

RESPONSE OF THE PS II FUNCTION OF *MORUS ALBA* VAR. 'QIUYU' SEEDLING LEAVES TO DIFFERENT NITROGEN FORMS

XU, N.^{1,2*} – YU, S. P.^{1,2*} – DING, J. N.^{1,2} – SHI, C. Q.^{1,2}

¹Harbin University, Harbin 150086, China

²Key Laboratory of Heilongjiang Province for Cold-Regions Wetlands Ecology and Environment Research, Harbin University, Harbin 150086, China

*Corresponding author

e-mail: wetlands1972@126.com; xunan0451@126.com

(Received 26th Aug 2021; accepted 3rd Dec 2021)

Abstract. In order to gain insights into the effect of different nitrogen forms on the photosynthetic ability of *Morus alba*, we studied chlorophyll fluorescence characteristics of one-year-old *M. alba* leaves treated with nitrate-nitrogen (NO_3^- -N), ammonium-nitrogen (NH_4^+ -N) and ammonium nitrate (NH_4NO_3). We found that the PSII photochemical activity, electron transport rate and light energy usage of the *M. alba* leaves were significantly lower in plants treated with NH_4^+ -N compared to those treated with NO_3^- -N and NH_4NO_3 . We did not observe a significant difference in photosynthetic parameters between the NO_3^- -N and NH_4NO_3 treatments. There was an increase in the relative variable fluorescence values at the J step (V_J) and I step (V_I) of *M. alba* leaves under the NH_4^+ -N treatment compared to the NO_3^- -N and NH_4NO_3 treatments. In addition, the values of V_k and V_L in *M. alba* leaves in plants treated with NH_4^+ -N were significantly lower than the NO_3^- -N and NH_4NO_3 treatments, suggesting that the inhibition of the oxygen-evolving complex (OEC) activity and the declined stability of the thylakoid membrane structure in *M. alba* leaves contributed to the decline of the photochemical activity in the PSII reaction center under the treatment of NH_4^+ -N.

Keywords: *Morus alba*, Chlorophyll fluorescence, nitrogen, photosynthetic, OJIP curves

Introduction

Nitrogen is one of the most important elements for plants. Over half of the nitrogen in plants mainly exists within chloroplasts in the form of enzymes, participating in the photosynthetic metabolic processes (Shangguan et al., 2000) and playing an important role in their growth and development (Wang and Baerenklau, 2014). Except for a small amount of amino acids absorbed by plants, nitrogen is mainly absorbed by the roots in the form of nitrate-nitrogen (NO_3^- -N), ammonium-nitrogen (NH_4^+ -N) and, organic nitrogen (Chapin et al., 1993; Kuelland, 1994). NO_3^- -N or NH_4^+ -N constitutes the major form of absorption (Lin and Lin, 2011). Different plants have unique preferences for the type of nitrogen that the roots absorb. For example, *Picea glauca* (Kim et al., 2002), *Pinus sylvestris* (Nordin et al., 2001) and *Oryza sativa* L. (Matsudo et al., 2009) prefer NH_4^+ -N, while excessive NH_4^+ -N can be toxic to the NO_3^- -N-preferring plants like *Haematococcus pluvialis* (Choi et al., 2003) and *Neochloris oleoabundans* (Li et al., 2008; Pruvost et al., 2009). A relatively high concentration of NO_3^- -N can significantly improve the stress resistance of *Indian mustard* (Nathawat et al., 2007) and *Spirulina platensis* (Danesi et al., 2002), while the application of NH_4^+ -N was proven to be more beneficial for *Hordeum vulgare* L. under salt stress (Ali et al., 2001). Many researches have also been conducted studying the absorption volume, absorption rate, and absorption proportion of NH_4^+ -N and NO_3^- -N in crops such as rice, wheat and

vegetables such as cabbage, lettuce, and spinach (Ambus et al., 2011; Sutton et al., 2012; Gruffman et al., 2012).

Morus alba L. is the most widely cultivated and utilized plant in China., *M. alba* can be classified depending on its applications as *M. alba* for silkworm, consumption, fruit, feed, or virescence and so on (Wang et al., 2004; Liu et al., 2019). *M. alba* leaves are highly nutritious, with protein content similar to alfalfa, which is 80%~100% higher than that of forage grass of the grass family, and 40%~50% higher than that of legume forage (Wang et al., 2004; Liu et al., 2019). *M. alba* leaves are rich in amino acids with appropriate proportions, especially rich in glutamic acid, making the *M. alba* leaves an excellent source of protein, and feed combined with *M. alba* leaves can have an enhanced nutritional value (Xu et al., 2012; Liu et al., 2019). With the development of the “South-north” relocation policy which is aimed to encourage planting *M. alba* trees in the Northern part of China, *M. alba* is being cultivated at an increasing rate. *M. alba* trees are resistant to salinity and drought (Hu et al., 2007; Zhang et al., 2012; Pang et al., 2014), therefore the cultivation acreage of *M. alba* in the saline-alkali soil area of the stock breeding grassland region of Song Nen Plain is relatively large, to help rehabilitate the degraded grassland and increase the forage capacity. However, the cultivation of *M. alba* in the saline-alkali regions of Northwestern Heilongjiang Province has resulted in slow growth before July, likely caused by the low temperature and less rainfall in the spring. In order to speed up the growth of *M. alba* at the early stages, a previous study has found that increasing nitrogen fertilization could promote the growth of *M. alba* (Zhang et al., 2012; Pang et al., 2014) and the appropriate amount of nitrogen application was determined as well.

The strong stress resistance ability of *M. alba* makes it an excellent tree species for vegetation restoration in degraded ecosystems (Sun et al., 2003; Chen et al., 2006; Liu et al., 2019). However, the lack of nitrogen in the soil of these degraded ecosystems limits plant growth, affecting vegetation recovery (Britto et al., 2001; Jan et al., 2013; Guo et al., 2017; Mu et al., 2021). Careful application of nitrogen fertilizer is one of the important measures that can be adopted to speed up the recovery of degraded ecosystems. In our previous study, we found that *M. alba* preferred NO_3^- -N. Application of NO_3^- -N to *M. alba* increased photosynthetic ability and electron transport rate of the leaves compared to plants that were treated with NH_4^+ -N (Nathawat et al., 2007). In addition to increased photosynthesis, the salt tolerance of *M. alba* also improved with the application of NO_3^- -N (Zhang et al., 2013). However, it remains unclear what causes the high electron transport rate and photosynthetic capacity of *M. alba* under the treatment of NO_3^- -N. In this study, chlorophyll fluorescence technology and fast chlorophyll fluorescence kinetics (OJIP) method were used to study the physiological responses of the leaves of *M. alba* seedlings, such as PS II photochemical reactivity, oxygen-evolving complex (OEC) function at the electron donor side, electron transport ability at the electron receptor side, electron storage capacity, thylakoid membrane structure state and light allocation parameters under different nitrogen forms; the reasons for the higher photosynthetic capacities under NO_3^- -N treatment compared to NH_4^+ -N treatment. In order to provide theoretical basis for the rational allocation of tree species in the process of vegetation restoration in the degraded ecological nitrogen deficient area, and provide some basic data and provide some basic data for rational fertilization for *M. alba*.

Materials and methods

Materials

Seeds of Mulberry ‘Qiuyu’ were received from the Heilongjiang Academy of Agricultural Sciences, China. Uniform mulberry seeds with orange color were selected for surface disinfection with 3.0% NaCl solution for 20 min, then washed with sterile water, soaked in sterilized saturated CaSO₄ solution for 6 h, and placed in an incubator at 37°C to accelerate germination. After blanking, the seeds were sown to the culture medium for seedling growth. The medium was fully mixed with fully mixed peatsoil and vermiculite in a ratio of 1:1(V/V). The medium was high-temperature sterilized, and the temperature was 25/23°C (light/dark), the light intensity was 400 μmol·m⁻²·s⁻¹, the photoperiod was 12/12 h(light/dark). Cultured in an artificial climate box with relative humidity of about 75%, and managed the seedling stage for about 25 days. When the main radicle of seed germination grew to about 3 cm, seedlings with relatively consistent growth were selected to pull out from the culture substrate, carefully washed the culture substrate on the root surface, and then moved into the culture substrate (length 50 cm; width 40 cm; high 30 cm). The culture medium was Hogland nutrient solution, and the pH value of the medium was 6.0± 0.1. In order to prevent the roots from seeing light, the hydroponic box was wrapped with black light-proof paper, and the electric air pump was used for continuous ventilation. After one week of culture, the seedlings germinated white new roots and began to be treated with nitrogen fertilizer. The molar ratios of (NH₄⁺-N) (NO₃⁻-N) in each treatment were 100:0, 50:50, 0:100, respectively. The concentration of other nutrient elements is completely the same, and the solution formula of each treatment is shown in *Table 1*. The culture medium was replaced every 3 days. The two upper fully expanded attached leaves of six to eight weeks old plants were used for experiments.

Table 1. Nutrition composition of the hydroponics solution with different percentage of ammonium and nitrate nitrogen

Nutrition Composition	NH ₄ ⁺ :NO ₃ ⁻		
	100:0	50:50	0:100
Ca(NO ₃) ₂ (mmol·L ⁻¹)	1.50	0.50	0.00
KNO ₃ (mmol·L ⁻¹)	2.00	1.50	0.00
(NH ₄) ₂ SO ₄ (mmol·L ⁻¹)	0.00	1.25	0.25
K ₂ SO ₄ (mmol·L ⁻¹)	0.00	0.25	1.00
CaSO ₄ (mmol·L ⁻¹)	0.00	0.50	1.00
CaCl ₂ (mmol·L ⁻¹)	0.00	0.50	0.50
KH ₂ PO ₄ (mmol·L ⁻¹)	1.00	1.00	1.00
MgSO ₄ (mmol·L ⁻¹)	1.00	1.00	1.00
NaCl (mmol·L ⁻¹)	0.50	0.50	0.50

Experimental design

The experiment was conducted using a simple randomized block design. There were three treatments of the experiment, with different percentage of ammonium and nitrate nitrogen (NO₃⁻-N : NH₄⁺-N) which were 100:0, 50:0 and 0:100 (*Table 1*). There were 3 seedlings in each treatment and 3 replicates in each treatment. The growth parameters and

photosynthetic parameters were measured after the seedlings were cultured in different nitrogen nutrient solution for 2 weeks.

Growth parameters determination

Select the processing relative consistent growth in mulberry branch contact with the ground diameter, the record for the ground diameter, each treatment for the plant, respectively will be filming for the root and aboveground 105°C for 30 min, 60°C after drying to constant weight according to its weight, or get the underground biomass and biomass on the ground, and calculate the total biomass (the sum of underground biomass and the ground biomass), root-shoot ratio = underground biomass/biomass on the ground.

Measurement of chlorophyll fluorescence

PSII Chl fluorescence measurements were done using an FMS-2 portable pulse modulated fluorometer (*Hansatch*, UK). The maximum PSII quantum yield (F_v/F_m) was determined in dark adapted (15 min) samples. After a short log phase, the fluorescence transient was induced by continuous actinic white light ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 2 h, provided by Fluorimeter light source. Maximum quantum yield of PSII (F_v/F_m), photochemical quenching coefficient (q_p), and the actual PSII efficiency under irradiance (Φ_{PSII}) were calculated according to Genty et al. (1989). Chlorophyll a fluorescence transient was measured with a Handy-PEA fluorometer (*Hansatch*, UK). Having been dark adapted for 1 h before heat treatment, all the leaves were immediately exposed to a saturating light pulse ($3000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PFD) for 2 s after heat treatment in the dark at different times. Each transient obtained from the dark-adapted samples was analyzed according to the JIP-test (Strasser et al., 1995) by utilizing the following original data: (1) the fluorescence intensity at 20 ms (F_o , when all RCs of PSII are open); (2) the maximum fluorescence intensity (F_m , when all RCs of PSII are closed) and (3) the fluorescence intensities at 300 ms (K-step), 2 ms (J-step) and 30 ms (I-step). The maximum quantum yield of PSII photochemistry (F_v/F_m) was calculated as: $F_v/F_m = (F_m - F_o) / F_m$, in this study, $F_m = F_p$. The relative variable fluorescence intensity at J-step (V_j) and I-step (V_i) were calculated as: $V_i = (F_i - F_o) / (F_m - F_o)$. V_K and V_L were the relative variable fluorescence on the V_{O-J} and V_{O-K} point at 0.3 and 0.15 ms.

According to the JIP-test (Strasser et al., 1995; Zhang et al., 2018a) could obtain such as that maximum quantum yield of PSII photochemistry (F_v/F_m), Performance index on absorption basis (PI_{ABS}), Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (at $t=0$) (Ψ_o), Quantum yield for electron transport (at $t=0$) (ϕE_o), quantum yield of absorption flux to dissipated energy (ϕD_o), Absorption flux per RC (ABS/RC), Trapped energy flux per RC (at $t=0$) (TR_o/RC), Electron transport flux per RC (at $t=0$) (ET_o/RC) and Dissipated energy flux per RC (at $t=0$) (DI_o/RC).

Statistical analysis

Excel and SPSS software (Version. 22) were used to conduct statistical analyses on the measured data. The data in the figure was denoted as mean $\pm$ standard deviation (SD). One-way ANOVA and least significant difference (LSD) were used to compare the differences among different data groups ($n=3$).

Results and analysis

The effect of different nitrogen forms on chlorophyll fluorescence parameters in the leaves of mulberry

When $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ is 50:50, the above ground biomass and underground biomass of mulberry seedlings reach the highest respectively (2.85 g/ plant and 0.993 g/ plant), and the total biomass from high to low is 50:50, 100:0, 0:100, the difference is very significant (Table 2) ($P<0.05$). The root-shoot ratio of the seedlings was the largest when $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ was 100:0, but the difference was not significant when it was 0:100, and the difference was the smallest when it was 50:50, which was 27.3% and 27.2% lower than the former two, respectively, and the difference was significant ($P<0.05$). The ground diameter is the largest when $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ is 50:50, which is about 1.5 times of that of 100:0 and 0:100, and the difference is extremely significant.

Table 2. Biomass on leaves of mulberry seedlings to different nitrogen forms

$\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$	Above ground biomass (g)	Underground biomass (g)	Total biomass (g)	Root-shoot ratio (%)	Ground diameter(mm)
100:0	1.21±0.02b	0.308±0.04b	1.519±3.11b	3.98±0.51a	0.31±0.14b
50:50	2.85±0.06a	0.993±0.10a	3.840±3.90a	2.89±0.37b	0.54±0.15a
0:100	1.21±0.06b	0.35±0.03bc	1.563±3.66b	3.97±0.19a	0.32±0.15b

The effect of different nitrogen forms on F_v/F_m , $\Phi_{PS II}$, q_p and NPQ in the leaves of mulberry

To characterize the effect of $\text{NO}_3^-\text{-N}$, $\text{NH}_4^+\text{-N}$, NH_4NO_3 treatment on mulberry leaf photosynthesis parameters, we measured the values of F_v/F_m , $\Phi_{PS II}$ and q_p of the *M. alba* leaves under various treatments. We observed a significant decrease in F_v/F_m , $\Phi_{PS II}$ and q_p in plants treated with $\text{NH}_4^+\text{-N}$ compared to $\text{NO}_3^-\text{-N}$ and NH_4NO_3 (Fig. 1). Specifically, we observed a decrease of 9.13% ($P<0.01$), 55.53% ($P<0.01$) and 26.27% ($P<0.01$) for F_v/F_m , $\Phi_{PS II}$ and q_p , respectively, when compared to $\text{NO}_3^-\text{-N}$, and 10.65% ($P<0.01$), 55.36% ($P<0.01$) and 38.87% ($P<0.01$) when compared to the NH_4NO_3 treatment respectively. All differences were highly significant. Under $\text{NH}_4^+\text{-N}$ treatment, the NPQ value of mulberry leaves was significantly higher than those treated with $\text{NO}_3^-\text{-N}$ and NH_4NO_3 . The differences of the chlorophyll fluorescence parameters of mulberry leaves under the treatments of $\text{NO}_3^-\text{-N}$ and NH_4NO_3 were insignificant.

Responses of $\Phi_{PS II}$ and ETR of mulberry seedling leaves to illumination intensity under the treatment of different nitrogen forms

We next tested the values of $\Phi_{PS II}$ and ETR of mulberry seedling leaves treated by different nitrogen forms under different illumination intensity conditions. In general, we observed that increasing the illumination intensity resulted in a decrease in $\Phi_{PS II}$ of mulberry seedling leaves (Fig. 2). ETR values first increased and then declined upon increased illumination, suggesting that there was light saturation. However, we observed significant differences between the different nitrogen treatments. Plants treated with $\text{NO}_3^-\text{-N}$ and NH_4NO_3 did not show difference in $\Phi_{PS II}$ and ETR at different illumination intensities. However, in plants treated with $\text{NH}_4^+\text{-N}$, the $\Phi_{PS II}$ was significantly lower at different illumination intensities ($P<0.01$). There was no difference in ETR of plants

illuminated with 100 and 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, but when the illumination intensity exceeded 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the ETR under $\text{NH}_4^+\text{-N}$ treatment was significantly lower than those under the treatment of $\text{NO}_3^-\text{-N}$ and NH_4NO_3 ($P<0.01$). Lastly, the light saturation point was above 1,000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for $\text{NO}_3^-\text{-N}$ and NH_4NO_3 treatments, and around 600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for the $\text{NH}_4^+\text{-N}$ treatment.

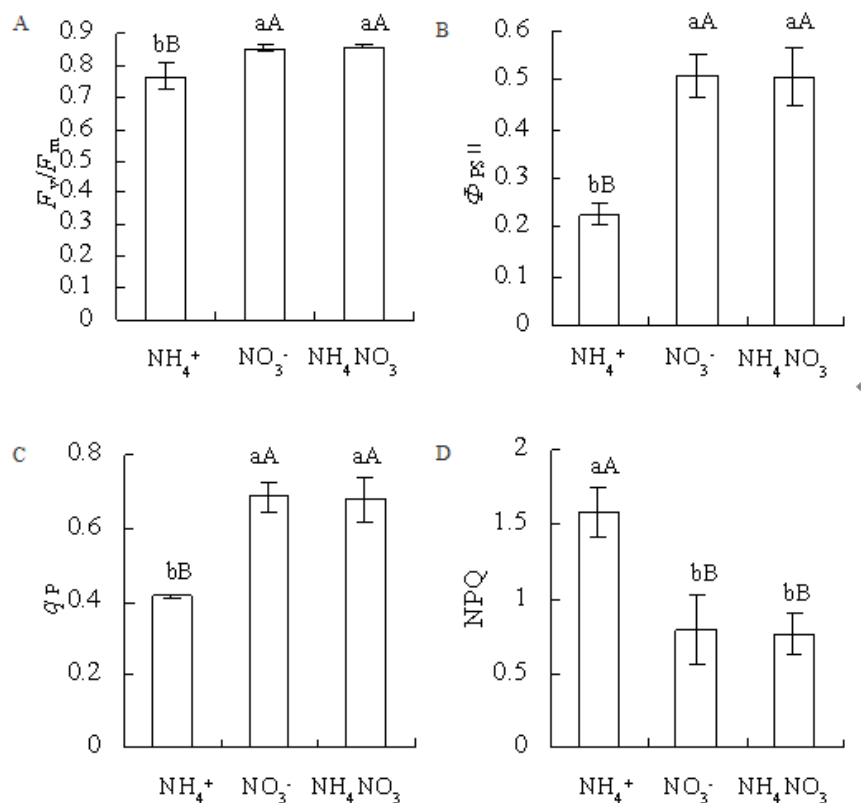


Figure 1. Responses of chlorophyll fluorescence parameters F_v/F_m , Φ_{PSII} , q_p and NPQ in the leaves of mulberry seedlings to different nitrogen forms. Means $\pm$ SD of three replicates are presented. Values followed by different small letters mean significant difference ($p<0.05$), values followed by different capital letters mean very significant difference ($p<0.01$). $n=3$

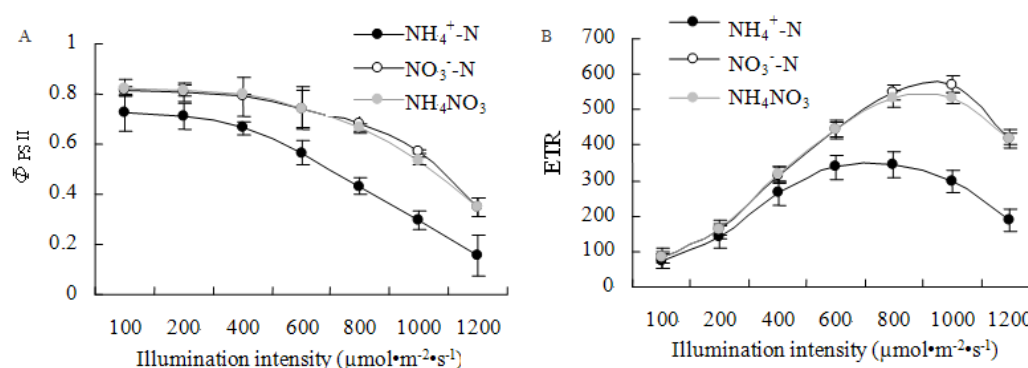


Figure 2. Responses of chlorophyll fluorescence parameters of mulberry seedling leaves to illumination intensity under the treatment of different nitrogen fertilizer treatments. Means $\pm$ SD of three replicates are presented. $n=3$

Responses of OJIP curves in leaves of mulberry seedlings to different nitrogen forms

Fig. 3-A shows that there were significant differences in terms of the OJIP curves of mulberry leaves under the treatments of different nitrogen forms. We did not observe a difference in the relative fluorescent intensities at the O and J step on the OJIP curve in plants treated with NO_3^- -N and NH_4NO_3 . With the extension of time, the relative fluorescent intensities of mulberry leaves at the I and P step under the treatment of NH_4NO_3 gradually became lower than those treated with NO_3^- -N. Under the NH_4^+ -N treatment, the relative fluorescent intensities of mulberry leaves at the O and J step were significantly higher than those treated with NO_3^- -N and NH_4NO_3 , but at the P step, the relative fluorescence intensity was lower than those under the treatments of NO_3^- -N and NH_4NO_3 .

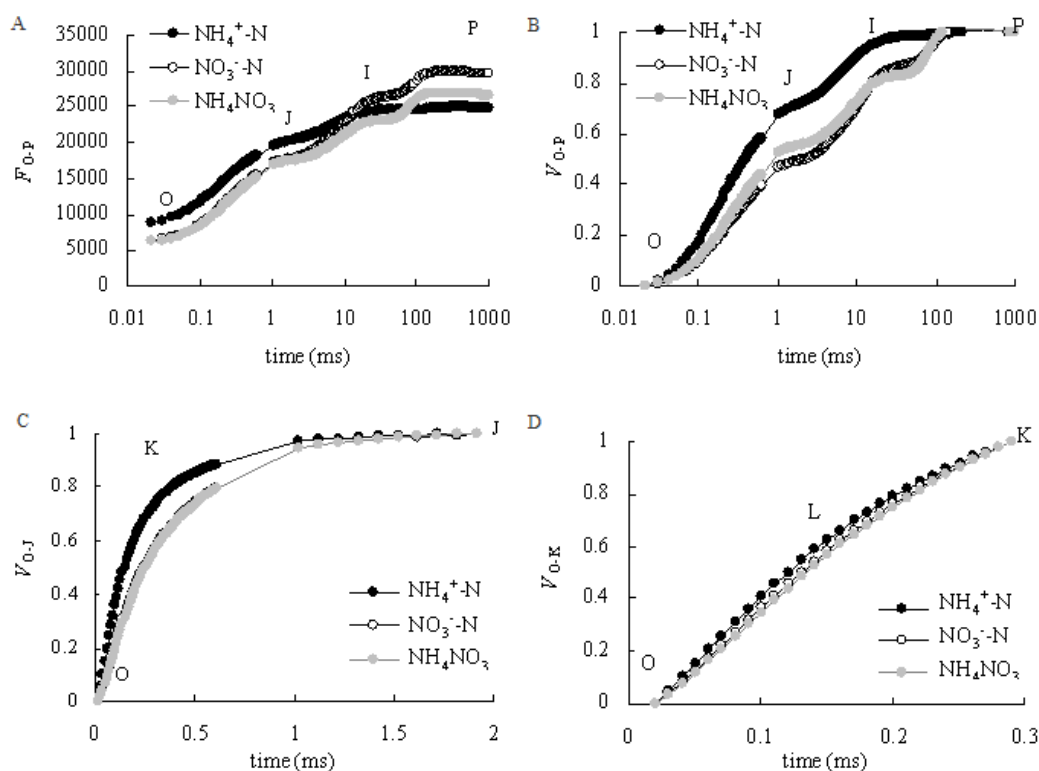


Figure 3. Responses of OJIP curve and the standardized O-P, O-J and O-K curves in leaves of mulberry seedlings to different nitrogen forms. Chlorophyll a fluorescence transients were analyzed with the JIP-test

After standardization of the OJIP curve according to O-P, O-J and O-K, we observed that under the NH_4^+ -N treatment, the relative variable fluorescences of the *M. alba* leaves at the J step (2 ms) and the I step (30 ms) were significantly increased compared to the NO_3^- -N and NH_4NO_3 treatments. Under the NH_4NO_3 treatment, the relative variable fluorescence of the mulberry leaves at the J step was slightly higher compared to the NO_3^- -N treatment, but there was no significant difference between the two treatments at the I step (Fig. 3-B). The relative variable fluorescence of the mulberry leaves at the K step (0.3 ms) was significantly higher in the NH_4^+ -N treatment compared to NO_3^- -N and NH_4NO_3 treatments, while there was no significant difference between the NO_3^- -N and

NH₄NO₃ treatments (Fig. 3-C). There were no major differences in the standardized O-K curves between the different treatments, however, the relative variable fluorescence of the mulberry leaves at the L step (0.15 ms) was higher in the NH₄⁺-N treatment compared to the NO₃⁻-N and NH₄NO₃ treatments (Fig. 3-D).

The effect of nitrogen forms on the variable fluorescence in the OJIP curve in mulberry seedling leaves

We next conducted a quantitative analysis of the differences in the relative variable fluorescence at each point, and found that the relative variable fluorescence of V_L , V_K , V_J , and V_I of *M. alba* leaves at the L, K, J and I step were all significantly higher in the NH₄⁺-N treatment compared to the NO₃⁻-N and NH₄NO₃ treatments. The increases were 7.19% ($P>0.05$), 29.97% ($P<0.01$), 46.39% ($P<0.01$) and 15.02% ($P<0.01$), respectively, when compared to NO₃⁻-N treatment, and 10.38% ($P>0.05$), 31.43% ($P<0.01$), 31.38% ($P<0.01$) and 19.83% ($P<0.01$) when compared to the NH₄NO₃ treatment (Fig. 4). All variables except for V_L showed highly significant differences. The differences of the relative variable fluorescence between the NO₃⁻-N and NH₄NO₃ treatments were relatively small. One notable difference was the 4.13% ($P<0.05$) increase of V_I in the NO₃⁻-N treatment compared to the NH₄NO₃ treatment.

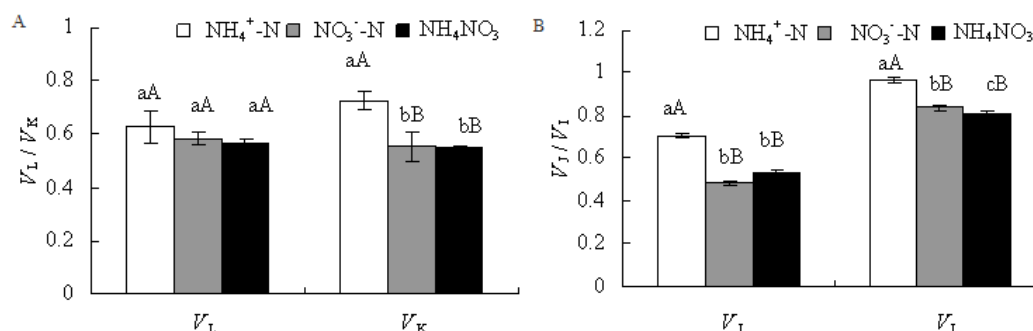


Figure 4. Responses of V_L , V_K , V_J and V_I in leaves of mulberry seedlings to different nitrogen forms. Means $\pm$ SD of three replicates are presented. values followed by different small letters mean significant difference ($p<0.05$), values followed by different capital letters mean very significant difference ($p<0.01$). $n=3$

Responses of S_m , N , ϕE_o and ϕD_o in leaves of mulberry seedlings to different nitrogen forms

We observed significantly higher S_m and N in mulberry leaves in plants treated with NH₄⁺-N compared to the NO₃⁻-N and NH₄NO₃ treatments. The S_m and N were, slightly lower under the NH₄NO₃ treatment compared to the NO₃⁻-N treatment, but the difference was not significant (Fig. 5). Under the NH₄⁺-N treatment, the ϕE_o of mulberry leaves were 54.27% ($P<0.01$) lower compared to the NO₃⁻-N treatment, and 47.27% ($P<0.01$) lower compared to the NH₄NO₃ treatment. The ϕE_o of mulberry leaves was 15.30% ($P>0.05$) higher under NO₃⁻-N treatment compared to the NH₄NO₃ treatment. Under NH₄⁺-N treatment, the ϕD_o of mulberry leaves were 68.64% ($P<0.01$) lower compared to the NO₃⁻-N treatment, and 54.11% ($P<0.01$) lower compared to the NH₄NO₃ treatment. There was no significant difference in ϕD_o between the NO₃⁻-N and NH₄NO₃ treatments.

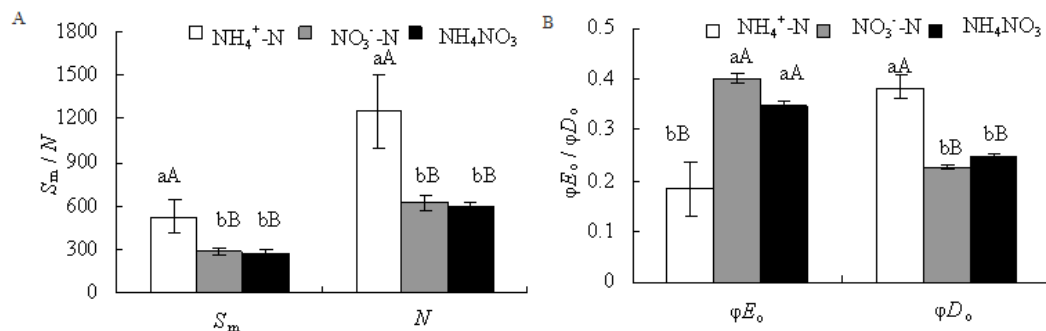


Figure 5. Responses of S_m , N , ϕE_0 and ϕD_0 in leaves of mulberry seedlings to different nitrogen forms. Means $\pm$ SD of three replicates are presented. values followed by different small letters mean significant difference ($p < 0.05$), values followed by different capital letters mean very significant difference ($p < 0.01$). $n = 3$

Discussion

In our study, compared with the single nitrogen nutrition, the proper $\text{NH}_4^+\text{-N}:\text{NO}_3^-\text{-N}$ ratio can promote mulberry growth. There are great differences in the uptake and utilization of nitrogen forms in plants, and the uptake of nitrogen is different in different plants or in different growth stages of the same plant. The pure supply of $\text{NH}_4^+\text{-N}$ will inhibit the absorption of K^+ and Ca^{2+} , and bring ammonia toxicity, limiting the growth of plants (Gruffman et al., 2012; Liu et al., 2019; Khan et al., 2020). However, the pure supply of $\text{NO}_3^-\text{-N}$ tends to increase the pH of rhizosphere, which is not conducive to the absorption and utilization of mineral nutrients (Ipperisiel et al., 1989; Guo et al., 2017; Cai et al., 2019). Combined with the results of previous studies and our study, the reasons for the highest growth of mulberry when the ratio of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ was 50:50 were as follows: on the one hand, it was conducive to root growth, increased the absorption and transformation of soil nutrients, and reduced ammonia poisoning (Omara, 1989; Zhang et al., 2019; Yang et al., 2020); On the other hand, under the condition of solution culture, ammonium nitrogen will not be quickly converted to nitrate nitrogen, which not only avoids the reduction of cation absorption under the condition of single ammonium nitrogen supply, but also avoids the low nitrogen utilization rate caused by the easy leaching of $\text{NO}_3^-\text{-N}$ in soil culture, which can save more energy and increase the absorption of phosphorus than single nitrate nitrogen. Therefore, mulberry grows best when $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ are properly proportioning.

Chlorophyll fluorescence analysis is important in understanding the absorption and utilization of light energy by plants, and the function of the photosynthesis system. The effects of different nitrogen forms on a plant's photosynthesis system can be different. For example, the study by Chen et al. showed that rice plants under water-stress had less inhibitory effect on the photosynthetic capability when treated with $\text{NH}_4^+\text{-N}$ compared to the plant treated with $\text{NO}_3^-\text{-N}$, and the content of Rubisco in the leaves was higher (Sun et al., 2003; Chen et al., 2006; Guo et al., 2017; Yang et al., 2020). Here, we found that treating *M. alba* with $\text{NH}_4^+\text{-N}$ resulted in significantly decreases in F_v/F_m , $\Phi_{\text{PS II}}$ and q_p in the leaves compared to the $\text{NO}_3^-\text{-N}$ treatment. From the illumination response curves of the chlorophyll fluorescence parameters, we also observed that mulberry leaves under different light intensities result in significantly lower $\Phi_{\text{PS II}}$ and ETR in the $\text{NH}_4^+\text{-N}$ treatment compared to $\text{NO}_3^-\text{-N}$. Under the treatment of $\text{NH}_4^+\text{-N}$, the ETR light saturation point was significantly lower than those under the treatment of $\text{NO}_3^-\text{-N}$. This suggests that

treating mulberry with $\text{NH}_4^+\text{-N}$ reduces the PS II photochemical activity and solar energy utilization capacity of the *M. alba* leaves compared to $\text{NO}_3^-\text{-N}$ treatment. Interestingly, we observed significantly higher NPQ in *M. alba* leaves treated with $\text{NH}_4^+\text{-N}$, indicating that the leaves of *M. alba* treated with $\text{NH}_4^+\text{-N}$ depended on the non-radiative energy dissipation mechanism of the xanthophyll cycle to reduce the excess excitation energy in leaves. However, this would also reduce the proportion of light energy used in photochemical reactions under $\text{NH}_4^+\text{-N}$ treatment, thus directly leading to a reduced supply of the assimilatory power such as ATP and NADPH, and further inhibiting the photosynthetic carbon assimilation ability, thereby reducing the amount of photosynthate accumulation. This is consistent with the results obtained by a large number of studies showing that application of $\text{NH}_4^+\text{-N}$ alone can have a toxic effect on plants (Britto et al., 2001; Cai et al., 2017; Mu et al., 2021).

In addition, rational application of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ can significantly mitigate ammonium salt poisoning in plants. The study by Jan et al. showed that, compared to the application of $\text{NO}_3^-\text{-N}$ alone, a mixed application of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ can significantly increase dry matter accumulation and protein content in plants (Jan et al., 2013; Mu et al., 2021). However, the study by Nathawat et al. found that a mixed application of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, the yield of *Brassica juncea* L. seeds showed no difference compared to single application of $\text{NO}_3^-\text{-N}$, but was significantly higher than a single application of $\text{NH}_4^+\text{-N}$ (Nathawat et al., 2007; Yang et al., 2019). A mixed application of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ can also benefit the growth of *Citrus reticulata* (Nathawat et al., 2007; Cai et al., 2017). In this study, we found that chlorophyll fluorescence parameters of mulberry leaves were significantly higher in NH_4NO_3 treated plants compared to $\text{NH}_4^+\text{-N}$, but showed no significant difference compared to $\text{NO}_3^-\text{-N}$ treatment. The treatment of NH_4NO_3 , which is equivalent to a treatment with 1 : 1 ratio mix of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, can significantly relieve ammonium salt poisoning in *M. alba*, and achieve an equimolar treatment level as $\text{NO}_3^-\text{-N}$.

The fast chlorophyll fluorescence kinetics technologies can qualitatively and quantitatively measure the functions of different sections of the photosynthesis process, and it is very important for the in-depth study on the photosynthetic function of plants. In this study, we observed significant differences in the OJIP curves between the different nitrogen treatments. Under the $\text{NH}_4^+\text{-N}$ treatment, the relative fluorescence intensities of the mulberry leaves at the O and J step were significantly higher compared to $\text{NO}_3^-\text{-N}$ and NH_4NO_3 , but at the P step, the relative fluorescence intensities were lower than those under the treatments of $\text{NO}_3^-\text{-N}$ and NH_4NO_3 . Therefore, the $\text{NH}_4^+\text{-N}$ treatment resulted in a less pronounced OJIP curve, which suggests that the PSII reaction center was less active. However, OJIP curve can be strongly influenced by environmental factors, and OJIP curves offend require standardization. In this study, we standardized the OJIP curve as O-P, O-J and O-K respectively, and under the $\text{NH}_4^+\text{-N}$ treatment, the relative variable fluorescences V_J and V_I at the J step (2 ms) and I step (30 ms) were significantly increased compared the $\text{NO}_3^-\text{-N}$ and NH_4NO_3 treatments. Under the NH_4NO_3 treatment, the relative variable fluorescence of the mulberry leaves at the J step was slightly higher than those under the treatment of $\text{NO}_3^-\text{-N}$, while there was no significant difference between the two treatments at the I step. V_I reflects the situation of electron transfer from Q_A to Q_B at the receptor side of the PSII reaction center. Increased relative variable fluorescence at the I step caused by the inhibition of the transport process from Q_A to Q_B (Zhang et al., 2018a,b). Therefore, the results from this experiment showed that treating mulberry with $\text{NH}_4^+\text{-N}$ reduced the capacity of electron transport from Q_A to Q_B at the receptor side of

the PSII reaction center compared to treatments of NO_3^- -N and NH_4NO_3 . However, under the treatment of NH_4^+ -N, the S_m and N of mulberry leaves were significantly higher, indicating that the PQ pool at the receptor side of the PS II reaction center was larger, and Q_A^- was reduced more often. These also indicate that the low Q_A to Q_B transport capacity under the treatment of NH_4^+ -N results from decreased electron acceptability of Q_B in the mulberry leaves, and not because of a reduced capacity to accept electrons by the PQ pool downstream of the electron transport. The relative instability of the Q_B function of mulberry leaves has been shown in previous experiments (Xu et al., 2012; Liu et al., 2019).

Under NH_4^+ -N treatment, the relative variable fluorescence V_K of the mulberry leaves at the K step (0.3 ms) was significantly higher than those under the treatments of NO_3^- -N and NH_4NO_3 , and there was no significant difference between the NO_3^- -N and NH_4NO_3 treatments. This suggests that under the NO_3^- -N and NH_4NO_3 treatments, the oxygen-evolving complex (OEC) activities of mulberry leaves were significantly higher than the NH_4^+ -N treatment. High OEC activity facilitates the water splitting function at the donor side and provides sufficient electron supply for the photosynthetic electron transport chain. Low OEC activity in mulberry leaves under the NH_4^+ -N treatment can lead to incomplete water splitting, resulting in the production of active oxygen molecules such as H_2O_2 , thus undermining the normal function of the photosynthetic system. The decrease in the capacity of accepting electrons by Q_B at the electron transport receptor side may be caused by the attack by active oxygen molecules. NH_4^+ -N treatment can change the ultrastructure of *Lycopersicon esculentum* leaf cells, and cause chloroplast swelling, fuzzy or even broken layers, and significant accumulation of osmiophilic granules. In contrast, treatment of NH_4NO_3 can significantly improve this situation (Zhang et al., 2012a, 2018a,b). The osmiophilic granule is an important sign of the dissociation of the thylakoid membrane (Zhang et al., 2012b, 2018; Penella et al., 2014). We found that the differences of the standardized O-K curves under different treatments were relatively small, but under the NH_4^+ -N treatment, the relative variable fluorescence V_L at the L step (0.15 ms) was higher than those under the NO_3^- -N and NH_4NO_3 treatments. These results suggest that NH_4^+ -N treatment alone results in lower structural stability of the thylakoid membrane of the mulberry leaf compare to NO_3^- -N and NH_4NO_3 treatments. However, the variation ranges of V_L under different treatments were all significantly smaller than those of V_K , V_J and V_I , indicating that the stability of the thylakoid structure of mulberry leaves is less vulnerable to damage under the treatments of different nitrogen comparing to the OEC activity and the electron transfer function at the receptor side of the PSII reaction center.

Conclusions

The PSII photochemical activity of the mulberry leaves was significantly higher in the NO_3^- -N treatment compared to NH_4^+ -N, and applying NH_4^+ -N alone was toxic to mulberry. It not only reduced the PSII photochemical activity and the electron transport capacity in the leaves, but also inhibited the oxygen evolving complex function. The light energy absorbed by leaves was mainly dissipated in the form of invalid heat energy. The proportion of the light energy used in photochemical reaction was decreased, and even the thylakoid structure was altered in the NH_4^+ -N treatment. However, under the treatments of NO_3^- -N and NH_4NO_3 , the photosynthetic capacity of mulberry leaves was significantly higher than the NH_4^+ -N treatment. There was no significant difference between the NO_3^- -N and NH_4NO_3 treatments; and the treatment of NO_3^- -N could

significantly relieve the ammonium toxicity in mulberry caused by applying $\text{NH}_4^+\text{-N}$ alone. Our results provide the basis for the development of new nitrogen fertilizer utilization schemes for *M. alba*. In order to further explain the decline of photosynthetic mechanism capacity, especially chlorophyll fluorescence, the enzymatic induced by sole nitrogen forms, but this needs further study.

Funding. This study was financially supported by China-Norway International Collaboration Project (Grant No.CHN-17/0019); The project of Heilongjiang Province Key Laboratory of Cold Region Wetland Ecology and Environment Research (No.201910) of the Harbin University; Young Doctoral Research Foundation of Harbin University (No.2020106); Harbin University of Dr Youth “Internet + double gen start-up fund project” (HUSC202108).

REFERENCES

- [1] Ali, A., Tucker, T. C., Thompson, T. L., Salim, M. (2001): Effects of salinity and mixed ammonium and nitrate nutrition on the growth and nitrogen utilization of barley. – Journal of Agronomy and Crop Science 186: 223-228.
- [2] Ambus, P., Skiba, U., Butterbach-Bahl, K., Sutton, M. A. (2011): Reactive nitrogen and greenhouse gas flux interactions in terrestrial ecosystems. – Plant and Soil 343(1): 1-3.
- [3] Britto, D. T., Siddiqi, M. Y., Glass, A. D. M., Kronzucker, H. J. (2001): Futile transmembrane NH_4^+ cycling: A cellular hypothesis to explain ammonium toxicity in plants. – Proceedings of the National Academy of Sciences of the United States of America 98: 4255-4258.
- [4] Cai, Y. T., Zhang, H., Qi, Y. P., Ye, X., Huang, Z. S., Guo, J. X., Chen, L. S., Yang, L. T. (2019): Responses of reactive oxygen species and methylglyoxal metabolisms to magnesium-deficiency differ greatly among the roots, upper and lower leaves of *Citrus sinensis*. – BMC Plant Biology 19(1): 76.
- [5] Chapin, F. S., Autumn, K., Pugnaire, F. (1993): Evolution of Suites of Traits in Response to Environmental Stress. – The American Naturalist 142: 78-92.
- [6] Chen, G., Zhou, Y., Guo, S. W., Shen, Q. (2006): Effects of Different Nitrogen Forms on Rice Seedlings Growth with Partial Roots Exposed to Water Stress. – Chinese Journal of Rice Science 20(6): 638-644.
- [7] Choi, S. L., Suh, I. S., Lee, C. G. (2003): Lumostatic operation of bubble column photobioreactors for *Haematococcus pluvialis* cultures using a specific light uptake rate as a control parameter. – Enzyme Microb. Technol 33: 403-409.
- [8] Danesi, E. D. G., Rangel-Yagui, C. O., Carvalho, J. C. M., Sato, S. (2002): An investigation of effect of replacing nitrate by urea in the growth and production of chlorophyll by *Spirulina platensis*. – Biomass Bioenerg 23: 261-269.
- [9] Genty, B., Briantais, J. M., Baker, N. R. (1989): The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim Biophys Acta 990: 87-92.
- [10] Gruffman, L., Ishida, T., Nordin, A., Nasholm, T. (2012): Cultivation of Norway spruce and Scots pine on organic nitrogen improves seedling morphology and field performance. – Forest Ecology and Management 276(15): 118-124.
- [11] Guo, P., Li, Q., Qi, Y. P., Yang, L. T., Ye, X., Chen, H. H., Chen, L. S. (2017): Sulfur mediated alleviation of aluminum toxicity in *Citrus grandis* seedlings. – International Journal of Molecular Sciences 18(12): 2570.
- [12] Hu, Y. B., Sun, G. Y., Wang, X. C. (2007): Induction characteristics and response of photosynthetic quantum conversion to changes in irradiance in mulberry plants. – Journal of Plant Physiology 164: 959-968.

- [13] Ipperisiel, D., Alli, I., Machenze, A. F., Mehuys, G. R. (1989): Nitrogen distribution, yield, and quality of silage corn after four nitrogen fertilization. – *Agron. J* 81: 783-789.
- [14] Jan, A., Osman, M. B., Amanullah (2013): Response of chickpea to nitrogen sources under salinity stress. – *Journal of Plant Nutrition* 36: 1373-1382.
- [15] Khan, A., Wang, Z., Xu, K., Li, L., He, L., Hu, H., Li, L. Y., Zhu, X. G. (2020): Validation of an Enzyme-Driven Model Explaining Photosynthetic Rate Responses to Limited Nitrogen in Crop Plants. – *Frontiers and Plant Science* 11: 533341.
- [16] Kim, T., Mills, H. A., Wetzstein, H. Y. (2002): Studies on effects of nitrogen form on growth, development, and nutrient uptake in pecan. – *Journal of Plant Nutrition* 25(3): 497-508.
- [17] Kuelland, K. (1994): Amino Acid Absorption by Arctic Plants: Implications for Plant Nutrition and Nitrogen Cycling. – *Ecology* 75: 2373-2383.
- [18] Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C. Q. (2008): Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. – *Appl. Microbiol. Biotechnol* 81: 629-636.
- [19] Li, Q., Chen, H. H., Qi, Y. P., Ye, X., Yang, L. T., Huang, R. Z., Chen, L. S. (2019): Excess copper effects on growth, uptake of water and nutrients, carbohydrates, and PSII photochemistry revealed by OJIP transients in citrus seedlings. – *Environmental Science and Pollution Research International* 26(29): 30188-30205.
- [20] Lin, Q., Lin, J. D. (2011): Effects of nitrogen source and concentration on biomass and oil production of a *Scenedesmus rubescens* like microalga. – *Bioresource Technology* 102(2): 1615-21.
- [21] Liu, X. J., Xu, N., Wu, Y. N., Li, J. B., Zhong, H. X., Zhang, H. H. (2019): Photosynthesis, chilling acclimation and the response of antioxidant enzymes to chilling stress in mulberry seedlings. – *Journal of Forest Research* 30(6): 2021-2029.
- [22] Matsudo, M. C., Bezerra, R. P., Sato, S., Perego, P., Converti, A., Carvalho, J. C. M. (2009): Repeated fed-batch cultivation of *Arthrospira* (*Spirulina*) *platensis* using urea as nitrogen source. – *Biochem. Eng. J.* 43: 52-57.
- [23] Mu, X., Chen, Y. (2021): The physiological response of photosynthesis to nitrogen deficiency. – *Plant Physiology and Biochemistry* 158: 76-82.
- [24] Nathawat, N. S., Kuhad, M. S., Goswami, C. L., Patel, A. L., Kumar, R. (2007): Interactive effect of N source on salinity on growth indices and ion content of Indian mustard. – *Journal of Plant Nutrition* 30: 569-98.
- [25] Nordin, A., Uggla, C., Näsholm, T. (2001): Nitrogen forms in bark, wood and foliage of nitrogen-fertilized *Pinus sylvestris*. – *Tree Physiology* 21(1): 59-64.
- [26] Omara, H. A. (1989): The effect of spacing, nitrogen and phosphorus application on growth and yield of maize (*Zea mays* L.). – M.Sc. Thesis, Univ. of Khartoum, Faculty of Agric.
- [27] Pang, H. S., Zhang, H. H., Tian, Y., Ao, H., Sun, G. Y. (2014): Effects of NO₃⁻-N on growth and photosynthetic characteristics of mulberry seedlings under Na₂CO₃ stress. – *Pratacultural Science* 31(8): 1515-1522.
- [28] Penella, C., Nebauer, S. G., Bautista, A. S., Lópezgalarza, S., Calatayud, A. (2014): Rootstock alleviates PEG-induced water stress in grafted pepper seedlings: physiological responses. – *J Plant Physiol.* 171(10): 842-51.
- [29] Pruvost, J., van Vooren, G., Cogne, G., Legrand, J. (2009): Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. – *Bioresour. Technol.* 100: 5988-5995.
- [30] Shangguan, Z. P., Shao, M. A., Dyckmans, J. (2000): Effect of nitrogen nutrition and water deficit on net photosynthetic rate and chlorophyll fluorescence in winter wheat. – *Journal of Plant Physiology* 156(1): 46-51.
- [31] Strasser, R. J., Srivastava, A., Govindjee, A. (1995): Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria. – *Photochemistry and Photobiology* 61(1): 32-42.

- [32] Sun, G. H., Zeng, X. P., Zhao, P., Peng, S. L. (2003): Comparison of photosynthetic parameters in leaves of Citrus grands grown under different forms of nitrogen source during photosynthetic acclimation to elevated CO₂. – Acta Ecologica Sinica 23(1): 14-21.
- [33] Sutton, M. A., Reis, S., Billen, G., Cellier, P., Erisman, J. W., Mosier, A. R., Nemitz, E., Sprent, J., Grinsven, H. V., Voss, M. (2012): Nitrogen and Global Change. – Biogeosciences 9(1): 1691-1693.
- [34] Wang, C. H., Xing, X. R., Han, X. G. (2004): Advances in study of factors affecting soil N mineralization in grassland ecosystems. – Chinese Journal of Applied Ecology 15(11): 2184-2188.
- [35] Wang, J., Baerenklau, K. A. (2014): Crop response functions integrating water, nitrogen, and salinity. – Agricultural Water Management 139: 17-30.
- [36] Xu, N., Zhang, H. H., Zhu, W. X., Li, X., Yue, B. B., Jin, W. W., Wang, L. Z., Sun, G. Y. (2012): Effects of nitrogen form on seeding growth and its photosynthetic characteristics of forage mulberry. – Pratacultural Science 29(10): 1568-1572.
- [37] Xu, C., Fisher, R., Wullschlegler, S. D., Wilson, C. J., Cai, M., McDowell, N. G. (2012): Toward a mechanistic modeling of nitrogen limitation on vegetation dynamics. – PLoS One 7: e37914.
- [38] Yang, X., Henry, H. A. L., Zhong, S., Meng, B., Sun, W. (2020): Towards a mechanistic understanding of soil nitrogen availability responses to summer vs. winter drought in a semiarid grassland. – Science Total Environment 741: 140272.
- [39] Yin, L., Xu, H., Dong, S., Chu, J., Dai, X., He, M. (2019): Optimised nitrogen allocation favours improvement in canopy photosynthetic nitrogen-use efficiency: Evidence from late-sown winter wheat. – Environmental and Experimental Botany 159: 75-86.
- [40] Zhang, H. H., Zhang, X. L., Hu, Y. B., Li, X., Xu, N., Tian, Y., Zhang, T., Sun, G. Y. (2012a): Growth characteristics and photosystem II functions of *Sorghum bicolor* × *S. sudanense* seedlings under drought stress. – Acta Agrestia Sinica 20(5): 881-887.
- [41] Zhang, Z. S., Li, G., Gao, H. Y., Zhang, L. T., Yang, C., Liu, P. (2012b): Characterization of photosynthetic performance during senescence in stay-green and quick-leaf-senescence *Zea mays* L. inbred lines. – Plos One 7(8): e42936.
- [42] Zhang, H. H., Zhang, X. L., Li, X., Xu, N., Sun, G. Y. (2013): Role of D1 Protein Turnover and Xanthophylls Cycle in Protecting of Photosystem II Functions in Leaves of *Morus alba* under NaCl Stress. – Scientia Silvae Sinicae 49(1): 99-106.
- [43] Zhang, H. H., Feng, P., Yang, W., Sui, X., Li, X., Gu, S. Y., Xu, N. (2018a): Effects of flooding stress on the photosynthetic apparatus of leaves of two Physocarpus cultivars. – Journal of Forest Research 29(4): 1049-1059.
- [44] Zhang, H. H., Xu, N., Sui, X., Zhong, H. X., Yin, Z. P., Li, X., Sun, G. Y. (2018b): Photosystem II function response to drought stress in leaves of two alfalfa (*Medicago sativa*) varieties. – International Journal of Agriculture and Biology 20(5): 1012-1020.
- [45] Zhang, K., Wu, Y., Hang, H. (2019): Differential contributions of NO₃⁻/NH₄⁺ to nitrogen use in response to a variable inorganic nitrogen supply in plantlets of two Brassicaceae species in vitro. – Plant Methods 15: 86.