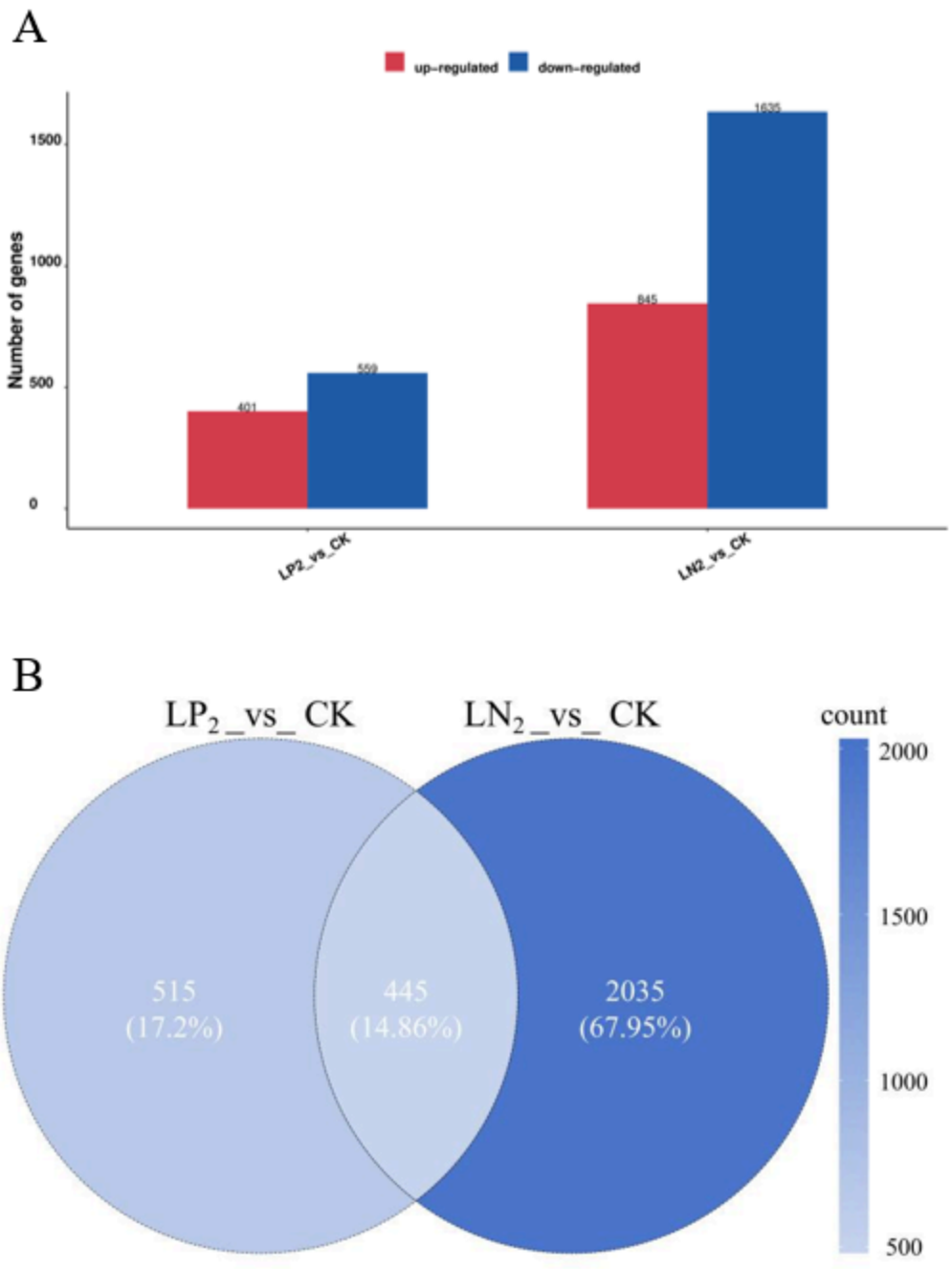


Figure 7

Comparison of differentially expressed genes in the transcriptome of *I. chinensis* under LN and LP treatment and control

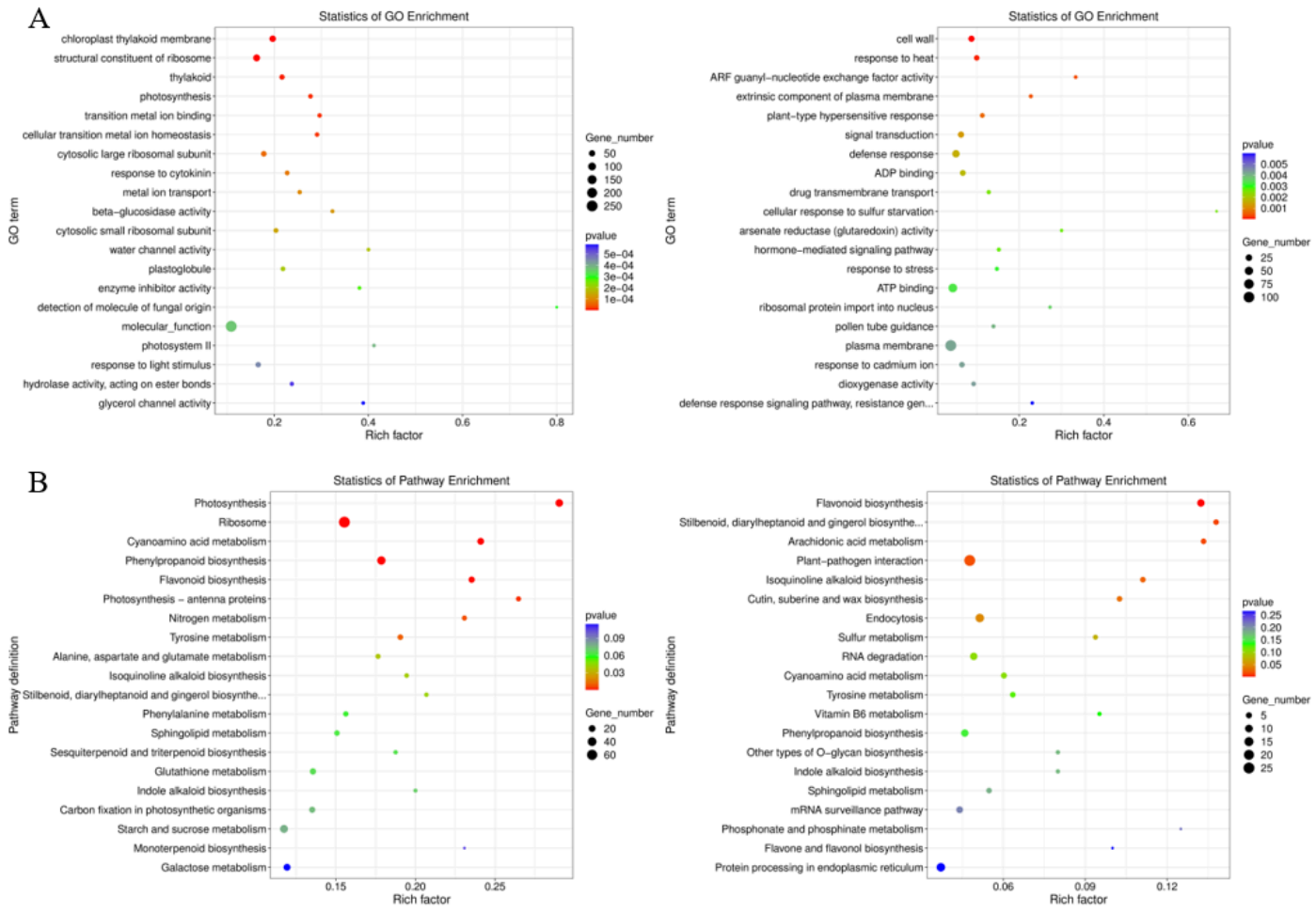


Figure 8

Bubble plot analysis of GO enrichment (A) and KEGG enrichment of differentially expressed genes under LN (left) and LP (right) treatment

Notes:

A: Results of the first 20 pathways for GO enrichment analysis of DEGs in LN_2 vs CK and LP_2 vs CK. The horizontal coordinate Rich Factor represents the proportion of the difference between the Unigenes located in the GO to the total Unigenes located in the GO (Rich.Factor = S/B). The larger the Rich factor, the higher the GO enrichment; the vertical coordinate is GO Term, that is, the GO function annotation; in the bubble chart, the size of the bubble represents the number of S, and the color of the bubble represents the P value of the enrichment analysis, that is, the significance of the enrichment. The smaller the P value, the more significant the enrichment is.

B: Results of the first 20 pathways for KEGG enrichment analysis of DEGs in LN2 vs CK and LP2 vs CK. The horizontal coordinate Rich Factor represents the proportion of the difference Unigene number located in the Pathway to the total Unigene number located in the Pathway. The larger the Rich Factor, the higher the degree of enrichment of the Pathway; the vertical coordinate is KEGG Pathway; in the

bubble chart, the size of the bubble represents S, and the color of the bubble represents the P value of the enrichment analysis, that is, the significance of the enrichment. The smaller the P value, the more significant the enrichment is.

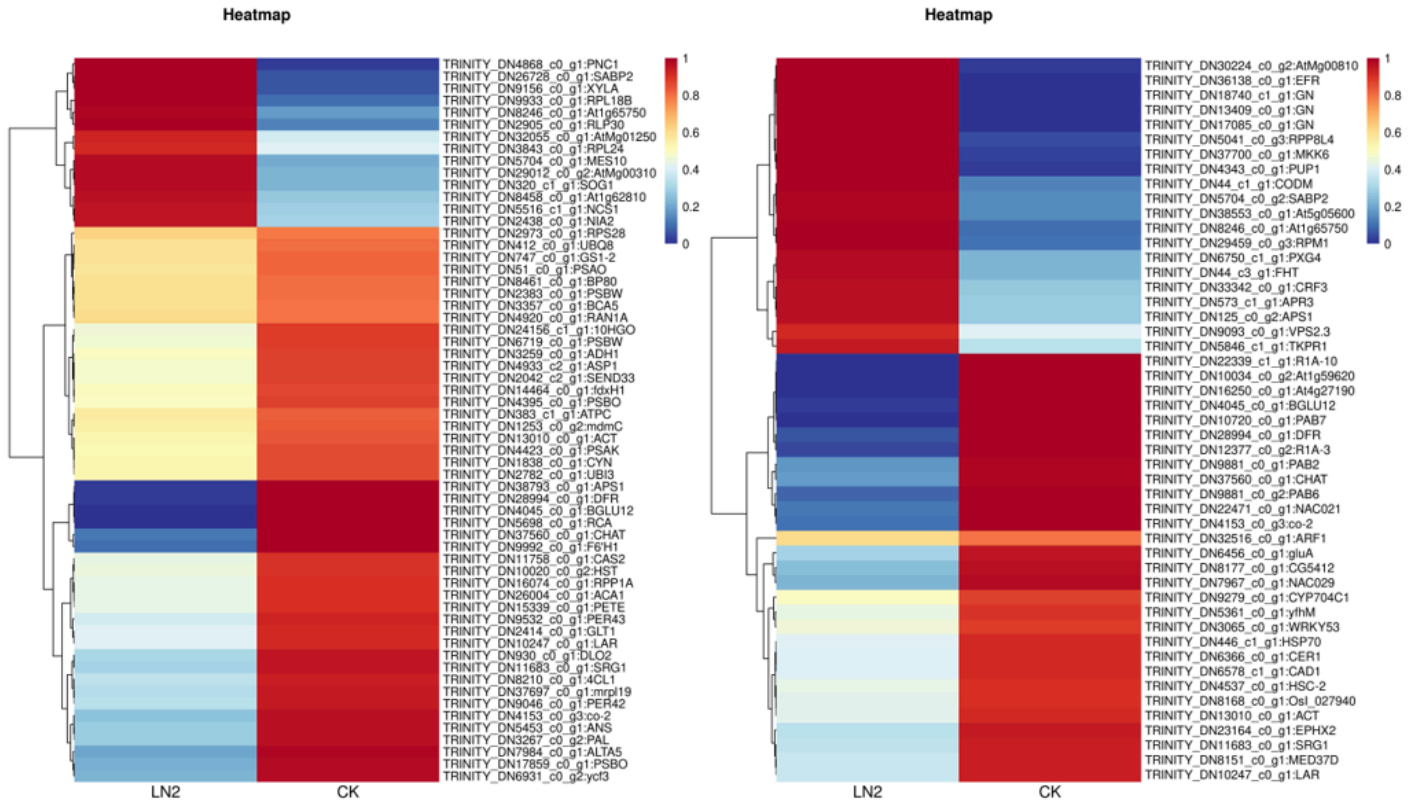


Figure 9

Heat map of differential gene expression in key KEGG pathways in the LN(left) and LP(right) treatment control group

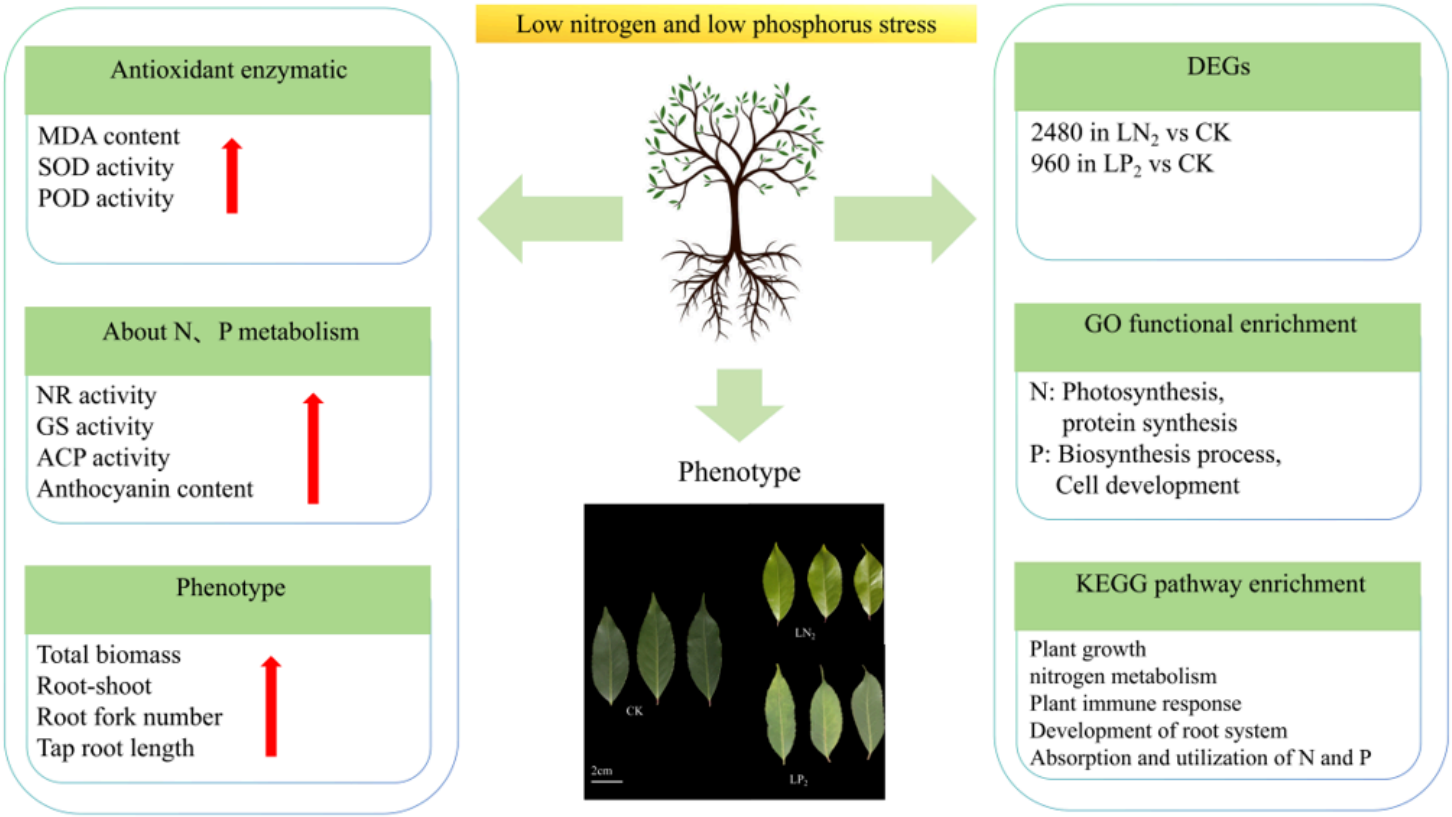


Figure 10

Schematic diagram of the multiple regulatory mechanisms of *I. chinensis* in response to nitrogen and phosphorus deficiency.