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Pretreating poplar cuttings with low nitrogen ameliorates salt stress responses by increasing stored carbohydrates and priming stress signaling pathways

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ABSTRACT

Soil salinity is a widespread stress in semi-arid forests worldwide, but how to manage nitrogen (N) nutrition to improve plant saline tolerance remains unclear. Here, the cuttings of a widely distributed poplar from central Asia, *Populus russikki* Jabl., were exposed to either normal or low nitrogen (LN) concentrations for two weeks in semi-controlled greenhouse, and then they were added with moderate salt solution or not for another two weeks to evaluate their physiological, biochemical, metabolites and transcriptomic profile changes. LN-pretreating alleviated the toxicity caused by the subsequent salt stress in the poplar plants, demonstrated by a significant reduction in the influx of Na^+ and Cl^- and improvement of the K^+/Na^+ ratio. The other salt-stressed traits were also ameliarated, indicated by the variations of chlorophyll content, PSII photochemical activity and lipid peroxidation. Stress alleviation resulted from two different processes. First, LN pretreatment caused a significant increase of non-structural carbohydrates (NSC), allowed for an increased production of osmolytes and a higher potential fueling ion transport under subsequent salt condition, along with increased transcript levels of the cation/H^+ ATPase. Second, LN pretreatment enhanced the transcript levels of stress signaling components and phytohormones pathway as well as antioxidant enzyme activities. The results indicate that early restrictions of N supply could enhance posterior survival under saline stress in poplar plants, which is important for plantation programs and restoration activities in semi-arid areas.

1. Introduction

Saline soils are widespread globally and they are expected to expand to over 50% of arable lands by 2050 (Jamil et al., 2011). Salt stress increases osmotic stress and induces ionic toxicity in plants due to the influx of excess Na^+ and Cl^- (Raddatz, 2020; Oliveira et al., 2019). Ion toxicity, in turn, favors cytosolic K^+ efflux, imbalance of ion homeostasis and nutrition deficiency, amongst others, ultimately leading to cellular oxidative damage, photosynthetic pigments degradation, growth inhibition and plant wilting (Garcia et al., 2017; Raddatz, 2020). Plants have developed a series of adaptations to tolerate salinity. For instance, membrane sodium antiporters, including Na^+/H^+ exchangers (NHXs and SOS1), seek to exclude excessive Na^+ from the cell cytoplasm. Similarly, the class I high affinity K+ transporters (HKT1s) mediates Na^+ removal from the xylem sap, to prevent shoot Na^+ excess (Zelm et al., 2020). Due to chemical similarity between Na^+ and K^+, one major aspect associated with salt toxicity is that Na^+ may replace K^+ within cellular functions. The previously reported salt adaptations strategy in plants generally come down to increase the cellular level of the K^+ / Na^+ ratio, avoiding the competition between Na^+ and K^+ within cellular functions (Yang et al., 2008).

Seedling "hardening" is a common practice in many nurseries

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worldwide, where exposure of a seedling to a mild stress improves its tolerance to subsequent stresses (Wojtyla et al., 2020). However, there still lack enough studies on the effects of earlier application of N deprivation on plant responses to subsequent stress in plants. It has been known that nutritional starvation alter the carbon/nitrogen (C/N) balance in plants, which plays an role in signal delivery (Maekawa et al., 2012). It is expected that the signaling pathways for stress-related phytohormones, such as ethylene and jasmonate, would be activated by LN and confer an advantage under subsequent salt stress.

Comparing the transcriptome profiles in response to different stresses (applied alone or in combination) is useful to understand which responses are specific to a given stress, or represent shared responses to the different stresses (Sharma et al., 2018). Along those lines, the present study has been conducted to jointly compare transcriptome responses to both, high salinity and LN concentrations. It is thus currently unknown to which extent are responses to high salinity similar to those under LN, and whether the former stress could be used to acclimate the plant to the latter.

Poplar species are important for the ecological restoration of saline areas in arid and semi-arid regions of Central Asia, where *Populus russikki* Jabl. grows naturally and is widely used in plantations (Ma et al., 2016). *P. russikki* shows a mild degree of salt tolerance and, under high nitrogen (N) concentrations, it shows fast-growing rates and a well-developed root system (Ma et al., 2016). Here, we sought to understand which of the responses in *P. russikki* to salt and N stresses are shared across both stresses, and which ones occur only in response to salt stress. In particular the general hypothesis would been tested that treating *P. russikki* with LN would induce an acclimation response and improve subsequent response to salt stress. To this end, a factorial experiment has been performed, and the responses to salinity in poplar cuttings would be examined for the plants previously exposed to either "normal" N or LN concentrations. More specifically, the hypothesis would be tested: 1) decreasing sink activities under LN would trigger an increase in non-structural carbohydrates (NSC) and, consequently, higher osmotic resistant by increased production of osmoprotectants; 2) LN would trigger a signaling response in some stress-related phytohormones pathways that would enhance salt tolerance. To this end, transcriptome profile changes have been used to assess metabolic traits, particularly the processes related to carbon and nitrogen metabolisms, and how LN-induced metabolic changes influence the responses to subsequent salt tolerance.

2. Materials and methods

2.1. Plant growth conditions and treatments

One-year old branch cuttings of *P. russikiki* in the lower reaches of the Tarim River, northwestern China (40′25′59″ N, 88′01′34″ E) were sampling and planted in 10 L pots containing a mixture of quartz sand and vermiculite (2:1). All the pots were watered regularly to field capacity with 1/2 hoagland solution. After 6 weeks of growth in pots, 96 cuttings with similar height (~0.5 m) and stem diameter (1.5 mm) were selected for the experiment in semi-controlled greenhouse, with temperature varying between 20 °C and 25 °C, a daylength of 14–16 h and photosynthetically active radiation of 350–900 μmol m^{-2} s^{-1}. The pots, containing the quartz sand/vermiculite mixture, were washed by soaking them for 4 h under tap water (constantly flowing water) in order to remove N and other soluble nutrients before implementing treatments. The plants were divided into two halves. The first received "normal" or control N application in a modified 1/2 hoagland solution [450 mg L^{-1} NH_4NO_3, 93.25 mg L^{-1} KCl, 172 mg L^{-1} $CaSO_4$, 34 mg L^{-1} KH_2PO_4, 125 mg L^{-1} $MgSO_4$, 9.325 mg L^{-1}, Fe-EDTA and other microelements were also added]. The second half of the plants received low nitrogen (LN) application with a modified 1/2 hoagland solution as above except limited N (2 mg L^{-1} NH_4NO_3). The solution was renewed every four days. After two weeks of growth in the different N treatments, salt solution (4383 mg L^{-1} NaCl (75 mM)) or similar amount of tap water were added to form a factorial design (Li et al., 2021; Yao et al., 2021). That is, there were 4 treatments, i.e. normal N and no salt addition (control), normal N and salt addition (S), low concentrations of nitrogen (LN), and also LN pretreatment followed with salt treatment (LNS). There were 8 replicates for each treatment. The 1/2 hoagland solution or LN solution were similar as before.

After 2 weeks of salt treatment, the fully-expanded leaves from the upper canopy and fine roots were harvested and washed with distilled water. Half of the harvested samples were immediately frozen in liquid N for RNA isolation and measurements of biochemical traits. The remaining half were dried to constant weight at 70 °C for determination of free inorganic ions, soluble sugar and total carbon and total nitrogen. Two days before harvest the PSII photochemistry traits were measured by chlorophyll fluorescence.

2.2. RNA extraction

Total RNA was isolated using the RNA Extraction Kit (BioTeke, Beijing, China). The quality and quantity of RNA were checked by a spectrophotometer (IMPLEN, CA, USA). Purified RNA samples were sent to BGI Co. Ltd. (Shenzhen, Chin) for library preparation and RNA-seq analysis in leaves were performed using an Illumina platform with a paired-end 150 bp sequencing strategy.

2.3. RNA-seq data assay

Primary sequencing data, or raw reads, were subjected to quality control (QC) and then filtered into clean reads that aligned to the *Populus trichocarpa* sequences by Bowtie 2. The reads numbers mapped to each gene were counted by HTSeq v0.6.1 (Anders et al., 2015). The FPKM method was used to measure the expression level. We then tested for differences in differentially expressed genes (DEGs) between two groups by the NOISeq method (Tarazona et al., 2011). We also developed a DEG heatmap among different treatments with the javaTreeview software (de Hoon et al., 2004). Genes usually interact with each other to play roles in certain biological functions, and pathway enrichment analysis of DEGs were constructed on the KEGG database (Kanehisa et al., 2008). The scattered plots for the top 20 of KEGG enrichment pathways have been demonstrated.

2.4. Quantitative real time PCR analyses

The gene expression patterns from the RNA-seq analysis in leaves was validated by quantitative real time PCR (qRT-PCR). Especially, the expression patterns of genes related to different ion transporters (K^+/Na^+, NO_3^-) were analyzed by qRT-PCR method in leaves and also in roots and bark (The bark is used for qRT-PCR analysis of *PtNRT1.7* and *PrNRT1.9* expressed in the phloem).

Total RNA in fully-expanded leaves was extracted by the RNAprep Pure Plant Kit (Invitrogen) and treated with DNase for removal of genomic DNA. Then the total RNA was reverse-transcribed by reverse transcriptase (Invitrogen). The transcripts of relative genes were determined by quantitative real time PCR (qRT-PCR), the poplar actin (GenBank accession BU879695) and polyubiquitin (UBQ, accession BU879229) were used as internal quantitative controls (Brunner et al., 2004). All gene accessions, gene-specific primers, and corresponding references are listed in Table S1. The PCR amplification procedure consisted of an initial step at 95 °C for 1 min followed by 39 cycles of 5 s at 95 °C, 10 s at 58 °C and 30 s at 72 °C. Amplification of the target gene in every cycle was quantified by SYBR Green (Qiagen, Hilden, Germany), and the relative expression abundance of the target genes was determined by the $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen, 2001).

2.5. Determination of total carbon, total nitrogen and ion concentration

Samples were dried to constant weight at 70 °C for 24 h, and then ground to fine powder. The contents of total N and total carbon were determined by the vario Max Organic Elemental Analyzer (Elementar Analysensysteme GmbH, Germany). Ion analyses were performed with homogenized dry matter for each treatment. After extraction with nitric acid for 0.2 g, the concentration of cations was determined by an Atomic Absorption Spectrometer (AA 700, shimadzu Corp. Japan) and of anions (except NO_3^-) by Ion Chromatography (ICS-5000, Thermo. Sci. Inc., Waltham). For the determination of NO_3^- concentration, it was extracted for the fine powder (ca 0.100 mg) with 1 ml of deionized water at 45 °C for 2 h in 1 ml deionized water at 45 °C for 1 h, and then measured according to Patterson et al. (2010).

2.6. Determination of the concentration of total amino acid, proline, photosynthetic pigments, anthocyanin, soluble sugar, and non-structural carbohydrates (NSC)

Total free amino acids were extracted according to Winter et al. (1992), and analysed by ninhydrin reaction method. More specially, proline concentration was determined according to Karolewski (1985). The soluble protein was extracted and determined by coomassie brilliant blue G-250 as in Bradford (1976). Anthocyanins were extracted by methanol:HCl (99:1, v/v) and determined at 630 nm using a UV–vis spectrophotometer. The photosynthetic pigments was extracted by 95% ethyl alcohol, and chlorophyll a, chlorophyll b and carotenoids were measured by UV/VIS spectrophotometer (UV-5500 PC) according to Nayek et al. (2014).

NSCs include total soluble sugar and starch per dry mater (mg g^{-1}). To measure them, the fresh sample was first boiled in distilled water as above to extract the soluble sugars, and then the extract solution was filtered to obtain the soluble solution. After that, the starch in the sample was hydrolyzed by $HClO_4$ (Kabeya and Sakai, 2003). Soluble sugars and hydrolyzed starch were measured at 630 nm using a UV–vis spectrophotometer (UV-1700, Shimadzu Crop., Japan) by anthrone colorimetric method.

2.7. Determination of PSII photochemical activity

The PSII photochemical activity was measured after 10 days salt treatment, between 09:00 and 11:00 h, using a portable pulse-modulated fluorometer (Mini-PAM II, Heinz Walz, Effeltrich, Germany). The minimal and maximal fluorescence (F_0 and F_M) were measured in dark-adapted leaves using a saturation pulse for weighing maximum quantum efficiency of PSII (Fv/Fm). For the light-adapted leaves, the chlorophyll fluorescence was measured by varying the photosynthetically active radiation (PAR) stepwise from 900, 800,700, 600, 500, 400, 300, 200, 100, 50 and 0 μmol m^{-2} s^{-1}. During the light response curves, it was measured for F_s, and light-adapted maximal fluorescence (Fm'). These parameter were used to calculate electron transport rate (ETR), quantum Yield (*Y*), photochemical quenching (Qp), and Non-photochemical quenching (NPQ). The ETR was determined to the rate of electron transport efficiency through photosystem II (PSII), *Φ*PSII to measure the quantum efficiency of PSII electron transport, Qp to provide an indication of the proportion of PSII reaction centers that were open, NPQ to monitors the apparent rate constant for heat loss from PSII (Murchie et al., 2013).

2.8. Determination of reactive oxygen species (ROS) and associated enzymes

Fresh young and old leaves (0.3 g) of each treatment were homogenized with 5% trichloroacetic acid (TCA) to extract the malondialdehyde (MDA), a marker of the degree of oxidative stress. The homogenate was centrifuged at 3000 r/min for 10 min to obtain a clear solution. MDA concentration was determined by thiobarbituric acid method according to Ohkawa et al. (1979). The Superoxide dismutase (SOD, EC 1.15.1.1), an enzyme that prevents antioxidant damage, was extracted by 0.05 mol L^{-1} sodium phosphate buffer (pH 7.8) and enzyme activity was determined according to (Beyer and Fridovich, 1987). Catalase (CAT, EC 1.11.1), an enzyme reducing H_2O_2 to H_2O, was extracted and determined according to Kar and Mishra (1976). Peroxidase (POD, EC1.11.1.7), an enzyme also involved in oxidative defense, was extract by 0.1 mol L^{-1} Tris-HCl (pH8.5), and enzyme activity was determined following Ohkawa et al. (1979).

2.9. Statistical analyses

The statistical program SPSS 13.0 (SPSS, Chicago, USA) was used for analyses. Significant differences among different treatments were tested using two-ways ANOVA. The differences across treatments were considered to be significant when the *P*-value of the ANOVA *F*-test was less than 0.05. For each parameter, pairwise comparisons among different treatment were conducted using LSD (least significance difference) test at $p < 0.05$ levels.

3. Results

3.1. Transcriptome analyses

RNA-seq analyses of fully-expanded leaf tissues in different treatments (control, LN, S, LNS) resulted in 271.8 million clean raw reads totally. 70,351 genes on the 12 samples (three replicates per treatment) were identified, and there were significant differences in global gene expression patterns across treatments (Fig. S1–S2). The number of different expressed genes (DEGs) were 237 in LN, 401 in S, 334 in LNS relative to control. Additionally, there were 338 DEGs in LNS in comparison with LN (Fig. 1; Tables S2–S5). The transcriptome profile changes were confirmed with qRT-PCR method for 10 DEGs related to the genes functioned in sodium or nitrate transport as well as nitrate assimilation (Fig. S3), and the high relation for the transcript analysis by two methods showed that the RNA-seq method can effectively reflect the transcriptome changes under salt and LN stresses.

Heatmap analyses of DEGs across pairwise treatment combinations (Fig. S4) indicated that the expression pattern was pronouncedly different between S and LN, relative to the control. KEGG pathway analyses showed that the DEGs induced by LN were concentrated on biosynthesis of secondary metabolites, which included up-regulation of flavonoid biosynthesis, flavone and flavonol biosynthesis, isoflavonoid biosynthesis, the down-regulation of diterpenoid, diaryheptanoid and stilbenoid biosynthesis (Fig. S5 a and S6; Table S2). The transcriptome weakening occurred in the processes associated with DNA replication,

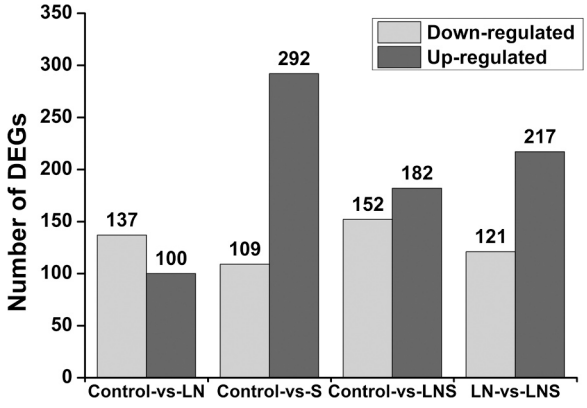

Fig. 1. Number of differentially expressed genes (DEGs) across pairwise treatment comparisons. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively.

base excision repair and homologous recombination. Biological processes associated with N metabolism were also affected by LN, like N-Glycan biosynthesis and cyanoamino acid metabolism.

The response to salt stress alone (S) was, at least to some extent, different to that in response to LN. The up-regulated responses for pathogen infection and insect attack was observed, like plant-pathogen interaction responses and insulin resistance (Fig. S5 b and S7; Table S3). Salt stress also induced the pathway of other stress responses, like signal transduction of stress-related hormones and endocytosis for compound detoxification. In addition, carbon metabolism was regulated by salt, as there was an up-regulation of galactose metabolism, citrate cycle pathway, starch/sucrose metabolism, pentose and glucuronate interconversions, but it showed a down-regulation of biological process in flavonoid biosynthesis and isoflavonoid biosynthesis. Amino acid metabolism was affected, including tyrosine metabolism, alanine, aspartate and glutamate metabolisms, cysteine and methionine metabolisms.

When the salt treatment followed a LN pretreatment (LNS), LN-induced biological metabolism primed the responses for subsequent salt stress. The transcript changes induced by the LNS treatment fall into intermediate of those changes induced by LN and by S treatments (Fig. S5 c and S7; Table S4). That is, KEGG analysis showed that DEGs of LN vs LNS concentrated on changes in metabolic pathways (mainly primary metabolism), down-regulation of biosynthesis of secondary metabolites, and up-regulation of plant-pathogen interactions (Fig. S5 d and S8; Table S5).

3.2. Ion balance

Na^+ concentrations in leaves and roots increased from 0.97 and 2.00 mg g^{-1} in the control to 16.72 and 21.64 mg g^{-1} after salt stress applications (S) (Fig. 2a,b). However, salt concentration in LNS (12.34 and 19.47 mg g^{-1} in leaves and roots) was significantly lower than in S, indicating that previous growth reduction under LN alleviated subsequent salt stress. Along with Na^+ enhancements, K^+ concentrations in both leaves and roots decreased by 50–33% after salt application (Fig. 2c,d). However, K^+ concentrations in both, LN and LNS treatment, were significantly higher than in the S treatment (Fig. 2c,d). We observed that Na^+ transporters, such as the high affinity Na^+/K^+ transporters *PrHAK1.1* and *PrHKT1.2,* were up-regulated under S in roots (Fig. 3d, f), whereas down-regulated in *PrHKT1.1*(Table S2-S4). The transcripts in the salt-stressed leaves were increased for *PrSOS1* (S and LNS-stressed leaves), *PrNHX1* (LNS-stressed leaves), one vacuolar cation/proton exchanger (Unigene24157_Prusskii) and for one vacuolar membrane H^+-transporting ATPase (Unigene36527_Prusskii, Table S3) (Fig. 3i; Table S3).

The concentrations of Ca^{2+} and Mg^{2+} in leaves and roots were significantly reduced (over 20%) in LN whereas no significant change in root Ca^{2+} level (Fig. 2e–h). This response is partly driven by the reduced demand for charge balance of the NO_3^- anion. Conversely, the concentrations of these two cations were significantly increased in leaves and roots (25–50%) after salt additions (except root Ca^{2+}, which stayed constant). Consistent with these results, an up-regulation caused by salt application occurred for the transcripts of Ca^{2+}/H^+ exchanger and Ca^{2+}/Na^+ exchanger (Unigene10416_Prusskii; CL1794. Contig2–4_Prusskii; Unigene2278_Prusskii) (Table S3). The up-regulation of Ca^{2+}-ATPase and Mg^{2+}-ATPase (Unigene11066_Prusskii) was also observed, which could provide salt-stressed plants with more energy for Ca^{2+} and Mg^{2+} absorption.

Regarding anion responses, there existed significant increased in Cl^- concentrations in response to S. LN did not significantly affect Cl^- concentrations in roots (relative to the control), but there was a positive effect in roots. LNS led to significantly lower Cl^- in leaves but to no change in roots (relative to S, Fig. 5a–b). SO_4^{2-} concentrations significantly increased after S, LN and LNS in both leaves and roots. However, the increase in SO_4^{2-} was largest in LN (79–127%), smallest in S (43–81%) and intermediate in LNS (59–82%; Fig. 5c–d). It was also observed for the up-regulation of the genes associated with sulfur relay

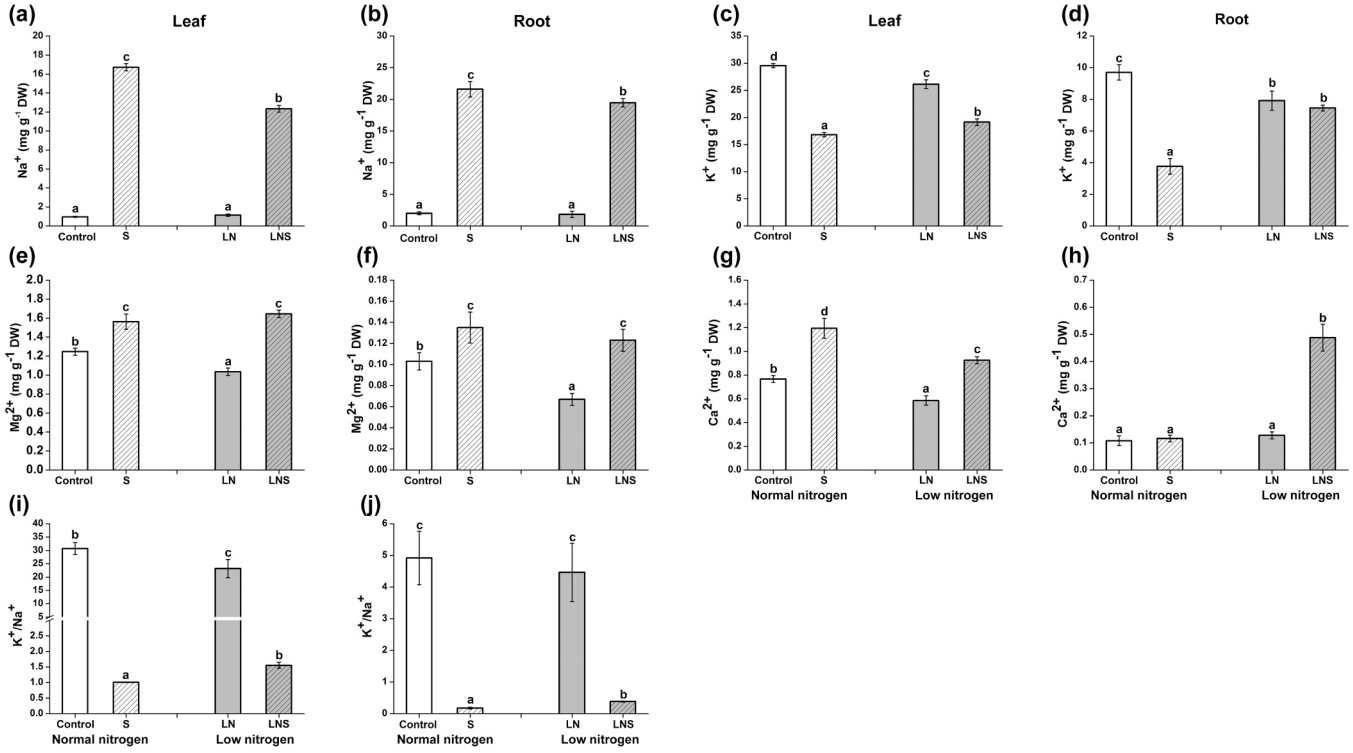

Fig. 2. Concentrations of inorganic cations and the K^+/Na^+ ratio in leaves and roots of *P. russikki* across different treatments. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

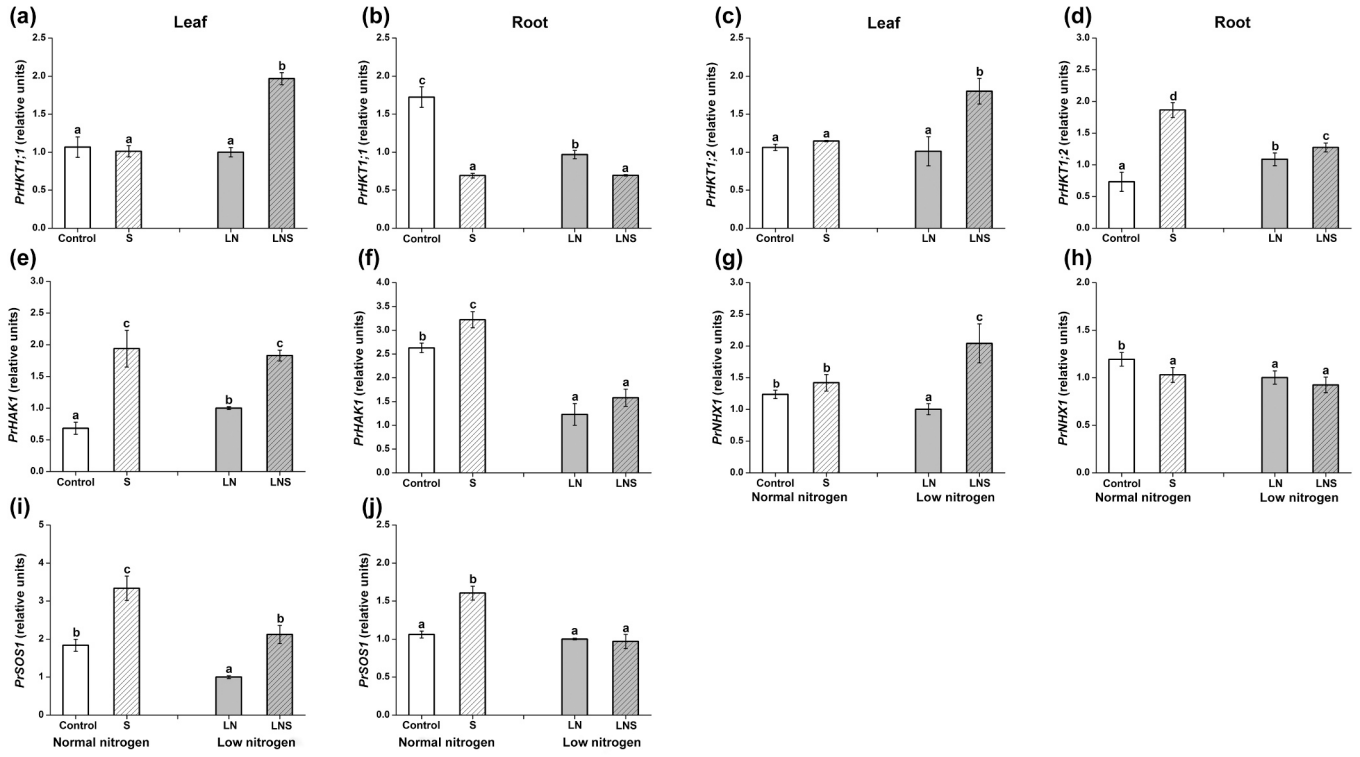

Fig. 3. Expression of genes involved in Na^+ absorption and transport in leaves and roots of *P. russikki* across different treatments. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

and sulfur transfer (Fig. S6–S7). The transciptome analyses also showed that the synthesis for sulfur-related amino acid or polypeptide (synthesis of glutamate, tyrosin, cysteine and GSH compounds) were up-regulated by salt,

3.3. Photosynthetical pigment and photochemical analyses

Chlorophyll concentrations (Chl*a*, Chl*b* and total chlorophyll) were significantly declined in all treatment applications, relative to the control. The decline was largest in LN (about 30%), smallest in S (about 15%), and intermediate in LNS (23–27%; Fig. 6). The ratio of Chl*a*/Chl*b* remained constant across treatments, indicating that the different N and salt treatments impacted both pigments to the same extent. Chlorophyll is a N-containing compound and its decline under LN is thus expected. Transcript levels for chlorophyll degradation were additionally increased by LN (Fig. S6). Total carotenoid concentrations, which are N-free molecules, were also significantly affected by LN, S and LNS. However, in this case the effect was larger in S (41% decline) and LNS (42%) than in LN (about 28%, Fig. 6e). On the other hand, anthocyanin concentrations significantly increased in S (13%), but the increase was significantly larger in LN (39%) and LNS (35%; Fig. 6f).

Leaf photochemistry was significantly affected by salt stress (Fig. S9), with pronounced reductions in F_v/F_m, *ΦPSII*, *ETR* and *qP* and a significant increase in *NPQ*. In particular, the negative effects of salt led to a peak *ETR* at a PAR of 65 μmol m^{-2} s^{-1} with subsequent declines at higher radiation (Fig. S9 a). These results indicate a strong photoinhibition in *P. russikki* even when PAR is as low as 65 μmol m^{-2} s^{-1} under saline soils. The effects of LN on leaf photochemistry were minor (relative to the control), except for *NPQ* which was marked lower. However, leaf photochemistry under LNS condition was modestly improved in comparison with sole salt treatment.

3.4. Carbon / nitrogen metabolism and C/N balance

Total N and NO_3^- concentrations significantly declined in roots and leaves under both LN and S treatments (Fig. 4a–d). Similarly, the transcript levels of the main nitrate transporter, *PrNRT1.1*, also declined in roots and leaves (Fig. S10). In particular, more N was allocated to leaves or roots separately by LN and salts (Fig. 4c, d), separately showing by the increased and reduced leaf N / root N ratio. This was driven by changes in the transcription of genes associated with N uptake (*PrNRT1.5* and *PrNRT1.8*, Fig. S12) or with the N assimilation pathway (*PrNIA*, *PrNIR*, *PrGS-1*, *PrGDH*, Fig. S13). The *PrNRT1.5*a/c in the roots was up-regulated by salt. The transcription of N assimilation-related genes were affected in roots to a lesser extent than in leaves in response to treatments, like *PrNIA*, *PrNIR*, *PrGS-1*, *PrGDH* (Fig. S13). Total leaf N concentrations were reduced by LN and further reduced in LNS leaves, but not in roots. It seemed that LN made the root N reach a threshold level so that the following salt treatment could not further reduce root N level. Accordingly, total soluble protein concentrations were reduced by either LN or salt stress in both roots and leaves, and that they were further reduced by LNS (Fig. 4e–f). On the contrary, free amino acid concentrations in both roots and leaves declined under LN, especially sharply in roots, but there was a significant increase under salt stress (Fig. 4g–h). LN-caused reduction in amino acid concentration was, at least partly, reversed by salt stress, as showed by modest increase in LNS treatment. In particular, the concentration of proline was increased significantly after salt application (Fig. 4i–j). On the other hand, in all the treatments (LN, S, LNS) there exhibited significant increase of the transcript of *PrNRT1.7*, which functioned in relocation of NO_3^- and amino acids to young leaves from old leaves (Fig. S11).

Due to the reduction of chlorophyll and photochemical efficiency, the total carbon concentrations in roots and leaves were significantly reduced by LN and S, while LNS slightly alleviated salt-caused carbon loss in both roots and leaves (Fig. 7e–f). The C/N ratio increased under N deprivation (regardless of salt stress) in both roots and leaves (Fig. 7h–i).

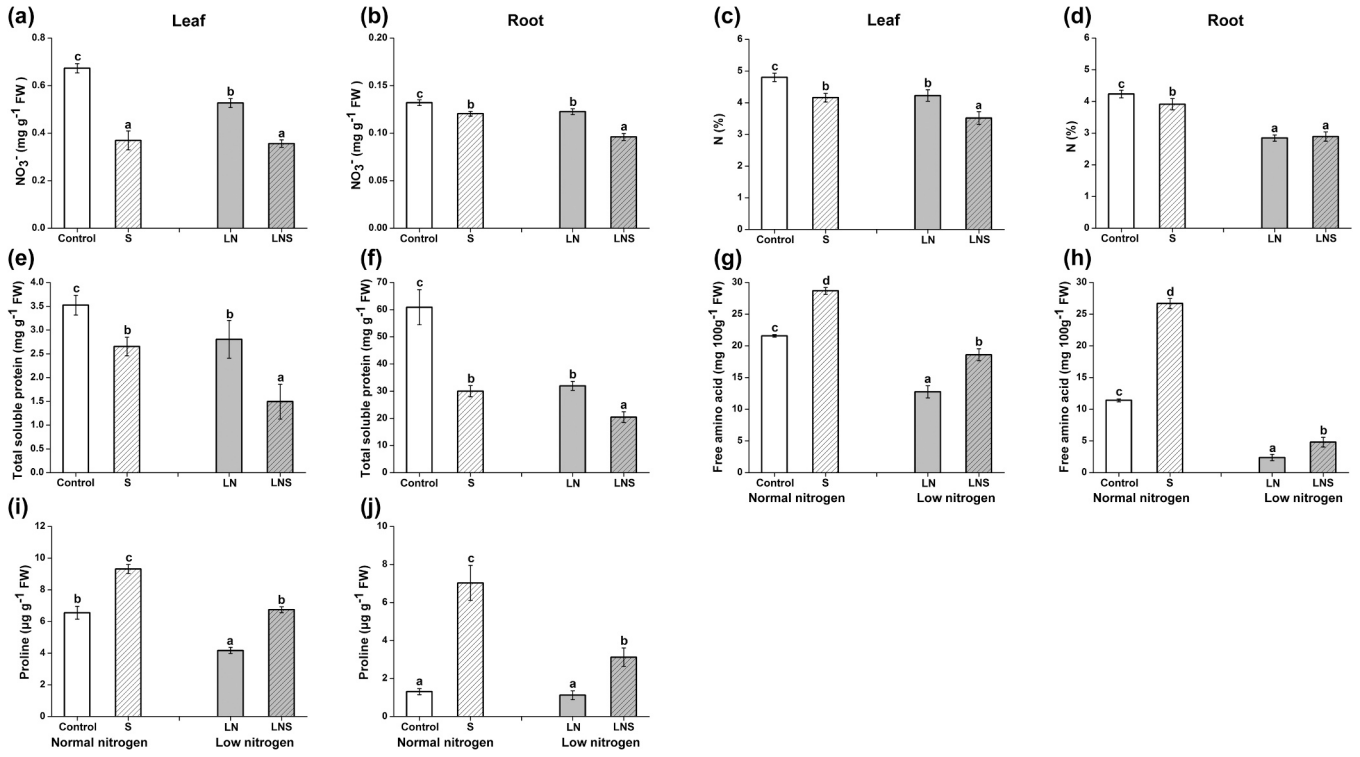

Fig. 4. Concentrations of different nitrogen compounds and total nitrogen (%) in leaves and roots of *P. russikki* across different treatments. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

NSC concentrations were increased by LN but decreased by salt in leaves (Fig. 7a–d). Plant growth was restricted in LN treatment and, consequently, the phytohormones for plant growth (gibberellin, auxin, brassinosteroid) were inhibited in their synthesis or signaling pathway (Fig. S6). The transcripts associated with the biosynthesis pathway of the different phenylpropanoid compounds were enhanced under salt stress (Fig. S7), especially those pathway related to the synthesis of isoflavone, lignin, and anthocyanin. This could explain the photoinhibition effects at a low radiation level (65 μmol m^{-2} s^{-1}, Fig. S9 a). On the other hand, salt caused significant increase in the transcription of genes associated with polysaccharide decomposition (including pectin, cellobiose and glucan decomposition), sugar metabolism and organic acid synthesis (glycolysis, citric acid cycling pathway and the synthesis of galactinol, galactose and pyruvate) (Fig. S7).

3.5. Plant ROS balance and transcript change of stress signaling pathway

The leaf MDA concentration, one membrane lipid peroxidation index, both increased in LN and, to a greater extent, in S treatments (Fig. S14). LN pretreatment alleviated the salt-induced enhancement of MDA. The catalase and superoxide dismutase enzyme, with both separately function in H_2O_2 and superoxygen quenching, are substrate-driven enzymes. Their activities were increased in LN and, to a greater extent, in S, indicating higher ROS pressure in the latter. LNS-stressed plants exhibited lower leaf CAT and SOD enzyme activity than S plants, demonstrating that pretreating plants with N deprivations lowered the ROS pressure. However, S and LNS treatments showed similar levels of MDA, CAT and SOD in roots. The activity of the POD enzyme, involved in H_2O_2 quenching using phenolic substrates, declined in salt-stressed leaves and declined markedly in the roots treated by S or LNS, implying lower phenol substrates in the stressed organs.

In addition, the gene expression was enhanced by LN and salt treatment for the key signaling components of stress-related phytohormone, like PrABF for ABA, AP2-like factor for ethylene and calcium-binding protein (Ca^{2+}-CaM/CML) for nitrate oxide (Figs. S6–S7; Tables S2–S3). Also, the stress signaling cascade pathway was intensified, such as the enhancement of FLS2 transcript for MAPK cascade pathway in plants. The transcript enhancement of these genes by LN pretreatment help build cross-tolerance for the later salt stress (Fig. S8; Table S5).

4. Discussion

Transcriptome analyses by RNA sequencing are being increasingly used as a comprehensive tool to understand the complex plant responses to various stresses (Mutz et al., 2013). Different studies have examined transcriptome changes when exposure to salt stress in *Salicornia europaea* (Ma et al., 2013), *Populus alba* × *Populus glandulosa* (Yao et al., 2018) and *Vitis vinifera* (Guan et al., 2018) amongst others. Global transcriptome analyses between two closely related Brassicaeae species, *Thellungiella salsuginea* and *Arabidopsis thaliana,* have demonstrated a mechanism of stress-anticipatory preparedness for the former species with high salt tolerance (Gong et al., 2005). In the present study, the relatively low proportion of DEGs in the transcriptome profile change (0.3–0.6% depending on treatment) indicated that the genes that encode proteins with fast turn-over have be detected in longer-term (e.g. 2 weeks) treatments. Generally the number of DEGs shortly after stress application is very high. Previous studies conducted briefly (30 min to 4 days) after stress application, often find that over 15% of the total number of identified genes are DEGs (Guan et al., 2018; Liu et al., 2019; Ma et al., 2013). However, the transcripts in the majority of DEGs gradually return to the same levels as those before the stress, as reported on poplar transcriptome variation after long-term of salt or LN stress or supplemented with some beneficial agents like silicon or fullerenol (Shafiq et al., 2021; Zhang et al., 2014). Consequently, analyses of the transcriptome after long-term treatment application, such as this study for 2 weeks of stress application, are ideal to observe acclimation responses in cellular metabolism. The intermediate change of

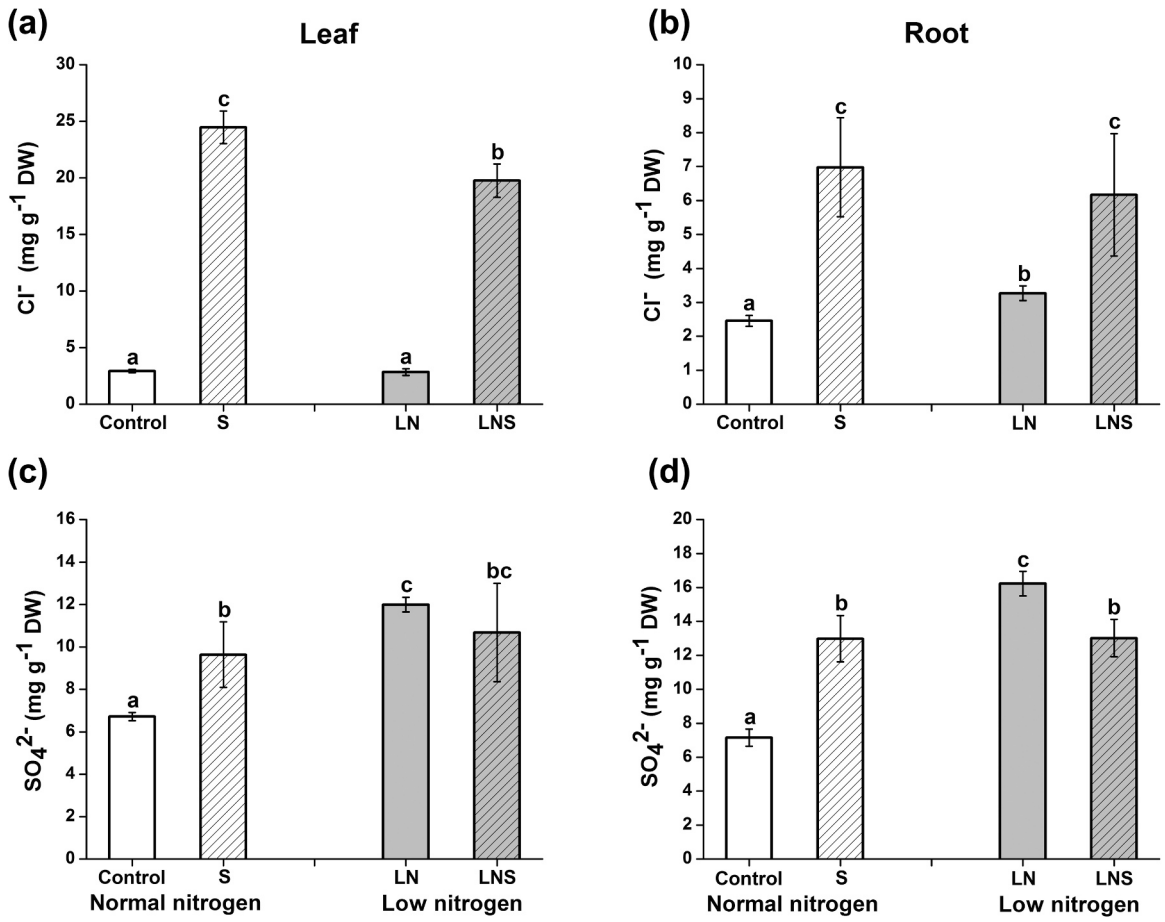

Fig. 5. Concentrations of inorganic anions (Cl^- and SO_4^{2-}) in leaves and roots of *P. russikki* across different treatments. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

stress-related transcriptome profile responded to LNS among the different treatments confirmed the alleviation effects of LN pretreatment.on physiological and biochemical performance of salt-stress poplar.

Compared with other poplar species (Ma et al., 2016), salt-stressed *P. russikki* shows higher levels of Na^+ concentration, and this can be partly explained by the salt-induced upregulation in root sodium transporters like *PrHAK1.1* and *PrHKT1.2* that functions in root Na^+ influx. But opposite transcript responses in HAK transporters have been documented for the salt-tolerant *P. euphratica* (down-regulation under salt, Ding et al., 2010), hinting that the response of sodium transporters to salt stress could be involved in inter-specific differences to salt tolerance within poplars. On the other hand, the excess sodium in the poplar cytoplasm can be excluded to apoplast by *PrSOS1* or compartmented into vacuole by *PrNHX1* and vacuolar cation/proton exchanger fueling by the vacuolar membrane H^+-transporting ATPase, the up-regulation of these genes help the poplar tolerate salt toxicity. The major cellular cation transporter, *PrSOS1*, prefers Na^+ compartmentation over K^+. The potassium concentration was decreased by sole LN or sole S treatment for the following reasons. First, in order to satisfy the charge balance required in ion transport for nutrition, a positive relation between the uptake and translocation of potassium and nitrate must exist (Raddatz et al., 2020). The limited nitrate (LN) treatment in the present study also restricted the co-transport of potassium under the non-salt conditions, explaining the lower K^+ level in LN-stressed plants. Second, low K^+ level in salt-stressed plants can influence the activation of the nitrate assimilation enzyme (Wang et al., 2018), explaining the low levels of [NO_3^-] and total N in the S treatment, despite sufficiency in nitrate supply (Fig. 4a–d). It is critically important to maintain ion homeostasis, particularly in the K^+/Na^+ ratio, for plants to survive under salt stress (Almeida et al., 2017). The significant increase in the K^+/Na^+ ratio by the LN pretreatment indicates that N deprivation helps poplar plants to counteract the salt stress (Fig. 2i–j), and similar responses were reported in *Poa annua* (Zhao et al., 2016). Previous studies demonstrated that Ca^{2+} and Mg^{2+} application could improve salt tolerance in winter wheat and honeysuckle (Huang et al., 2019; Mansour et al., 1998). The up-regulation of Ca^{2+}-ATPase and Mg^{2+}-ATPase transcipt by the LN pretreating provide salt-stressed plants with more energy fueling for Ca^{2+} and Mg^{2+} absorption. Furthermore, the excess cellular [Cl^-] can cause cellular dysfunction, necrotic lesions and chlorotic discolorations (Zelm et al., 2020), the reducing Cl^- levels, at least in leaves, can improve the plant treated by LNS avoiding Cl^- toxicity. On the contrary, increases in SO_4^{2-} concentrations can help plants restore the cellular charge balance in the condition of Na^+ influx or NO_3^- deprivation (Carnol et al., 1997; Sun et al., 2018). The upregulation of genes involved in sulfur relay/transfer and the synthesis for sulfur-related amino acid or polypeptide demonstrated that the synthesis of sulphur-related compound were strengthened, like glutamate, tyrosin, cysteine and GSH compounds. These compounds play a chelation role against toxic ions (Anjum et al., 2014), and helped poplar plants acclimate to salt stress in this study. Therefore, LN pretreatment showed priming effects in the ion balance by the upregulation of genes functioned in cation transport and chelation and ATPase.

N deficiency and salt stress separately preferentially allocated N to the upper part and roots in the poplar, and this is related to the contrast transcript changes induced by salt and LN treatment in the root

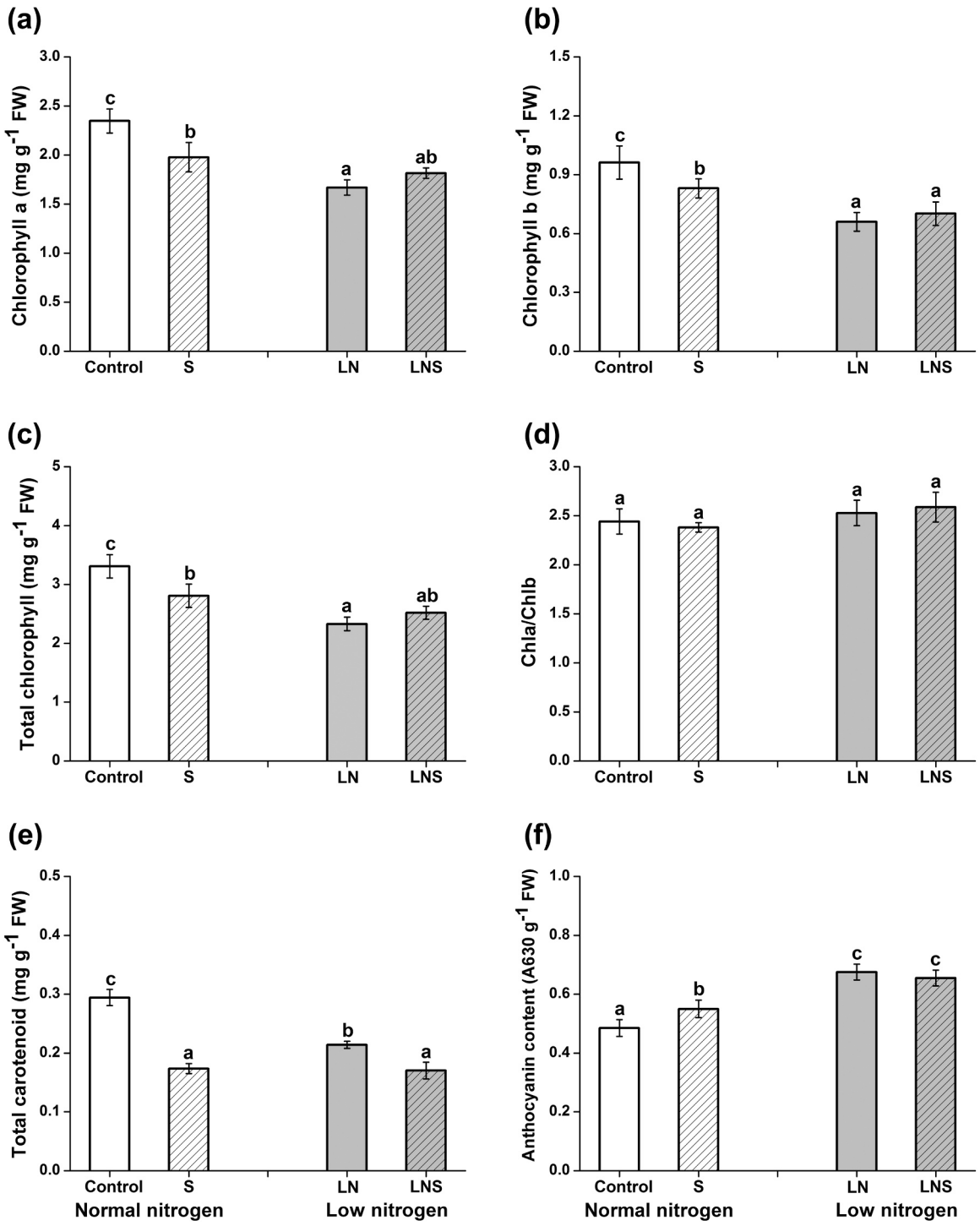

Fig. 6. Concentration of photosynthetic pigments and anthocyanin in the leaves of *P. russikki* across different treatments. LN, S and LNS indicate low nitrogen, salt and low nitrogen and salt, respectively. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

PrNRT1.5a/c as well as root *PrNRT1.8*. *PrNRT1.5a/c* in poplar restricts the influx of NO_3^- and of total N into roots, showed contrary function with its Arabidopsis homologue (Yao et al., 2021). The salt-induced up-regulation of root *PrNRT1.5a/c* was favoured the restriction of NO_3^- and total N into roots, and this made the amino acid, an important compound against osmotic stress, be enhanced in the roots, especially proline. Earlier studies reported that amino acid concentrations increased under salt conditions in grey poplar (Ehlting et al., 2007). On the other hand, *PrNRT1.8* in poplar functions in root-to-shoot nitrate transportation, and LN-induced increase of its transcript was favoured the N allocation to the shoots.

In a recent study, Gao et al. (2019) found that N deficiency priming enhanced photosynthesis adaptation to drought stress in wheat seedlings by increasing the electron flux to photorespiration and the Mehler pathway and reducing photoinhibition, and *ΦPSII*, *qL* (Photochemical quenching), and *Fv/Fm* was recovered faster by LN priming, with lower NPQ level than sole drought stress, which is similar with the present result. The lower requirement for NPQ likely derived from the low

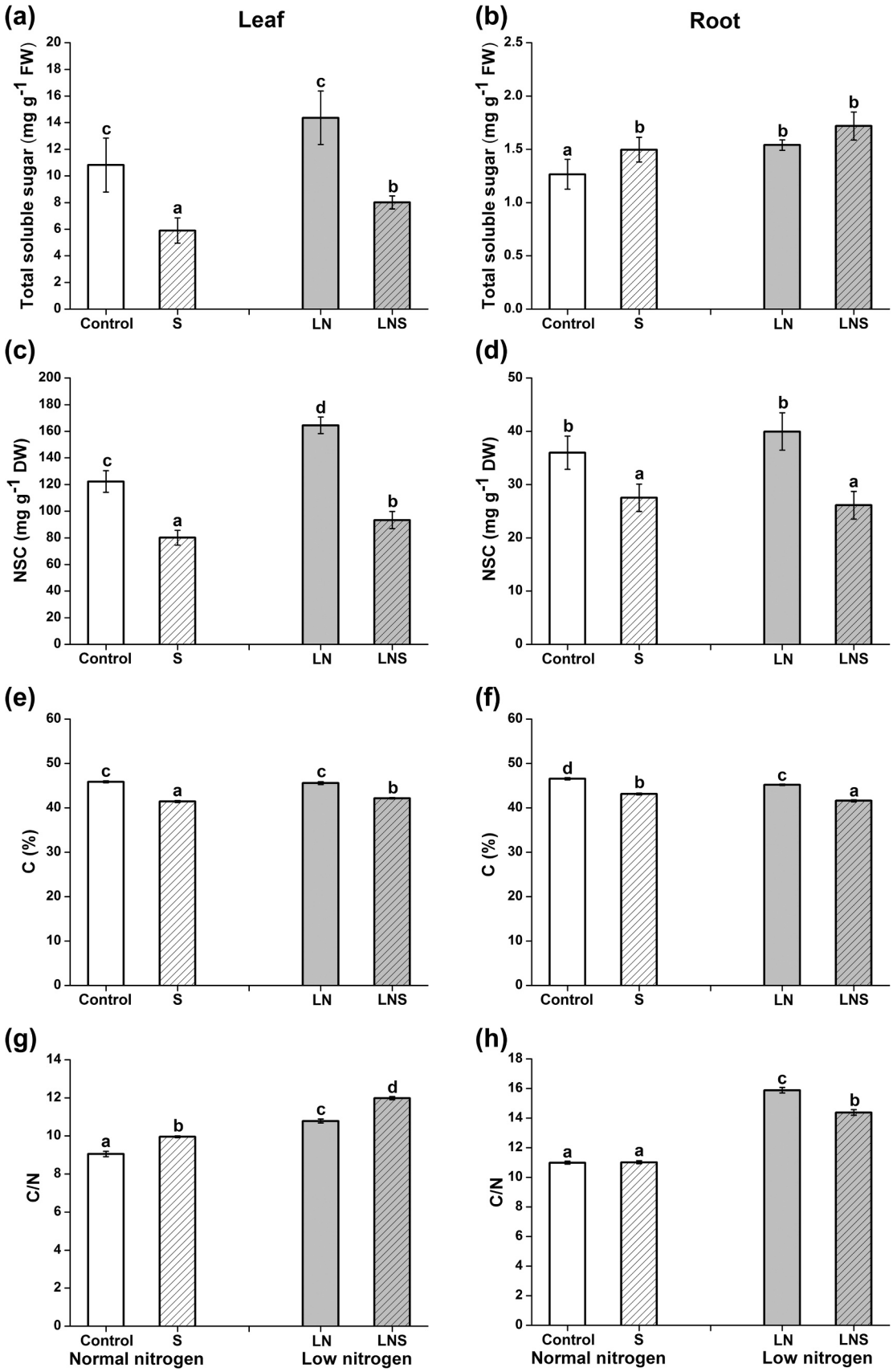

Fig. 7. The concentrations of carbon compounds (total soluble sugar and non-structural carbohydrates (NSC)) and total carbon as well as C/N ratios in leaves and roots of *P. russikki* across different treatments. Columns represent mean values (± SD) of three biological replicates. Different letters indicate significant differences across treatments according to LSD.

chlorophyll concentrations under LN condition. The reduced photochemical activity in different stress conditions reduced total carbon concentration in plants, but the C/N ratio was still increased for the bigger drop of total N concentration which sharply reduced plant growth and make the sink limitation. Under LN, sink limitations led to the accumulation of NSCs as sugar, starch and phenolic compounds, similar to the drought study (Resco de Dios and Gessler, 2021). The increased NSC by LN pretreatment provide the building blocks for osmotic protectants for the subsequent salt stress application, and it also had the potential to fuel the subcellular compartmentalization or efflux of Na^+ by providing more energy. Also, NSC by LN could consequently strengthen cell wall and increase cell wall elasticity to protect against the subsequent osmotic pressure. Therefore, the results validated the first specific hypothesis on the NSC accumulation and preparation for osmotic protectants. On the other hand, organic acid production was strengthened by salt application, as showed by the transcriptome profile change. Previous studies on metabolomic profiles in poplar demonstrated that these pathways for organic acid production also increased under salt or alkali stress (Janz et al., 2010; Sun et al., 2018). In an earlier study, Gong et al. (2005) found that salt cress (*Thellungiella halophila*) exhibited much higher levels of organic acids, amino acids and sugar concentration than its relative Arabidopsis thaliana, and similar responses have been observed in studies comparing salt-tolerant wild soybean vs its cultivated salt-sensitive relatives (Zhang et al., 2016). These organic molecules with low molecular weight can serve as effective osmolytes under salt stress (Zelm et al., 2020). Consequently, the increased levels of transcription in the pathway of organic and amino acid formation are likely to help the *P. russikk*i acclimate to salt stress. Recent study also confirmed that salt stress caused enhancement of soluble sugar and free amino acid by the activating the GABA shunt pathway (Ji et al., 2020).

Generally, the stress-affected trees are known to increase carbon allocation of to roots and increased C/N ratio for acclimating to different stresses (Aaltonen et al., 2017). This study provided another evidence on the C/N effects on plant stress tolerance by the N starvation priming method. In last ten years, several studies have been reported to improve plant tolerance by increased C/N ratio through modification of ATL31/ATL36, 14–3–3λand growth regulation factor 4 (GRF4) in arabidopsis, rice, tomato (Gao et al., 2019; Maekawa et al., 2012). The plant nitrogen usage efficiency (NUE) is closely correlated with C/N ratio of whole plants. Sadras and Richards (2014) found that the wheat germplasms with high level of NUE and relative growth rate (RGR) have low levels of water use efficiency and drought tolerance under N deficiency condition, showing the priority of growth or stress tolerance of different wheat germplasms. In this study, the *P. russikki* has a higher RGR than other species in Asia arid region, the growth priority traits makes it more sensitive to salt stress (Sun et al., 2018), in this study the N deficiency pretreating provide the cues for priming with stress resistance upon reducing its growth rate. Under climate change condition, warming temperatures and increased levels of atmosphere carbon dioxide cause a decrease of N availability for terrestrial plants (Craine et al., 2018), and this seems to help the plant species with high RGR, like *P. russikki*, tolerate soil salinity. On the other hand, as another major components of global change, the increasing atmospheric N deposition increases plant N nutrition and RGR, and this implies that N limitation treatment should be applied in sampling fostering in favor of their saline tolerance in the afforestation practice. In the field condition, arbuscular mycorrhizal fungi (AMFs) are usually colonized with many tree species like poplar, pines, et al., and their colonization mediate the activation of stress defense mechanisms that confer priming (Mauch-Mani et al., 2017). AMFs also use plant carbohydrates for their own metabolism and influence N uptake efficiency, the alteration of carbon/nitrogen status in the symbiont serve as another important stimulus for stress priming.

Plants have an intricate network of stress signals including signaling components, hormones, et al., which activate the suitable defensive mechanisms against the stresses. This intricate network and suitable mechanism can be stimulated to activate the plant defenses and get them ready for subsequent stresses (Mauch-Mani et al., 2017). Generally cross-tolerance is induced by a mild primary stress, such as N deficiency in the present study, and it activates common defenses to reinforce tolerance to different stresses. Carvalho and Silveira (2020) discussed that H_2O_2-retrograde signaling serve as a pivotal mechanism for priming and cross stress tolerance in plants. In this study, the key retrograde signal, such as carotenoid, ABF, AP2, MAPK and CaM/CML (Kleine and Leister, 2016), were significantly regulated (Table S2), showing its priming effects. These signaling component at last resulted in crosstalk with SOS3-SOS1 pathway that prefers Na^+ compartmentation over K^+ and improves plant tolerance to salt. In the present study, the ROS pressure was significantly reduced in LN pretreating plants. The results completely validated the general and second specific hypothesis on the LN priming effects on the triggering of stress signaling favoured for subsequent salt tolerance. Particularly, ?

5. Conclusions

In conclusion, *P. russikki* was sensitive to salt and to LN stresses. N deficiency caused inhibition of photochemistry and the accumulation of non-structural carbon compounds (NSC), consequently induced ROS harm. However, LN pretreatment alleviated the adverse effects caused by the subsequent application of salt stress. That is, N deprivation reduced the influx of toxic ions like Na^+ and Cl^-, increased the ratio of K^+/Na^+, it led a smaller reduction in chlorophyll content and in photochemical activity, and in lower lipid peroxidation level. The alleviation effects can be attributed to two reasons. First, LN led to higher NSC concentrations which consequently allow for the production of more osmolytes (soluble sugars and organic acids) that can then be used to counteract the osmotic stress induced by salt and to provide more energy for fueling the subcellular Na^+ compartmentalization. Second, LN pretreatment provides cross tolerance for the subsequent salt stress by enhancing antioxidant enzyme activity, and by increasing the transcript levels of signaling components in stress-related phytohormones and cascade pathways. The results indicate that restricting N supply in poplars may serve for priming responses to salt tolerance and to consequently increase survival, at least under moderate saline stress.

CRediT authorship contribution statement

Yongfeng Gao: Investigation, Formal analysis, Writing. **Yufang Sun:** Investigation, Data analysis. **Yongbin Ou:** Validation, Visualization, Writing – review & editing. **Xinhua Zheng:** Data analysis, Writing – review & editing. **Qian Feng:** Writing – review & editing. **Hao Zhang:** Writing – review & editing. Yang Fei: Writing – review & editing. **Víctor Resco de Dios:** Project administration, Writing – review & editing. **Yinan Yao:** Design, Supervision, Writing – original draft.

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.ecoenv.2021.112801.

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