*Pak. J. Bot.*, 50(1): 179-187, 2018.

# EFFECTS OF NITROGEN DEFICIENCY ON PHYSIOLOGY AND GROWTH OF *FRAXINUS MANDSHURICA*

**YUZHU ZHOU[1,2], YONGPING HE[1,2], JIAYAO SUN[1,2], JIAWEI ZHANG[1,2] AND YAGUANG ZHAN[1,2*]**

*[1]Life Science College, Northeast Forestry University, Harbin150040, P.R. China*
*[2]National Key Laboratory of Forest Genetics and Breeding, Northeast Forestry University, Harbin150040, P.R. China*
**Corresponding author's email: yaguangzhan@sina.com; Tel: +86-15774606215*

**Abstract**

This study was to investigate expression changes in biochemical molecules and physiology of *Fraxinus mandshurica* seedlings under nitrogen deficiency stress. The biochemical and physiological parameters together with gene expression levels undernitrogen deficiency stress were investigated. Differences in nitrogen metabolism key enzymes, ROS-scavenging genes, circadian rhythm genes, and endogenous hormones between nitrogen deficiency treatment and adequate nitrogen treatment were compared. Resistance to stress of the two groups was determined. The results showed that under the nitrogen deficiency condition, biomass and relative growth rate markedly decreased, and *Fraxinus mandshurica* had greater biochemical and physiological changes in nitrogen metabolism key enzymes and endogenous hormones. Additionally, expression of genes involved in biological clock and scavenging of reactive oxygen species (ROS) in nitrogen deficiency treatment were greatly changed while compared with that of total nitrogen treatment. In order to resist stress and maintain basic life activities, *Fraxinus mandshurica* increased its metabolic control, regulated oxidative stress and antioxidant systems, delayed aging, and inhibited their growth viachanging expression of circadian rhythm genes, reactive oxygen species scavenging genes, nitrogen metabolism, and endogenous hormones.

**Key words:** Nitrogen deficiency stress; *Fraxinus mandshurica*; Endogenous hormone; Nitrogen metabolism; Gene expression.

## Introduction

Nitrogen (N) is one of the essential elements of crops, and is involved in the synthesis and metabolism of a variety of substances (McAllister *et al*., 2012; Xu *et al*., 2012). The main symptoms of plant N deficiency are leaf senescence caused by lipid peroxidation and photosynthetic capacity inhibition caused by protein degradation. ROS rises and the N metabolism disrupts during senescence induced by N-deficiency (Casano *et al*., 1994; Grossman, 2001; Zhang *et al*., 2011; Zhang *et al*., 2012; Hassan *et al*., 2017). Plant responds to N-deficiency through its circadian clock (Nefissi *et al*., 2015), which contains an input pathway, a core oscillator, and an output pathway (Surez-Lpez *et al*., 2001; Yanovsky & Kay, 2002). The reaction is based on the transcription and translation of negative feedback loop oscillators, which contain a set of clock genes. Translation of these genes form negative feedback loopscan directly or indirectly inhibit their self-transcription and affect the transcription of other genes (Pokhilko *et al*., 2012, Dunlap, 1999). LHY/CCA1 and TOC1 encode highly conserved single MYB transcription factors, which are transcriptional repressors of oscillator genes. These transcription factors participate in the negative feedback regulation cycle of circadian clock oscillator. Organism can sense environmental signals and then regulate growth and development through circadian rhythms to adapt to seasonal and environmental changes (Ryosuke & George, 2003).

N stress causes ROS production and hence leading to DNA incision, lipid peroxidation, and cell death. ROS-scavenging enzymes, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) play important roles in N deficiencytolerance. Additionally, growing evidence shows that GDH are important in the balance of N metabolism with dual function. GDH assimilates ammonium when the plants have excessive external inorganic N sources, and GDH breaks down glutamate when plants lack inorganic N sources or organic carbon source (Melo-Oliveira *et al*., 1996). Furthermore, many endogenous hormonal responses to abiotic stress have been demonstrated in some species. These hormones function as plant-mediated signaling and could regulate oxidative stress and antioxidant systems (Milborrow, 2001; Sun *et al*., 2013; Shi *et al*., 2014; Rubio-Wilhelmi *et al*., 2011; Varvara & Bernard, 2001).

*Fraxinus mandshurica*, a rare species in North China and Northeast China and has been declared a national endangered speciesdue to its long-term overexploitation. N is very important for *Fraxinus mandshurica*, however, it is difficult to apply N in the wild, and it is very important to increase the efficiency of N fertilizer for *Fraxinus mandshurica* in the wild. At present, most studies on the effects of N stress on plant N metabolism are mainly focused on crops, but in terms of woody plants, especially trees, there are few reports. As for *Fraxinus mandshurica* , previous studies were mainly focused on ecology and evolution (Wallander, 2008; Hinsinger *et al*., 2014), spatial genetic structures (Hu *et al*., 2010), somatic embryogenesis (Kong *et al*., 2011), drought, salt tolerance, and DNA methylation in interspecific hybrids (Zeng *et al*., 2014; Zeng *et al*., 2015; He *et al*., 2015). However, there are no reports on the morphological, physiological, and molecular mechanisms of *Fraxinus mandshurica* under N deficiency stress. As a result, in this study, we investigated the impacts of N-deficiency stress on the growth and physiology of *Fraxinus mandshurica* seedlings.

## Materials and Methods

**Plant materials and N treatment:** *Fraxinus mandshurica* seeded for 60-days were obtained from greenhouse in Northeast Forestry University in Heilongjiang Province, China (N45° 43', E126° 37'). Seedlings with similar growth status were chosen for N treatment. Forty seedlings were transplanted in one pot (55cm long, 35cm width, and 15cm high) filled with sandy soil (Sterilized for 121°C, 2 h). Plants were divided into two groups, 4 pots for treatment and 4 pots for control. Plants were cultivated in a climate chamber (light intensity, 3000lux, 14/10 h and 30/18°Cday/night; relative humidity, 80%) and provided with Hoagland nutrient solution (8 mM $NH_4NO_3$, 1 mM $KH_2PO_4$, 1 mMKCl, 1 mM $CaCl_2$•$6H_2O$, 0.6 mM $MgSO_4$•$7H_2O$, 0.02 mM $FeCl_3$•$6H_2O$, 6μM $MnCl_2$•$4H_2O$, 0.016 mM $H_3BO_3$, 0. 3 μM $ZnCl_2$, 0.3 μM $CuCl_2$• $2H_2O$, and 0.3 μM $NaMoO_4$• $2H_2O$) every 2 days. After 14 days, 0 mM $NH_4NO_3$ and 8 mM $NH_4NO_3$ was designated as the variables for the N-deficiency stress treatment and the control condition (CK), then $NH_4NO_3$ was added again for 3 d for N treatment on experimental group. Samples were collected according to the needs of different measurement parameters after treatment in 8-9 am. The samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Each harvest was repeated for three times.

**Determination of enzyme activities:** Samples were collected for 1-2 g after 0, 0.5, 1, 3, 5, 7, 14, 21, and 24d after treatment for biochemical and physiological indicator measurement or for RNA isolation. All the operations were performed at 4°C. Samples of 0.5 g in 6 mL of 50 mM Imidazole-HCl buffer (pH 8.0) containing 2 mM $MgSO_4$, 0.5 mM EDTA, and 10 mM 2-mercaptoethanolwere homogenized using a chilled pestle and mortar. The extract was centrifuged for 20 min at 15,000 g, with the supernatant used for GS, GDH, and GOGAT activity determination. All of the enzyme activities were measured on a TU-1900 double-beam UV spectrophotometer (PERSEE, Beijing, China).

**GS activity measurements:** GS activity was determined according to Luo (Luo *et al*., 2013). The reaction mixture contained 1.6 ml of 50 mM imidazole-HCl buffer (pH 7.8) with 20 mM $MgSO_4$, 0.5 mM EDTA, 60 mM Na-glutamate, 10 mM ATP, and 10 mM hydroxylamine hydrochloride. The reaction was initiated by adding 0.7 ml enzyme extract, incubated for 3 min at 30°C, and terminated by the addition of 1 ml reagent (0.2 M TCA, 0.37M FeCl3, and 0.6 M HCl). After centrifugation at 5000 gand 4°Cfor 15 min, the absorbance of the supernatant was read at 540 nm. One unit of GS activity was defined as the formation of 1 μmol of γ-glutamyl hydroxamate per minute.

**GDH activity measurements:** The activity of GDH was determined according to Wang (Wang *et al*., 2008) with slight modifications. The 3.0 ml assay mixture consisted 0.3 ml of 0.1 mM α- ketoglutarate, 0.3 ml of 1 M $NH_4Cl$, 0.2 ml of 3 mM NADH, and 1.2 ml of 0.2 mol/L Tris-HCl buffer (pH 8.0). The reaction was started by the addition of 1 ml enzyme extract and assayed spectrophotometrically at 340 nm. One unit of GDH activity was defined as the formation of 1.0 μmol of NADH per minute.

**GOGAT activity measurements:** Activity of GOGAT was assayed on the basisof Lin's method (Lin & Kao, 1996). The 3.0 ml assay mixture was made up with 25 mM Tris-HCl buffer (pH 7.6) by adding 0.4 ml of 20 mM L-glutamine, 0.05 ml of 0.1 mM α- ketoglutarate, 0.1 ml of 10 mMKCl, 0.2 ml of 3 mM NADH, and 0.5 ml enzyme extract in a final volume of . The decrease in absorbance was measured by spectrophotometry at 340 nm.

**Determination of $NH_4$+, $NO_3$- concentration:** Samples were collected at 0, 1, 4, 7, 14, 21, and 24 d after treatment for the measurement of $NH_4$+ and $NO_3$- concentration. $NO_3$- concentration in samples was measuredaccording to Luo (Luo *et al*., 2013). Fresh tissue (1-2 g) was extracted with deionized water at 45°C for 1 hin a 20 ml tube. After centrifugation (5000 g, 20°C, 15 min), the supernatant(0.1 ml), used for nitrate quantification, was mixed thoroughly with 0.4 ml of 5% (w/v) salicylic acid in concentrated $H_2SO_4$.and incubated for 20 min at room temperature. Then the pH was raised to above 12 by the addition of 9.5 ml of 8% NaOH. The solution cooled to room temperature was used to determine the $NO_3$- concentration by spectrophotometry at 410 nm.

As for $NH_4$+ concentration determination, briefly, fine power (c.100 mg) was homogenized in an extraction solution of1 ml 100 mMHCl and 500 ul chloroform. After shaking at 4°C for 15min, the extract was centrifuged (10000 g, 4°C, 10 min). The aqueous phase was transferred to a 2 ml tube with 50 mg activated charcoal and mixed well and centrifuged (12000 g, 4°C, 5 min) again. $NH_4$+ concentration in the supernatant was determined by spectrophotometry at 620 nm.

**Extraction and determination of phytohormones:** Samples were collected for 0.5 g at 0, 1, 4, 7, 14, 21, and 24 d after treatment for the measurement of phytohormones content. The samples with fresh weight (DW) of 0.5±0.01 g were homogenized in 4.5 ml ice-cold 50 mM phosphate buffer (pH=7.2-7.4) for extraction. The extract was centrifuged at 3,000 rpm for 30 minat 4°C, and the supernatant was then frozen at −20°C. Approximately 100 μl was used for analyzing GA, ABA, IAA, CKT, and ETH by ELISA using assay kits (Shanghai Enzyme-linked Biological Technology Co., Shanghai, China) according to the manufacturer's instructions. Detection of different hormones was performed at 450 nm within 15 min after the termination of the reaction. A standard curve was established for each micro-titer plate, and GA, ABA, IAA, CTK, and ETH contents of each sample were measured three times.

**Gene expression analysis by real-time PCR:** Total RNA samples were extracted using CTAB Reagent and treated with RNase-free DNase I (Promega) to remove contaminated DNA. The mass and concentration of the extracted RNA were assessed using 1% (w/v) agarose gels and measured at 260 nm on a TU-1900 spectrophotometer

(PERSEE, Beijing, China). Quantitative RNA was reverse-transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real-Time) (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative-real time PCR was performed in an ABI7500 system with SYBR ® Premix Ex Taq™ II (Takara Bio, Inc., Otsu, Japan). The PCR procedure was as follows: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds, and a final melting curve at 70-94°C. All RT-PCR expression assays were calculated at least three times in independent experiments using 7500 Software v 2.0.6 (Applied Biosystems, Forster City, CA, USA) according to previously reported methods(Zhu *et al*., 2016). The α tubulin gene was used as an internal control to estimate the expression levels in three biological replicates.

**Plant growth measurements:** Seedlings were harvested carefully separated from the soil. The buds and roots were washed with tap water, rinsed with distilled water and dried with filter paper. We measured the fresh weight (FW), height, and stem diameter, then separated leave and roots for further plant physiological analysis.

**Statistical analysis:** All experiments were repeated at least three times and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences were assessed usingone-way ANOVA and Duncan's test. $p<0.05$ was considered as significant difference. The figures were created using Origin 8.0 software (Origin Lab Corporation, USA).

## Results

**Effects of N-deficiency stress on biomass of *Fraxinus mandshurica*:** To investigate the effects of N-deficiency stress on biomass of *Fraxinus mandshurica*, a number of plants were used to measure the biomass at 1 d and 21 d. The results showed that the plant biomass was significantly inhibited (Fig. 1). Plant height and fresh weight were more obviously affected while the diameter stem was less inhibited.

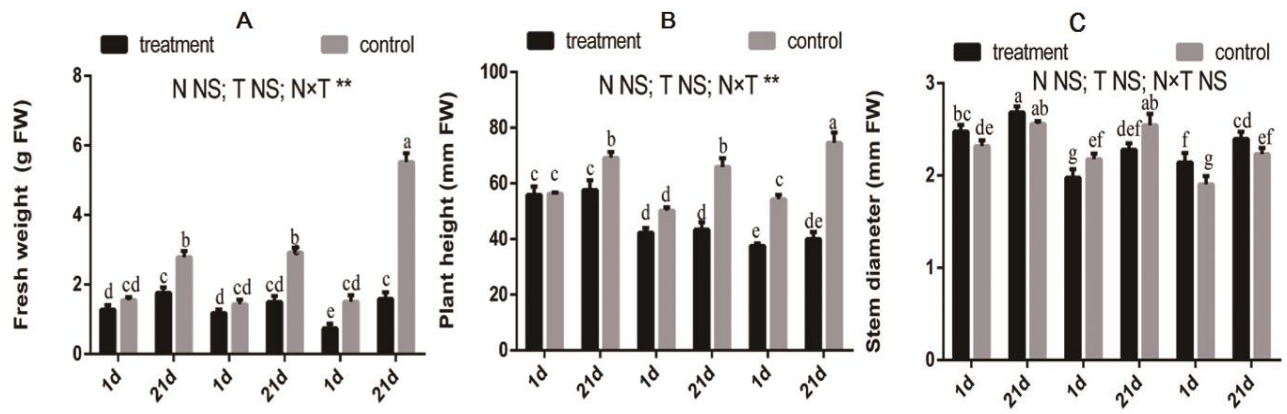


Fig. 1. Change of fresh weight (A) height (B) and stem diameter (C) under nitrogen-deficiency stress at different time.
Data represent the mean ± SE. Different letters abovebars within a statistically significant differences among developmental stages at the $p<0.05$ level according to Duncan's multiple range test. *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; NS: no significant. T, Time effect; N, Nitrogen deficiency effect; T×N, Time × Nitrogen deficiency interaction. The followings are the same as this figure.

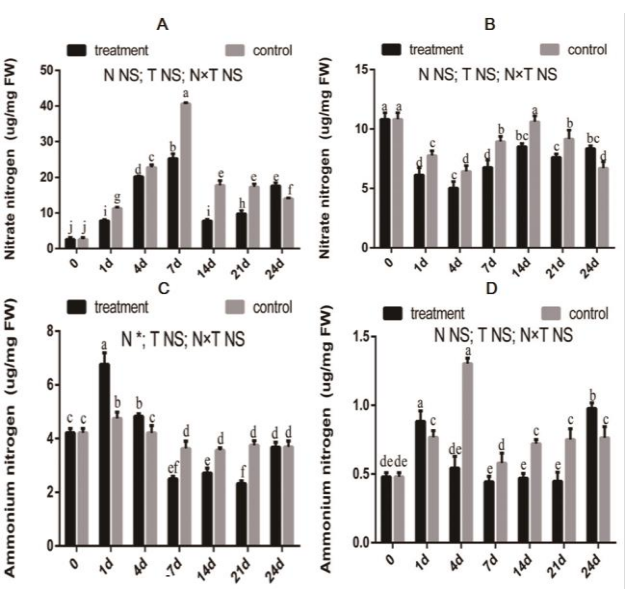


Fig. 2. Content of leaf $NH_4+$ (A), root $NH_4+$ (B), leaf $NO_3-$ (C), and root $NO_3-$(D) under nitrogen-deficiency stress at different time.

**The effect of N-deficiency stress on N metabolism of *Fraxinus mandshurica* seedling:** In order to identify the effects of N-deficiency stress on N metabolism of *Fraxinus mandshurica* seedling, $NO_3-$ and $NH_4+$ concentration measurement in samples was analyzed.$NO_3-$ content decreased during N-deficiency stress (Fig. 2A, B). $NO_3-$ content in root(Fig. 2B) made response at 1 d, and was lower than in the CK after 1d, whereas in leaf (Fig. 2A), the reduction of $NO_3-$ content was not large in 1- 4d, and had a large change in 4d-7d, which was about half of that in the CK from 14 to 21d. $NH_4+$ content increased firstly and then decreased (Fig. 2C, D), which in root (Fig. 2D) was reduced after 1-4d, but it in leaf (Fig. 2C) decreased slightly after 4-7d. The content of $NH_4+$ and $NO_3-$ increased after the recovery of normal N supply in root and leaf.

The enzyme activity and gene expression of GS increased and were higher than in the CK at 12h under N-deficient stress (Fig. 3A, B), and they decreased lately. GS enzyme activity was lower than in the CK after 3d. From then on, it kept lower level than in the CK, but the

difference between them was not significant. The change of GS gene expression was similar to that of the enzyme activity, was lower than in the CK after 12h, and the difference was large between experimental group and control group. GOGAT enzyme activity, it was inhibited by N-deficiency stress, which was lower than in the CK from 12h to 21d, and it was about half of that in the CK during N-deficiency stress (Fig. 3C). GOGAT gene expression was similar to that of GS (Fig. 3D), which had reaction after 2h, increased at first and then followed by decreased, about 1.5 times as much as the CK in 4h, about 33% of the CK at 1d, about half of that in the CK from 2d to 21d. When restoring normal supply of N, GS, and GOGAT gene expression and enzyme activity increased.

NR and NIR gene expression were reduced during N-deficiency stress (Fig. 3G, H). NR gene expression decreased sharply at 2h (Fig. 3G), and NIR also showed the same trend at 4h (Fig. 3H). From then on, the gene expression of NR and NIR were lower than that in control group, and NR gene expression was close to zero. After restoring normal N supply, the degree of recovery of NR and NIR gene expression was not large. Under N-deficiency stress, the GDH enzyme activity (Fig. 3E) increased from 0h to 3d, and decreased from 3d to 21d. GDH gene expression (Fig. 3F) was higher than that in the CK in 0-2d, but which was lower than that in the CK from 4d to 21d.

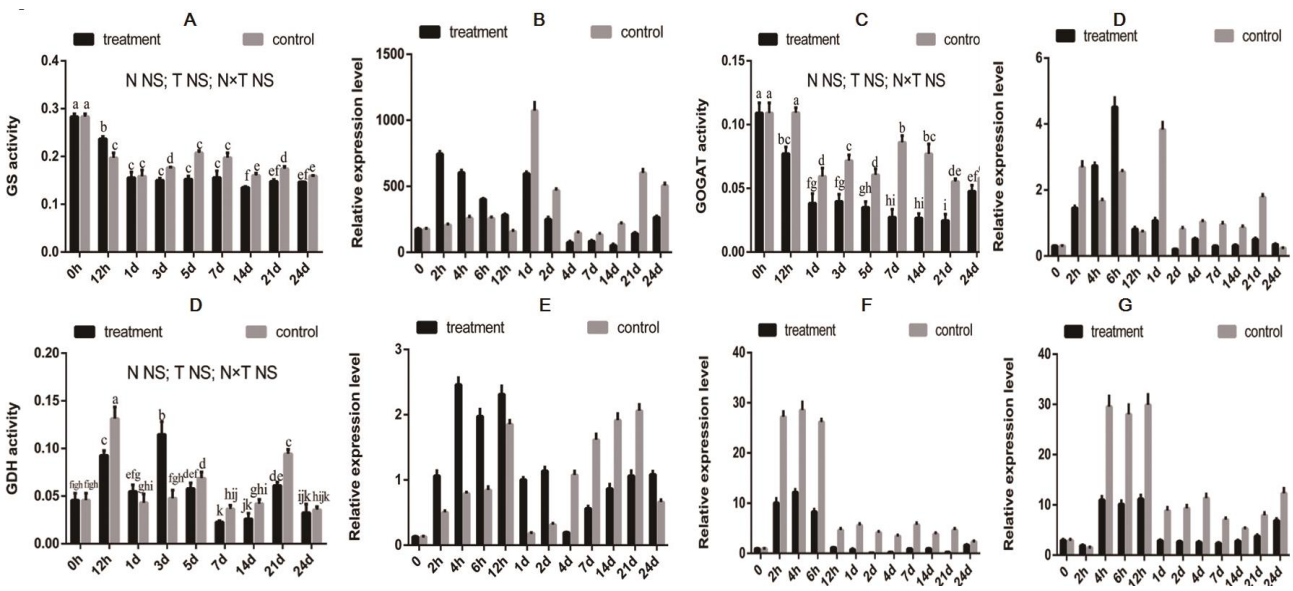


Fig. 3. Change of GS (A), GOGAT (C), GDH (E) and expression of nitrogen cycle related genes GS (B), GOGAT (D), GDH (F), NR (G), and NIR (H) under nitrogen-deficiency stress at different time.

**Effects of N-deficiency stress on circadian gene expression of *Fraxinus mandshurica*:** To testify the ability of plant anti N-deficiency stress, the expression of circadian gene was detected. According to transcriptome data (Fig. 4A, B), TOCl and LHY expression were promoted under N-deficiency stress. TOCl expression (Fig. 4A) was higher than that in the CK during N-deficiency stress, while LHY expression was lower than that in the CK from 2h to 4h and in 2-4d, and was higher than that in the CK at 1d (Fig. 4B) and from 7 to 21d. Plant seemed to receive the stress signal, and LHY was induced, so that LHY expression gradually increased. But its response speed than TOC1 had a daily cycle of backward.

**Effects of N-deficiency on expression of antioxidant-related genes of *Fraxinus mandshurica*:** Adversity often leads to increased ROS, which in turn brings damage to the plant. In order to further understand the ability of plants resistant to stress, the activity of the anti-active oxygen enzyme was measured. As shown in the Fig. 5, under N-deficiency stress, POD, SOD, and CAT genes responded rapidly with a high level of gene expression. That level of gene expression was higher than in the CK from 0 to 24d. The POD expression (Fig. 5A) was about 2 to 5 times of that that in the CK within 4d. CAT expression (Fig. 5C) was significantly higher than that in the CK from 4h to1d. SOD expression (Fig. 5B) was significantly higher than that in the CK from 6h to7d. In other time periods, there was little difference between them. After restoring to normal supply of N, the level of antioxidant-related gene expression was still higher than in the CK.

**Effects of N-deficiency on endogenous hormones of *Fraxinus mandshurica*:** Plants can regulate their growth and development through the changes of the content of hormones in their own body. In order to understand ability of plants resistant to stress, the content of hormones in plants was determined. During N-deficiency stress, the ABA content in leaf was higher than that in the CK (Fig. 6A), and was decreased after plant was restored to normal N supply, but itwas lower in root than that in the CK (Fig. 6B). The expression of NCED was increased during N-deficiency stress (Fig. 6C) and always higher than that in the CK from 6h to 21d. It reached the maximum at 1d, was about 4 times of that in the CK. PYR1 was related to the synthesis of ABA receptor gene, and its change rule was similar to NCED (Fig. 6D). From 4h, the expression of PYR1 kept high level, and it was much higher than that in the CK at 6h and in 4-7d. PYR1 expression showed a downward trend after the recovery of N supply.

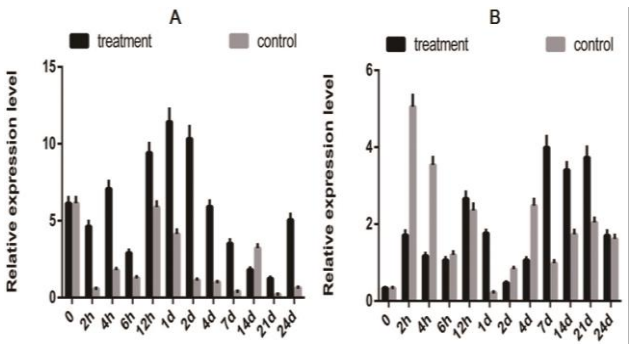


Fig. 4. Expression of Circadian genes (A) TOC1 and (B) LHY under nitrogen deficiency stress and control (CK) or different times.

N-deficiency stress promoted the synthesis of CTK in plants. CTK content in leaf was increased after 7d and was higher than that in the CK from 14d to 21d (Fig. 6E), however, in root, it was higher than that in the CK within 21d (Fig. 6F). In leaf (Fig. 7A), IAA content began to change after 1d to 4d under N- deficiency stress. IAA content increased first, was higher than that in the CK at 4d, then decreased in 4-21d, and it was lower than that in the CK at 21d. In root (Fig. 7B), IAA content was decreased and it was lower than that in the CK after 4d. After restoring the normal supply of N, IAA content increased and was higher than that in the CK at 24d. GA content was consistent with the change of leaf and root system (Fig. 7C, D). Under N-deficiency stress, they changed after 1 to 4d. GA content was lower than in the CK at 4d, but was always higher than that in the CK after 4d. ETH content increased under N-deficiency stress. In leaf, (Fig. 7E) ETH content increased at first and followed by a decrease, beginning to decline after 7-14d, which was lower than that in CK at 14d. In root (Fig. 7F), ETH content increased and was higher than in the CK after 1d, but declined after the recovery of N supply.

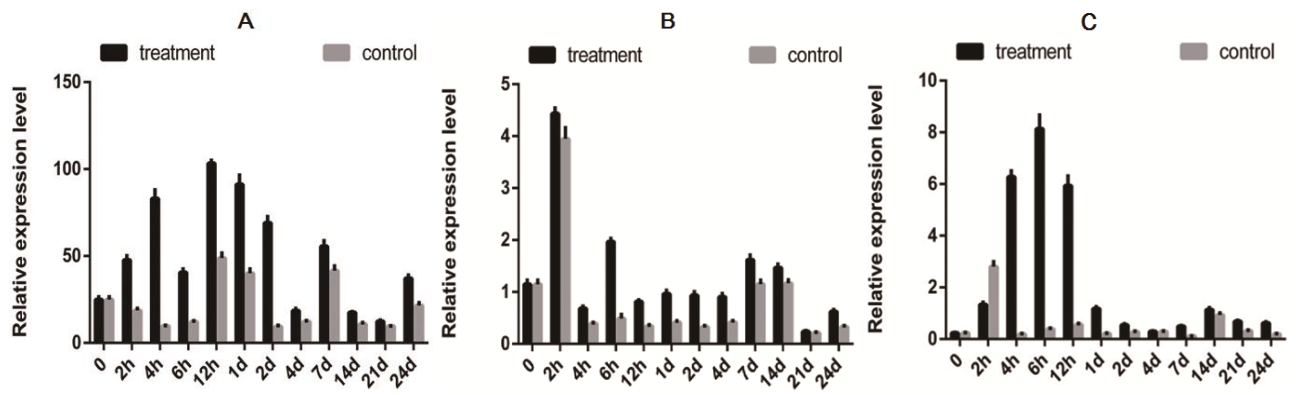


Fig. 5. Expression of antioxidant-related genes (A) POD, (B) SOD and (C) CAT during nitrogen-deficiency stress.

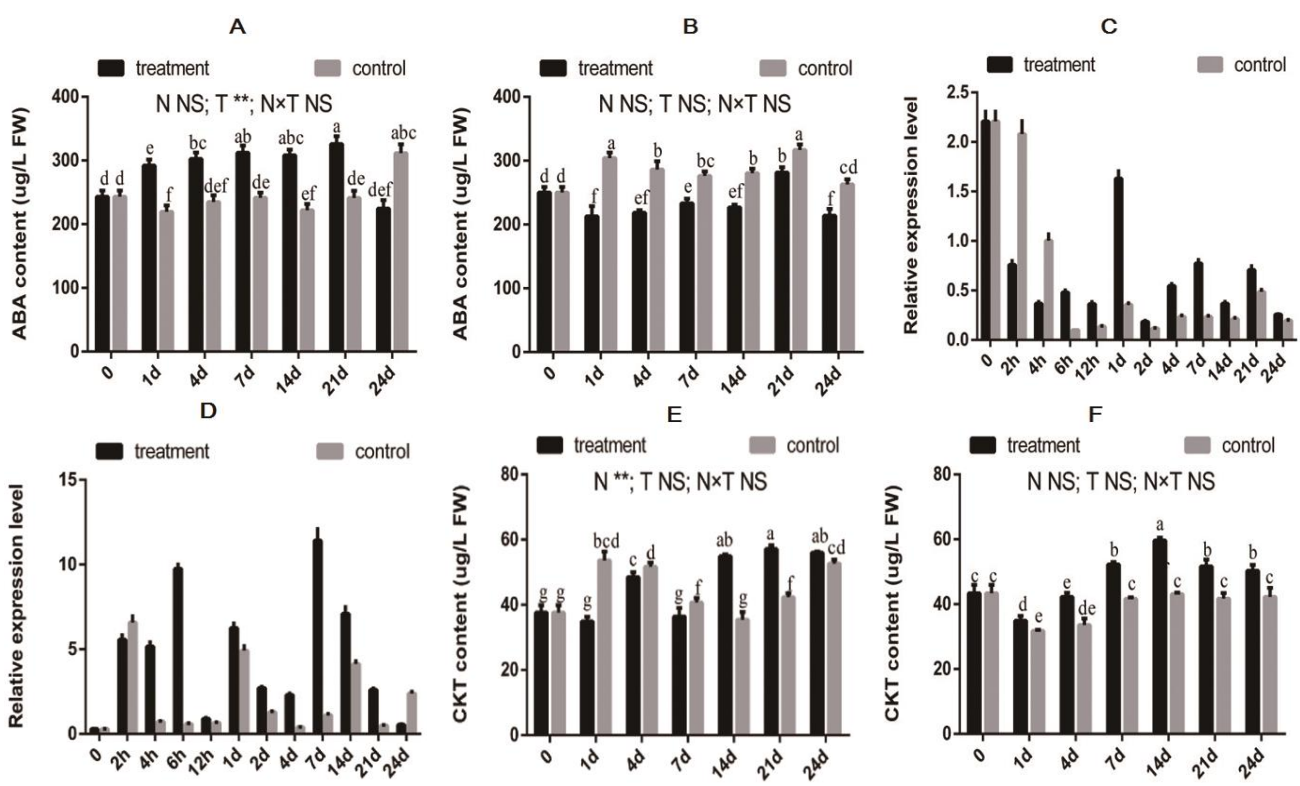


Fig. 6. The content of leaf ABA (A), root ABA (B), leaf CKT (E), root CKT (F), and the expression of NCED(C), PYR1 (D) during Nitrogen deficiency stress.

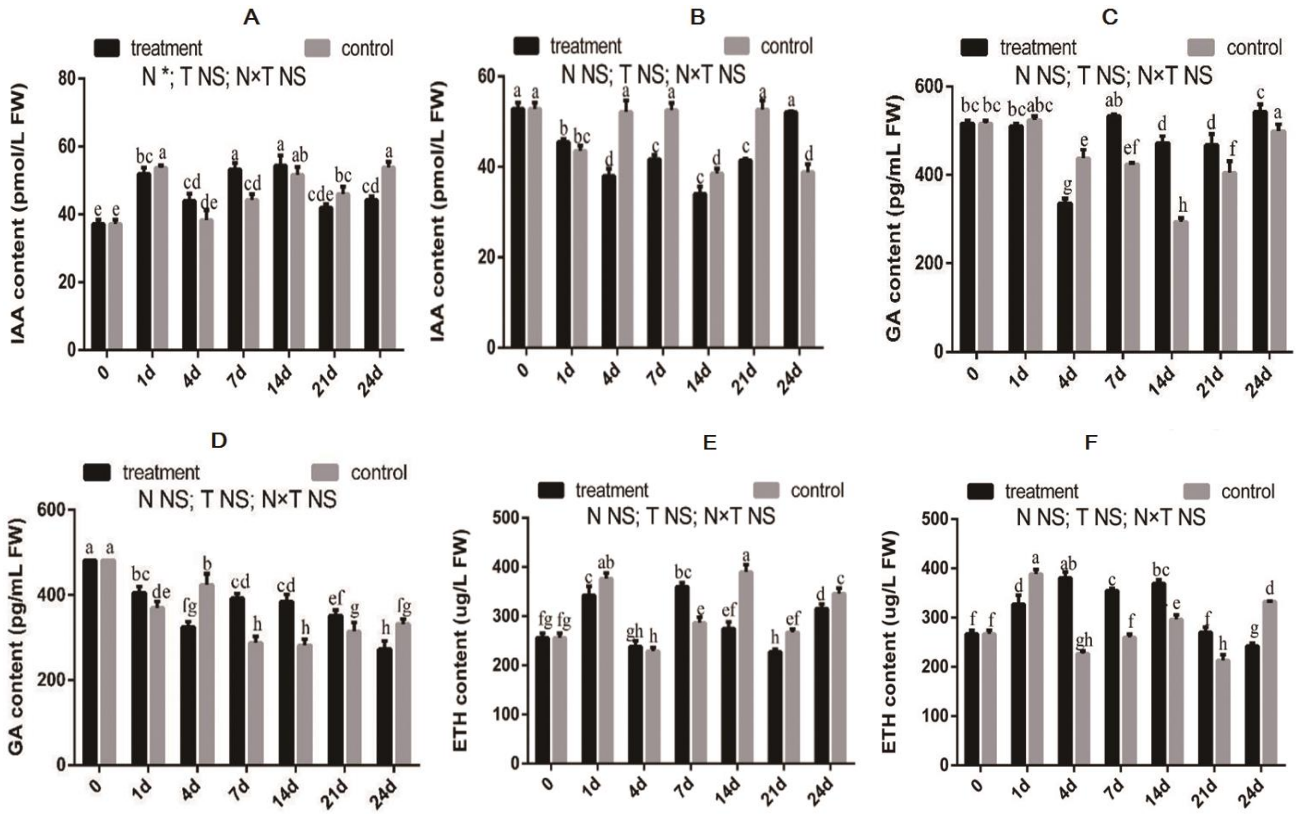


Fig. 7. The content of leafIAA(A), root IAA (B), leaf GA(C), root GA (D), leaf ETH (E), and root ETH.

## Discussion

The results of this study showed that there was no obvious deficiency of N status*in Fraxinus mandshurica* in experimental group compared to the CK. There were significant differences in physiological and morphological parameters as well as the expression of related genes between experimental group and control group, indicating the existence of ways to resist adversity. We supported the view that *Fraxinus mandshurica* could increase the control of their own material metabolism, regulate oxidative stress and the antioxidant system, delay aging and inhibit their growth for saving energy through circadian gene, reactive oxygen species scavenging genes, N metabolism, and endogenous hormone. As a result, it could resist stress and maintain basic life activities.

Our results showed that the plant biomass was significantly decreased. Plant height and fresh weight were more obvious, indicating that *Fraxinus mandshurica* inhibited their growth for saving energy. $NO_3$- and $NH_4$+ are two primary forms of inorganic N which can be absorbed by plants. Some $NO_3$- absorbed by the roots is assimilated into the roots, but the larger part is transported into the leaf, where it is firstly reduced to $NO_3$- by NR and then further converted to $NH_4$+ by NIR. The $NH_4$+, derived from nitrate or directly absorbed by ammonium transporters, is further assimilated by the GS/GOGAT pathway into amino acids (Xu *et al*., 2012). In this study, short term N-deficient stress which reduced the plant N supply, could promote plant N metabolism in the GS/GOGAT pathway. The results suggested the availability of a critical amount of N is important for optimal growth and development of the plants, and the amount of N greater or less than the required may slow or inhibit the growth. Furthermore, GDH enzyme activity and gene expression were increased first and then decreased, which showed *Fraxinus mandshurica* could maintain the balance of N metabolism through GDH pathway under N deficiency stress. These observations explained why the content of $NH_4$ + in the early stage increased during N-deficient stress. But the plant had a certain limit to the maintenance of N balance in the body, GS/GOGAT pathway was inhibited after 3d, GDH pathway was inhibited after 5d to 7d. In Addition, its expression was very low under N deficiency conditions, which was consistent with previous studies on other plant species (Grossman, 2001; Piwpuan & Zhai, 2013; Jampeetong & Brix, 2009). Our results indicated that NR is a kind of inducible enzyme and rate limiting enzyme, which is sensitive to environmental conditions.

As an important factor in the biological clock, LHY and TOC1 can identify changes in the external environment and control the response pathway of plant under stress. For example, controlling the expression of a series of gene and the synthesis of the material to help plant to adapt to the environment (Shor & Green, 2016; Anwer & Davis, 2013; Kinmonth-Schultz *et al*., 2013). In this study, we found that, under N-deficiency stress, the expression of TOC1 was higher than in that the CK, and LHY express was higher than the CK at some time. This result was similar to the previous study (Pokhilko *et al*., 2013; Lau *et al*., 2011) which showed that TOC1 inhibits the transcription of multiple target genes. LHY, as an inhibitor of TOC1 transcription, helps *Fraxinus mandshurica* to regulate the expression of TOC1 through varying its expression in different time periods. This

result implies that *Fraxinus mandshurica* regulates the biological clock to adapt to the environment through circadian gene of TOC1.

It is known that N-deficiency stress causes ROS accumulation (Wang *et al*., 2016). In Our results, the gene expression of ROS-scavenging enzyme (pod, sod, cat) had a significant increase under conditions of N deficiency, and they can help *Fraxinus mandshurica* to clear excess ROS in the body. Additionally, a variety of endogenous hormones also have antioxidant effects, therefore, *Fraxinus mandshurica* will decrease the expression of reactive oxygen resistance enzyme in late. When the N supply of plant is for normal restoration, *Fraxinus mandshurica* still has gene expression, because it has a large amount of ROS that need to be cleared. Plant maintained a high level of enzyme activity which could help plants to recover better from the stress state. The results showed that in order to improve the environment adaptability, the plants must maintain high gene expression of ROS-scavenging enzyme and start this way to activate other reactive oxygen species during the period of N deficiency and after the recovery of N supply.

Plant hormones play a role in alleviating salt stress in plants (Javid *et al*., 2011). Our result showed that when *Fraxinus mandshurica* resisted N deficiency stress, plant hormones also had the same effect. ABA can be used as an anti-reverse signaling molecule transmitting from root to leaf (Wilkinson & Davies, 2002), which has been proved in crops exposed to salt stress and drought stress (Keskin *et al*., 2010; Amjad *et al*., 2014). In our study, the content of ABA in leaf was higher than that in the CK, while the content of ABA in root was less than that in the CK. As with other stresses, when woody plants (*Fraxinus mandshurica*) was exposed N deficiency stress, ABA played the role of plant resistance signal. NCED is ABA synthase gene, therefore its trend change is consistent with the content of ABA.PYR1, as a plant receptor gene, can be used to enhance the response of rice to drought stress and salt stress (Kim *et al*., 2014). In our study, the expression of Pyr1 was much higher than that in the CK at some time. The result demonstratedPYR1 could also help woody plants (*Fraxinus mandshurica*) to enhance response to N deficiency stress.

CKT can regulate N-deficiency stress responses of plant by delaying the senescence of plants, enhancing the antioxidant activity, and inhibiting the decomposition of chlorophyll (Zardo *et al*., 2015; Zhang *et al*., 2015). CKT can be specifically synthesized in roots under stress (Lubovska *et al*., 2014), and functions as local and long-distance signals of N status in plants in both directions between root and leaf (Xu *et al*., 2012). In this study, the synthesis of CTK in root increased and was higher than that in the CK from 1d to 21d, while the content of CTK in leaf was higher than that in the CK after 7d.The result showed that *Fraxinus mandshurica* could alleviate the pressure caused by N deficiency stress by increasing CKT synthesis, and CKT was firstly specifically synthesized in the roots and than transported to the various parts of the plant.

Previous studies have shown that IAA can enhance the tolerance of corn and poplar to salt stress and drought adversity by delaying senescence, reducing chlorophyll loss and protein degradation, and suppressing the transcription of senescence-associated genes directly or indirectly (Zoerb *et al*., 2013; Ke Q *et al*., 2015). In addition, the precursor of IAA is an amino acid, IAA is transported basipetally and mediates the N signals from leaf to root (Xu *et al*., 2012). In our study, the content of IAA in leaf increased at the earlier stage and decreased at the later stage, while the content of IAA in root decreased. After the plant was restored to N supply, the content of IAA was more in the experimental group than in the CK. The result suggested, as with other stress, IAA played an important role in N deficiency stress. In addition, *Fraxinus mandshurica* gave priority play to own function of IAA in leaf during N-deficiency stress. Furthermore, *Fraxinus mandshurica* preferentially synthesized IAA in the root after stress relief and made up for the deficiency in root.

Predecessors observed that GA can help pea seedlings to resist salt stress by regulating oxidative stress and the antioxidant system (Gangwar*et al*., 2011). In the present study, the synthesis of GA was first increased and then decreased during N deficiency stress. Our result showed that, under N deficiency stress, *Fraxinus mandshurica* reduced the synthesis of GA, so that it could inhibit the growth of itself for saving energy. But when peas were exposed salt stress, *Fraxinus mandshurica* could also regulate oxidative stress and the antioxidant system by GA, which increased after 4-7d.

ETH plays a role not only in plant growth and development, but also in plant responses to abiotic stresses such as heat, Fe-deficiency, wounding, ozone and salt stress (Chen & Jin-Song, 2006; Chen *et al*., 2005; Wan-Hong *et al*., 2007). It could influence N metabolism, help leaf mustard to resist salt stress (Iqbal, 2015), and scaveng the excess of reactive oxygen species in tobacco (Golemiec *et al*., 2014). In addition, ETH plays an irreplaceable role in the process of tomato resistance to salt stress, especially in the root system (Poor & Borbely, 2015). On the other hand, the destruction of the N metabolism makes the synthesis of ETH precursors blocked. In this study, under N-deficiency stress, the content of ETH in leaf increased in the early stage and decreased in the late stage, and the content of ETH in root had a higher amount of synthesis. The result showed that woody plants (*Fraxinus mandshurica*), like other plants, increased the amount of ETH synthesis when exposed to N deficiency stress. In addition, N deficiency stress will cause greater damage to the roots of *Fraxinus mandshurica* by ROS, and therefore *Fraxinus mandshurica* is in short supply of ETH precursor and still maintains a high level of ETH in roots.

## Conclusion

In the present study, we determined the morphological, biochemical, and physiological characteristics of *Fraxinus mandshurica* under N deficiency stress, as well as its response to N deficiency stress on gene expression. Our study supports the hypothesis that N deficiency stress can affect the growth and development of *Fraxinus mandshurica*, and it will adapt to the environment through a variety of ways. First of all, the higher gene expression of circadian gene (TOC1 and LHY) and ROS-scavenging gene, the higher changes of endogenous hormones ABA, ETH, IAA, GA

and ETH in experimental group compared with control group, demonstrating that *Fraxinus mandshurica* could adapt to N deficiency environment by them. In addition, short-term lack of N could promote N metabolism of *Fraxinus mandshurica* and maintain N balance in a certain extent by GDH pathway. Furthermore, *Fraxinus mandshurica* resist N deficiency by ways of inhibition of growth for saving energy, regulating oxidative stress and antioxidant system, delaying aging, and strengthening control of the substance metabolism. This study revealed that under N stress, *Fraxinus mandshurica* could treat stress by multiple biological pathways and it was useful to study the survival of *Fraxinus mandshurica* in the wild.

**Acknowledgements**

This work is supported by National Natural Science Foundation (No. 3127069) and the National Innovation Training Program (No. 201510225028) of the Northeast Forestry University.

**References**

Amjad, M., J. Akhtar and Q. Anwar-ul-Ha. 2014. Integrating role of ethylene and ABA in tomato plants adaptation to salt stress. *Scientia Horticulturae*., 172: 109-116.

Anwer, M.U. and S.J. Davis. 2013. An overview of natural variation studies in the *Arabidopsis thaliana* circadian clock. *Seminars in Cell & Developmental Biology*, 24: 422-429.

Casano, L.M., M. Martin and B. Sabater. 1994. Sensitivity of superoxide dismutase transcript levels and activities to oxidative stress is lower in mature-senescent than in young barley leaves. *Plant Physiology*, 106: 1033-1039.

Chen, T. and Z. Jin-Song. 2006. Ethylene biosynthesis and signaling pathway. *Chin. Bull. Bot.*, 5: 519-530.

Chen, Y, F., N. Etheridge and G.E. Schaller. 2005. Ethylene signal transduction. *Annals of Botany*, 95: 901-915.

Dunlap, J.C. 1999. Molecular bases for circadian clocks, *Cell*., 96: 271-290.

Gangwar, S., V.P. Singh, P.K. Srivastava and J.N. Maurya. 2011. Modification of chromium (VI) phytotoxicity by exogenous gibberellic acid application in *Pisum sativum* (L.) seedlings. *Acta Physiol. Plant*., 33: 1385-1397.

Golemiec, E., K. Tokarz and M. Wielanek. 2014. A dissection of the effects of ethylene, H2O2 and high irradiance on antioxidants and several genes associated with stress and senescence in tobacco leaves. *J. Plant Physiol*., 171: 269-275.

Grossman, A. 2001. Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Ann. Rev. Plant Physiol. Plant Mol. Biol*., 52: 163-210.

Hassan, I.A., N.S. Haiba, R.H. Badr, J.M. Basahi, T. Almeelbi, I.M. Ismail and W.K. Tata. 2017. Effects of ambient ozone on reactive oxygen species and antioxidant metabolites in leaves of pea (*Pisum sativum* L.) plants. *Pak. J. Bot*., 49: 47-55.

He, Z.L., Y.G. Zhan, F.S. Zeng, X. Zhao and X. Wang. 2015. Drought physiology and gene expression characteristics of *Fraxinus* interspecific hybrids. *Plant Growth Reg*., 78: 179-163.

Hinsinger, D.D., M. Gaudeul, A. Couloux, J. Bousquet and N Frascaria-Lacoste. 2014. The phytogeography of Eurasian *Fraxinus* species reveals ancient transcontinental reticulation. *Mol. Phylogenet. Evol*., 77: 223-237.

Hu, L.J., K. Uchiyama and H.L. Shen 2010. Multiple-scaled spatial genetic structures of *Fraxinus mandshurica* over a riparian mountain landscape in Northeast China. Conserv. *Genet*., 11: 77-87.

Iqbal, N., S. Umar and N.A. Khan. 2015. Nitrogen availability regulates proline and ethylene production and alleviates salinity stress in mustard (*Brassica juncea*). *J. Plant Physiol*., 178: 84-91.

Jampeetong, A. and H. Brix. 2009. Nitrogen nutrition of Salvinianatans: Effects of inorganic nitrogen form on growth, morphology, nitrate reductase activity and uptake kinetics of ammonium and nitrate. *Aquat Bot*., 90: 67-73.

Javid, M.G., A. Sorooshzadeh and F. Moradi. 2011. The role of phytohormones in alleviating salt stress in Crop plants. *Aust. J. Crop Sci*., 5: 726-734.

Ke, Q.B., Z. Wang and C.Y. Ji. 2015. Transgenic poplar expressing Arabidopsis YUCCA6 exhibits auxin-overproduction phenotypes and increased tolerance to abiotic stress. *Plant Physiol. Bioch*., 94: 19-27.

Keskin, B.C., A.R. Memon, A.T. Sarikaya and B. Yüksel. 2010. Abscisic acid regulated gene expression in bread wheat. *Aust. J. Crop Sci*., 4: 617-625.

Kim, H., K. Lee and H. Hwang. 2014. Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *J. Exp. Bot*., 65: 453-464.

Kinmonth-Schultz, H.A., G.S. Golembeski and T. Imaizumi. 2013. Circadian clock-regulated physiological outputs: Dynamic responses in nature. *Semin Cell Dev. Biol*., 24: 407-413.

Kong, D.M., J.E. Preece and L.H. Shen. 2011. Somatic embryogenesis in immature cotyledons of Manchurian ash (*Fraxinus mandshurica* Rupr.). *Plant Cell Tis. Org*., 108: 485-492.

Lau, O.S., X. Huang and C. Jean-Benoit. 2011. Interaction of Arabidopsis DET1 with CCA1 and LHY in mediating transcriptional repression in the plant circadian clock. *Mol. Cell*., 43:703-712.

Lin, C.C. and C.H. Kao. 1996. Disturbed ammonium assimilation is associated with growth inhibition of roots in rice seedlings caused by NaCl. *Plant Growth Regul*., 18: 233-238.

Lubovska, Z., J. Dobra and H. Storchova. 2014. Cytokinin oxidase/dehydrogenase over expression modifies antioxidant defense against heat, drought and their combination in *Nicotiana tabacum* plants. *J. Plant Physiol*., 171: 1625-1633.

Luo, J., H. Li and T. Liu. 2013. Nitrogen metabolism of two contrasting poplar species during acclimation to limiting nitrogen availability.*J. Exp. Bot*., 64: 207-4224.

McAllister, C.H., P.H. Beatty and A.G. Good. 2012. Engineering nitrogen use efficient crop plants: the current status. Plant *Biotechnol. J*., 10: 1011-1025.

Melo-Oliveira, R., I.C. Oliveira and G.M. Coruzzi. 1996. Arabidopsis mutant analysis and gene regulation define a no redundant role for glutamate dehydrogenase in nitrogen assimilation. *Proc. Natl. Acad. Sci. USA*., 93: 4718-4723.

Milborrow, B.V. 2001. The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. *J. Exp. Bot*., 52:1145-1164.

Nefissi, R., Y. Natsui and K. Miyata. 2015. Roles of circadian clock in developmental controls and stress responses in Arabidopsis: Exploring a link for three components of clock function in Arabidopsis. *Adv. Genet*., 92:107-184.

Piwpuan, N., X. Zhai and H. Brix. 2013. Nitrogen nutrition of *Cyperus laevigatus* aeviphormium tenax: Effects of ammonium versus nitrate on growth, nitrate reductase activity and N uptake kinetics. *Aquat. Bot*., 106: 42-51.

Pokhilko, A., A.P. Fernandez, K.D. Edwards, M.M. Southern, K.J. Halliday and A.J. Millar. 2012. The clock gene circuit in Arabidopsis includes a repressilator with additional feedback loops. *Mol. Syst. Biol*., 8: 574-587.

Pokhilko, A., P. Mas and A.J. Millar. 2013. Modelling the widespread effects of TOC1 signaling on the plant circadian clock and its outputs. *BMC Sys. Biol*., 7: 23.

Poor, P.K.J. and P. Borbely. 2015. Salt stress-induced production of reactive oxygen-and nitrogen species and cell death in the ethylene receptor mutant never ripe and wild type tomato roots. *Plant Physiol. Bioch*., 97: 313-322.

Rubio-Wilhelmi, M.M., E.S. Anchez-Rodriguez and M.A. Rosales. 2011. Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. *Environ. Exp. Bot*., 72: 167-173.

Ryosuke, H. and C. George. 2003. Light on the circadian clock and the photoperiodic control of flowering. *Curr. Opin. Plant Biol*., 6: 13-19.

Shi, H., L. Chen, T.T. Ye, X.D. Liu, K.J. Ding and Z.L. Chan. 2014. Modulation of auxin content in Arabidopsis confers improved drought stress resistance. *Plant Physiol. Biochem*., 82: 209-217.

Shor, E. and R.M. Green. 2016. The impact of domestication on the circadian clock. *Trends Plant Sci*., 21: 281-283.

Sun, S., H. Wang, H. Yu, C. Zhong, X. Zhang and J. Peng. 2013. GASA14 regulates leaf expansion and abiotic stress resistance by modulating reactive oxygen species accumulation. *J. Exp. Bot*., 64: 1637-1647

Surez-Lpez, P., K. Wheatley and F. Robson. 2001. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature*, 410: 1116-1120.

Varvara, P.G. and R.G. Bernard. 2001. Ethylene and flooding stress in plants. *Plant Physiol. Biochem*., 39: 1-9.

Wallander, E. 2008. Systematics of *Fraxinus* (Oleaceae) and evolution of dioecy. *Plant Syst. Evol*., 273: 25-49.

Wang, F., X.M. Wang and C.Z. Zhao. 2016. Alternative pathway is involved in the tolerance of highland barley to the low-nitrogen stress by maintaining the cellular redox homeostasis. *Plant Cell Rep*., 35: 317-328.

Wang, L., Q. Zhou and L. Ding. 2008. Effect of cadmium toxicity on nitrogen metabolism in leaves of *Solanum nigrum* L., as a newly found cadmium hyperaccumulator. *J. Hazard Mater*., 154: 818-825.

Wan-Hong, C., J. Liu and H. Xin-Jian. 2007. Modulation of ethylene responses affects plant salt-stress responses. *Plant Physiol*., 143: 707-719.

Wilkinson, S. and W.J. Davies. 2002. ABA-based chemical signaling: the coordination of responses to stress in plants. *Plant Cell Environ*., 25: 195-210.

Xu, G., X. Fan and A.J. Miller. 2012. Plant nitrogen assimilation and use efficiency. *Ann. Rev. Plant Biol*., 63: 153-182.

Yanovsky, M.J. and S.A. Kay. 2002. Molecular basis of seasonal time measurement in Arabidopsis. *Nature*, 419: 308-312.

Zardo, D.M., A. Alberti and A. Nogueira. 2015. Phenolic compounds and antioxidant capacity of brazilian apples. *Food and Nutrition Sciences*, 6: 727-735.

Zeng, F.S., L.L. Li, N.S. Liang, X. Wang, and X. Li.2015. Salt tolerance and alterations in cytosine methylation in the interspecific hybrids of Fraxinus velutina and *Fraxinus mandshurica*. *Euphotic*., 205: 721-737.

Zeng, F.S., Y.G. Zhan and J. Dong. 2014. Drought resistance and DNA methylation of interspecific hybrids between *Fraxinus mandshurica* and *Fraxinus* Americana. *Trees-Structurer and Function*, 28: 1679-1692.

Zhang, L.T., Z.S. Zhang, H.Y. Gao, X.L. Meng, C. Yang, J.G. Liu and Q.W. Meng. 2012. The mitochondrial alternative oxidase pathway protects the photosynthetic apparatus against photodamage in Rumex K-1 leaves. *BMC Plant Biol*., 12: 40.

Zhang, L.T., Z.S. Zhang, H.Y. Gao, Z.C. Xue, C. Yang, X.L. Meng and Q.W. Meng. 2011. Mitochondrial alternative oxidase pathway protects plants against photo inhibition by alleviating inhibition of the repair of photo damaged PSII through preventing formation of reactive oxygen species in Rumex K-1 leaves. *Physiologia plantarum*, 143: 396-407.

Zhang, Y., I. Han and P. Dawson. 2015. Antioxidant activity assessment and color analysis of skin from different peach varieties grown in south carolina. *Food & Nutr. Sci*., 6: 18-28.

Zhu, Z., F. Qi and C. Yan. 2016. Sexually different morphological, physiological and molecular responses of *Fraxinus mandshurica* flowers to floral development and chilling stress. *Plant Physiol. Biochem*., 99: 97-107.

Zoerb, C., G. Christoph-Martin and K.H. Muehling. 2013. The influence of salt stress on ABA and auxin concentrations in two maize cultivars differing in salt resistance. *J. Plant Physiol*., 170: 220-224.

(Received for publication 12 February 2017)