

1 **Short Title: High irradiance and NH₄⁺ stress**

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High irradiance induces photoprotective mechanisms and a positive effect on NH_4^+ stress in *Pisum sativum*

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Summary

Photosynthesis provides plant metabolism with reduced carbon (C) but is also the main source of oxidative stress in plants. Likewise, high doses of NH_4^+ as sole N source have been reported to be toxic for most plants, resulting in reduced plant growth and restricting C availability. The combination of high photosynthetic photon flux densities (PPFD) and NH_4^+ nutrition may provide higher C availability but could also have a detrimental effect on the plants, therefore the objective of this study is to evaluate whether NH_4^+ induces photooxidative stress that is exacerbated under high light conditions.

Pea plants (*Pisum sativum* cv Sugar Snap) were grown hydroponically with NH_4^+ (0.5, 2.5, 5 and 10 mM) under high (750 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$) or low PPFD conditions (350 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$). High PPFD contributes to a higher tolerance to ammonium by pea plants, as it originated higher biomass content due to higher photosynthetic rates. However, a deficit of N (0.5 and 2.5 mM NH_4^+) under high PPFD conditions caused an anti-oxidant response, as indicated by increased photoprotective pigment and chloroplastic superoxide dismutase contents. Plants grown with higher doses of N and high PPFD showed less need for photoprotection. An increase in the specific leaf weight (SLW) ratio was observed associated not only with high PPFDs but also with the highest NH_4^+ dose. Overall, these results demonstrate that, despite the activation of some photoprotective responses at high PPFD, there were no photoinhibitory symptoms and a positive effect on NH_4^+ toxicity, thus suggesting that the harmful effects of NH_4^+ are not directly related to the generation of photooxidative stress.

Keywords: Ammonium stress, chlorophyll, carotene, superoxide dismutase, violaxanthin cycle.

Abbreviations: A: antheraxanthin; Chl: chlorophyll; HI: high irradiance; LI: low irradiance; PPFD: photosynthetic photon flux density; ROS: reactive oxygen species; SLW: specific leaf weight; SOD: superoxide dismutase; S.E.: standard error; V: violaxanthin; Z: Zeaxanthin

Introduction

In order to attain the goal of sustainable agriculture it is necessary to maintain an equilibrium between the best possible yield and product quality whilst ensuring a minimal environmental impact. Two of the most important environmental problems resulting from agricultural practices that rely on nitrate fertilizers are water pollution due to nitrate leaching and the increase in global warming due to atmospheric N-containing gas emissions. The use of stabilized- NH_4^+ -based fertilizers is a promising strategy to avoid this pollution as they are formulated with specific compounds such as nitrification inhibitors, which delay the nitrification of ammonium, thus maintaining soil N as NH_4^+ for a longer period of time. Also It is known that NH_4^+ nutrition, as a sole N source, can be toxic to many plants, although a broad degree of tolerance to NH_4^+ stress can be achieved in plants within certain NH_4^+ concentration ranges (Domínguez-Valdivia et al., 2008).

NH_4^+ is a direct precursor of nucleic acids, proteins and other organic molecules, as well as a product of their catabolism. It is the preferred N source when NH_4NO_3 is available, and it is well known that combined-N nutrition, including NO_3^- and NH_4^+ , generally guarantees higher crop yields than NO_3^- or NH_4^+ as the sole N source. Traditional plant breeding has, however, been performed under nitric or combined nutrition, therefore commercial varieties are not usually adapted to NH_4^+ -based nutrition.

C-skeleton availability, which depends on photosynthetic activity and is therefore directly linked to light intensity, may affect NH_4^+ tolerance. Photosynthesis is also the main source of oxidative stress in plant tissues, which is associated with negative effects on plant growth (Mittler, 2002). To counteract the generation of reactive oxygen species (ROS), chloroplasts possess highly efficient photoprotection defence systems that operate by two main mechanisms. The first of these prevents ROS production by enhancing the dissipation of excess excitation energy. This process depends on the presence of zeaxanthin (Z), which is formed by light-induced de-epoxidation of violaxanthin (V) via the intermediate antheraxanthin (A) in the so-called violaxanthin cycle, or xanthophyll cycle (Demmig-Adams & Adams, 1992). The second line of defense is the detoxification of ROS by other antioxidant mechanisms, including superoxide dismutases (SOD) as the first enzymatic protective barrier, and/or through a system of small molecules [e.g. α -tocopherol (Munné-Bosch, 2005)], which deal with ROS produced in photosynthetic membranes. All these mechanisms can be triggered in response to the generation of ROS caused by an environmental stress, such as high radiation.

Most of the previous studies regarding the effect of light intensity and its interaction with N nutrition have focused on studying the effect of light-induced stress (Zhu et al., 2000)

rather than its effect on N nutrition, and have obtained widely differing results as regards its effect on NH_4^+ tolerance (Magalhaes & Wilcox, 1983 a and b; Zhu et al., 2000). It has been shown recently that NH_4^+ nutrition, despite having a negative effect on plant growth, does not produce sufficient oxidative stress to explain such a toxic effect in pea plants (Domínguez-Valdivia et al., 2008). N-deficiency, however, leads to reduced plant growth and photosynthetic ability, which has been considered to produce photoinhibition (Kato et al., 2002). Finally, most studies on NH_4^+ tolerance are carried out at relatively low photosynthetic photon flux density (PPFD) compared to usual light irradiances outside the growth chamber (Zhu et al., 2000; Domínguez-Valdivia et al., 2008).

The objective of this work was therefore to study the effect of two PPFDs (350 and 750 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$), at increasing doses of NH_4^+ as sole N source, on the overall growth of the plant and the photoprotection mechanisms in pea leaves.

Materials and methods

Plant material and growth conditions. Pea seeds (cv. sugar snap) were surface-sterilized according to Labhilili et al. (1995). They were then germinated at 26 °C in darkness for 96 h, in perlite:vermiculite (1:2) and dampened with deionized water, prior to placement in a growth chamber (22/18 °C day/night, 70 % RH, 150 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light intensity and 14 h light/10 h dark photoperiod). One-week-old seedlings were transferred into tanks in groups of eight (8 L volume) and grown for three weeks in controlled-environment chambers under different conditions of NH_4^+ dose and light (two irradiances, see below). The hydroponic vessels contained aerated (0.4 L air min^{-1} L^{-1}) and N-free modified Rigaud and Puppo (1975) solution. The solution was buffered with CaCO_3 (5 mM) to pH 7–7.5, K_2HPO_4 was added instead of KH_2PO_4 and NH_4^+ was supplied as $(\text{NH}_4)_2\text{SO}_4$ during the treatment period, as the only N source, at different concentrations (0.5; 2.5; 5 and 10 mM). Ammonium sulphate was chosen since sulphate is known to have little effect on the uptake of other ions (Mengel and Kirkby, 1987). The nutrient solution was exchanged weekly. Two photosynthetic photon flux densities (PPFD) were applied during these three weeks: a high irradiance (HI) of 750 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ and a low irradiance (LI) of 350 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$. LI treatment was achieved by shading (50% of the incident light) with shade cloth. Plants were cultured in a growth chamber at 22/18°C (day/night) temperature, 60/70% relative humidity and 14 h light/10 h dark photoperiod. Plants were harvested by separating the shoot and root of each plant. The dry weight of several pea plants was obtained after weighing and keeping them in an oven at 75-80 °C for 72 h. Samples were collected, frozen in liquid N_2 and stored at -80 °C for further analysis of the remaining fresh pea plants.

Physiological parameters: CO₂ assimilation was determined using a portable IRGA (LI-6200, Li-Cor, Lincoln, NE, USA) at LI and HI. Measurements were performed with the last fully expanded leaf and with various different plants in each treatment. The foliar area was obtained by using a LICOR LI-3000 area measurer (Li-Cor). Specific leaf weight ratio (SLW) was calculated as the ratio leaf dry weight to its surface, and it was expressed in g DW · m⁻².

Fluorescence measurements were performed with a portable modulated light fluorimeter (OS 5-FL, Optisciences, Tyngsboro, U.S.A.). The maximal photochemical efficiency of the PSII was calculated using the ratio F_v/F_m , where $F_v = F_m - F_o$ (Genty *et al.*, 1989). This was calculated from initial (F_o) and maximum fluorescence (F_m) measured *in vivo* on the last fully expanded leaf pre-acclimatised to the dark for approximately 40 minutes. F_m was estimated by applying a light saturating flash with an intensity of 8000 μmol photons m⁻² s⁻¹.

Pigment and antioxidant content: Lipophilic antioxidants and photosynthetic pigments were extracted in acetone (100%) and analysed by reversed phase HPLC (García-Plazaola & Becerril 1999) following the modifications of García-Plazaola & Becerril (2001). Photosynthetic pigments were measured with a PDA detector (PDA996, Waters). Retention times and conversion factors for carotenoids were as described by García-Plazaola & Becerril (1999; 2001).

Rubisco content: Frozen leaves (0.2 g) were homogenized in a mortar with liquid N₂ in a phosphate buffer 0.1 M, pH 7. Samples were centrifuged at 20,000 g and 4 °C for 20 min, then 5 μg of protein from the supernatants was loaded onto SDS-PAGE gel (12.5% p/v), and stained with Gel-Code Blue Stain reagent (Pierce Biotechnology, Inc., Rockford, USA). The total protein was calculated according to Bradford (1976). To estimate the Rubisco content, densitometry analysis was conducted using the programme Quant 1 in GelDoc 2000 (Bio-Rad), taking 100% as the Rubisco content in the samples treated with 0.5 mM NH₄⁺ and LI.

Superoxide Dismutase activity (SOD, EC. 1.15.1.1). Frozen leaves (0.2 g approx.) were homogenized in a mortar with liquid N₂ and potassium phosphate buffer 50 mM pH 7.8, mannitol 300 mM, MgCl₂ 20 mM and EDTANa₂ 2 mM (1: 10 w/v). Samples were centrifuged at 20,000 g and 4 °C for 20 min. Supernatants (30 μg of protein) were electrophoresed on PAGE-native gels (15%) and SOD activity was detected *in gel* (Beauchamp and Fridovich, 1971; Larrainzar *et al.*, 2008). Isoenzyme identity was assigned according to Gómez *et al.* (2004).

Statistical analysis. The SPSS programme (v.15.0) was used. Statistical calculations were performed using unifactorial MANOVAS (factor: dose of NH₄⁺), including as dependent variables all those that could present a relationship between themselves (e.g. photosynthetic pigments and antioxidant pigments). The Levene test was used to determine variance

homogeneity. The LSD and Dunnett T3 post-hoc statistical tests were applied to determine the homogeneity and non-homogeneity of variances cases, respectively. Student's *t* test was performed to compare the irradiances for each NH_4^+ dose, taking into account whether it complied with the principle of homoscedasticity upon applying the Levene test. All statistical analyses were conducted at a significance level of 5% ($P \leq 0.05$).

Results

Plant growth under HI resulted in the highest biomass (shoot and root; dry weight), with the highest value being obtained for 2.5 mM NH_4^+ (Fig. 1 A and B), and highest photosynthesis rate (Fig. 2 A), with a maximum rate for 5 mM NH_4^+ . However, important growth restrictions were found at lowest and highest NH_4^+ doses under HI, and hence maximal differences in plant growth between HI and LI are seen at intermediate NH_4^+ concentrations (Fig. 1 A and B). Plants grown under LI did not show a notable variation between NH_4^+ treatments in terms of biomass or photosynthesis, with the highest values being obtained at an NH_4^+ dose of 2.5 mM. No major changes were detected in leaf area between irradiances or among NH_4^+ concentrations (Fig. 1 C). However, the different PPFDs had an important effect on the specific leaf weight index (SLW; Fig. 1 D). Thus, the plants exposed to HI were thicker and showed higher SLW values than those exposed to LI. On the other hand, the SLW ratio tended to increase with external NH_4^+ concentration under LI (Fig. 1 D). The F_v/F_m ratio was independent of PPFD and NH_4^+ dose applied (Fig. 2 B). Although both HI and LI treatments showed a similar PSII photochemical efficiency (Fig. 2 B), pea plants cultured under HI showed a far higher photosynthetic rate than those exposed to LI (Fig. 2 A).

The content of chlorophyll a and b, which are the main components in the photosynthetic antenna, did not vary between HI and LI (Fig. 3 A), although the chlorophyll content increased notably (threefold) on going from 0.5 to 5 mM NH_4^+ dose under HI. A similar, although slightly lower (twofold), increase was also detected under LI. The Chl a/b ratio remained constant in LI at all NH_4^+ doses tested, whereas it increased with NH_4^+ dose under HI (Fig. 3 B). The total carotenoid content based on Chl was higher for HI than for LI treatment (Fig. 4 A), with this increase being more evident at 0.5 mM NH_4^+ . Pea plants grown at 0.5 mM NH_4^+ showed the highest total carotenoid content under HI, whereas the carotenoid content at 2.5, 5 and 10 mM NH_4^+ was essentially the same. The total carotenoid content in plants exposed to LI remained constant regardless of NH_4^+ dose (Fig. 4 A) (Statistic not shown). The proportion for each individual carotenoid based on Chl differed with

light treatment and N applied, with the largest differences being found for the extreme NH_4^+ treatments (0.5 and 10 mM NH_4^+ ; Fig. 4 A).

The content of other anti-oxidant molecules such as α -tocopherol varied considerably for all measurements (LI and HI in all NH_4^+ doses). However, the effect of different PPFDs was only significant at 2.5 mM NH_4^+ , where α -tocopherol content was far higher in plants exposed to HI than those cultivated at LI (Fig. 4 B). Xanthophyll cycle pigment content (violaxanthin-V, antheraxanthin-A and zeaxanthin-Z) was also higher in plants exposed to HI (Fig. 5). Furthermore, HI changed the proportion of V cycle components, increasing the de-epoxidated forms (A+Z) with respect to those observed at LI at NH_4^+ doses of 0.5 and 2.5 mM (Fig. 5B). V cycle component content increased with NH_4^+ dose under LI (total cake area), whereas it tended to decrease with NH_4^+ dose at HI; the highest total V content was observed at 0.5 mM NH_4^+ (Fig. 5, total cake areas).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) content from leaf extracts was visualized in coomassie blue-stained SDS gel by loading equivalent amounts of total protein in each line (Fig 6 A). Rubisco content decreased at high NH_4^+ concentrations (approx. 33% for 5 mM NH_4^+ and 28% for 10 mM NH_4^+ , under both LI or HI, with respect to 0.5 mM NH_4^+). The highest Rubisco content was observed at 2.5 mM NH_4^+ and HI treatment (34% higher compared than that for 0.5 mM NH_4^+ under LI; Fig. 6 A and B1). Rubisco percentage values, either based on Chl or based on leaf area, tended to increase with NH_4^+ dose for both irradiances (Fig. 6 B2 and B3, respectively). Relative Rubisco content per μmol of Chl or per unit of leaf area showed higher values under HI, with the exception of 0.5 mM NH_4^+ , (Fig. 6 B2 and B3 respectively).

The native gel SOD activity test showed the different SOD isoenzymes present in pea leaves. The bands observed on the gels were identified on the basis of the band pattern obtained previously for pea (Gómez et al., 2004). At least four bands, which corresponded, by order of migration, to MnSOD, FeSOD and cytosolic and chloroplastic CuZnSOD isoenzymes, were detected. SODs presented greater activity under HI than under LI. However, irrespective of PPFD applied, the highest CuZnSOD isoenzyme (cytosolic and plastidial) activity was observed at 0.5 mM NH_4^+ , with even greater intensity in the chloroplastic isoform (CuZnSOD 2). FeSOD and CuZnSOD activity decreased slightly at high NH_4^+ concentrations (5 and 10 mM) with respect to low NH_4^+ doses (0.5 and 2.5 mM), whereas MnSOD activity (isoenzyme typically mitochondrial) remained unchanged for all treatments (Fig. 7).

Discussion

We have confirmed that NH_4^+ nutrition at high concentrations (5 and 10 mM) has a negative effect on biomass accumulation in pea plants (Fig. 1 A and B), as reported previously (Domínguez-Valdivia et al., 2008; Cruz et al., submitted). However, high PPFD (HI) was found to have a positive effect on plant growth and photosynthetic rate at intermediate NH_4^+ concentrations (Fig. 1 A and B and 2 A, respectively), where NH_4^+ stress is partly alleviated. This finding is in contrast to previous reports, which found that the negative effect of NH_4^+ nutrition on plant growth is accentuated under HI conditions in several species (Zhu et al., 2000; Guo et al., 2007). However, numerous studies have shown that photosynthetic rate and photosynthetic electronic transport responses to PPFD vary with species, N dose and light intensity applied throughout the culture period (Gastal & Lemaire, 2002; Kato et al., 2002).

The greater total biomass (shoot and root DW) found under HI compared to LI (except 0.5 mM of NH_4^+ ; Fig. 1 A and B) may well be due to the greater photosynthetic ability developed during the pea plants' adaptation to HI, as noted by Kato et al. (2002), which translates into greater photosynthetic rate (Fig. 2 A). Indeed, the higher SLW observed in plants grown under HI (except at 10 mM NH_4^+ ; Fig. 1 C) is associated with a thickening of the leaves under HI conditions. Thicker leaves (Fig. 1 D), higher net photosynthesis (Fig. 2 A) and higher Chl a/b ratios at 5 and 10 mM NH_4^+ under HI (Fig. 3 B), are common features of HI-acclimatised leaves and those exposed to sunlight. Interestingly, similar SLW levels were obtained at 10 mM NH_4^+ by plants under both PPFDs, thus indicating an effect of high NH_4^+ dose on the foliar thickness increase (Fig. 1 D).

In line with observations by Demmig-Adams et al. (1995), the relative lutein and neoxanthin contents were lower in HI plants than in LI plants (Fig. 4 A), whereas V cycle component (Fig. 4 and Fig. 5) and β -carotene levels (Fig. 4 A) increased under HI.

As documented in the literature (Verhoeven et al., 1997; Kato et al., 2002), the application of low N doses (0.5 mM) leads to a photoinhibitory effect that leads to a plant biomass reduction irrespective of the light intensity supplied (Fig. 1 A and B). Furthermore, N-deficiency has a remarkable effect on other parameters, such as lower photosynthesis rate (Fig. 2 A) and lower Chl a+b content (Fig. 3 A), which is probably related to a lower foliar N content. The 0.5 mM NH_4^+ dose also showed high values for parameters related to high photoprotective demand as a result of N-deficiency and HI exposure. The carotenoid content (Fig. 4 A), especially xanthophyll cycle components (Fig. 4 and 5), and CuZnSOD activity (Fig. 7) were far higher at 0.5 mM NH_4^+ than at other N doses. Moreover, plants grown at 0.5 mM NH_4^+ and HI also presented higher Rubisco content than those at 5 and 10 mM NH_4^+

(Fig. 6 A and B1). However, despite showing a higher photosynthetic rate (Fig. 2 A) than those cultivated under LI, these plants did not show higher growth based on dry weight (Fig. 1 A and B). This behaviour is likely related to the higher total carotenoid and V content (Fig. 4A and 5, respectively) and to the higher de-epoxidation index ($A + Z / V$) observed at 0.5 mM NH_4^+ and HI (Fig. 5B). All these results reflect a high degree of photoprotection and dissipation of non-photochemical energy (Müller et al., 2001) caused by N-deficiency, which could be explained by the risk of photo-oxidative damage being worsened by excessive light application.

Previous studies on *Pisum sativum* have found observed that 2.5 mM NH_4^+ is an inflection point in the response of a large number of parameters to external N (Domínguez-Valdivia et al., 2008; Cruz et al., submitted for publication). This may suggest that this dose represents a transition point in the metabolic response of the plant to the external NH_4^+ concentration, which may involve a compromise between the N requirement for photosynthesis and photoprotection and NH_4^+ toxicity. Some of the antioxidant system indicators, such as $\alpha\text{-Toc/Chl}$ (Fig. 4 B) (Iturbe-Ormaetxe et al., 1995) and the de-epoxidation index ($A + Z / V$) (Fig. 5 B), also presented significantly higher values here compared to other treatments.

The higher Rubisco content observed at 2.5 mM NH_4^+ for both irradiances (Fig. 6 A) is a remarkable effect which seems to be related to the inflection point in pea metabolism at this dose. Thus, an NH_4^+ dose of 2.5 mM and HI gave the highest expression of Rubisco (28% more than 2.5 mM NH_4^+ and LI; Fig. 6 A and B1), which matches the highest biomass peak (Fig. 1A and B). Warren et al. (2000) have examined several native Australian plants under different N nutrition conditions and found widely differing Rubisco contents. However, the amount of Rubisco increases with N dose applied, irrespective of its source (NO_3^- , NH_4NO_3 or NH_4^+) in most species (Makino et al., 1997; Bungard et al., 1997; Belastegui-Macadam, 2004; Fig. 6 B2 and B3). Guo et al. (2007) have found that Rubisco values follow different trends depending on how they are expressed ($\text{Rubisco}\cdot\text{protein}^{-1}$, $\text{Rubisco}\cdot\text{Chl}^{-1}$ or $\text{Rubisco}\cdot\text{cm}^{-2}$; Fig. 6 B). The higher Rubisco content based on total protein at low NH_4^+ doses (0.5 and 2.5 mM; Fig 6 A and 6 B1) may contribute to greater photorespiration rates, as reported by Muraoka et al. (2000), and an increase in photorespiratory ability has been found to be one of the photoprotective non-photochemical mechanisms in leaves exposed to excess light (Muraoka et al., 2000).

SODs catalyze the dismutation of superoxide radicals, whose production may be important in chloroplasts through Mehler's reaction (Asada, 1999). This flux of electrons into the oxygen may increase under conditions of stress, although it also occurs under physiological photosynthetic conditions (Foyer et al., 1994). FeSODs are chloroplastic

isoenzymes in pea (Moran et al., 2003) and have often been associated with protection against light radiation (Kliebstein et al., 1998; Kaminaka et al., 1999), which could explain their higher activity under HI conditions (Fig. 7). However, the major isoenzymes detected correspond to cytosolic and plastidial CuZnSOD isoforms (1 and 2 respectively), which show higher induction under HI and at low N doses (i.e. 0.5 and 2.5 mM NH_4^+ ; Fig. 7). This induction is related to the greater de-epoxidation index seen (Fig. 5 B) and the greater photoprotective ability, as shown by the higher V/carotenoid ratio (Fig. 4). These results therefore highlight the high level of photoprotection and greater anti-oxidant potential in pea leaves under such conditions (García-Plazaola et al., 2004).

Despite showing greater growth (Fig. 1 A and B), plants grown with an NH_4^+ dose of 2.5 mM under HI treatment could also be N-deficient, as is the case with 0.5 mM NH_4^+ . Both treatments showed higher levels of photoprotection and energy dissipation, which is usually proportional to the level of light stress to which leaves are exposed (García-Plazaola et al., 2008b). Furthermore, the Chl a/b ratio, which is a good indicator of a plant's acclimatization to light intensity (sun/shade), showed lower values at HI and low N doses (0.5 and 2.5 mM NH_4^+) than at 5 and 10 mM NH_4^+ (Fig. 3 B), which also points to an N-deficiency in those treatments. The ratio of Chl a to Chl b typically increases with irradiance once a plant becomes acclimated to a light environment (Demmig-Adams and Adams 1992; Demmig-Adams 1998; Thayer and Bjorkman 1990). The values reported herein correspond to those typically found for shaded leaves but are consistent with the trend of a higher Chl a/b ratio at higher irradiances. Despite light regulation of Chl a/b, an additional positive NH_4^+ effect was observed, thus suggesting an N-deficiency in the 0.5 and 2.5 NH_4^+ treatments.

Despite the activation of photoprotection systems in pea plants treated with high NH_4^+ doses (5 and 10 mM), as confirmed by the variation in carotenoid pigments and V cycle components under HI, not only were no photoinhibition symptoms observed, but there was a beneficial effect of the HI plants' adaptation in terms of NH_4^+ toxicity in comparison to LI. Furthermore, the activation of other antioxidant mechanisms, such as detoxification of ROS production (i.e. SOD), is not required at these N doses under HI.

Conclusions

Although higher carotenoid and V contents are induced by HI, this does not have a negative effect on the overall development of pea plants (cv. sugar-snap) grown in the presence of NH_4^+ . Indeed, a greater photosynthetic rate and improved plant growth are observed. However, N-deficiency triggers an increase in the elimination of ROS, which becomes increasingly evident under HI conditions. HI-exposed plants cultivated at high NH_4^+

doses show lower photoprotective demand than those cultivated at low NH_4^+ doses, possibly because they suffer a greater energy drain in photosynthesis.

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Legends to figures

Fig. 1. Effect of NH_4^+ doses on growth parameters: **(A)** shoot dry weight (g), **(B)** root dry weight (g), **(C)** total leaf area (cm^2) and **(D)** SLW ($\text{g DW} \cdot \text{m}^{-2}$) in pea plants cv. sugar-snap grown on LI (●) or HI (○). Data represent average values \pm S.E. (A and B: $n=14$; C and D: $n=3$). Letters show significant differences ($P \leq 0.05$) among NH_4^+ doses for HI (A, B, C and D) and LI (a, b, c and d). The asterisk (*) denotes significant differences ($P \leq 0.05$) for each NH_4^+ concentration between HI and LI.

Fig. 2. Effect of NH_4^+ doses on physiological parameters: **(A)** Photosynthesis ($\mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), **(B)** Maximum photochemical efficiency of PSII (F_v/F_m) in pea plants cv. sugar-snap grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM) and adapted to LI (●) or HI (○). Data represent average values \pm S.E. (A: $n=5-8$; B: $n=8$). Letters show significant differences ($P \leq 0.05$) among NH_4^+ doses for HI (A, B, C and D) and LI (a, b, c and d). The asterisk (*) denotes significant differences ($P \leq 0.05$) for each NH_4^+ concentration between HI and LI.

Fig. 3. Effect of NH_4^+ doses on physiological parameters: **(A)** total Chl content ($\mu\text{mol Chl (a+b)} \cdot \text{m}^{-2}$), **(B)** Chl a/b ratio, in pea plants cv. sugar-snap grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM) and adapted to LI (●) or HI (○). Data represent average values \pm S. E. ($n=4-5$). Letters show significant differences ($P \leq 0.05$) among NH_4^+ doses for HI (A, B, C and D) and LI (a, b, c and d). The asterisk (*) denotes significant differences ($P \leq 0.05$) for each NH_4^+ concentration between HI and LI.

Fig. 4. Carotenoids and α -tocopherol relative contents in pea leaves cv sugar-snap, grown with increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM) and adapted to low and high light intensities: **(A)** content of carotenoids. LI (left) and HI (right), respectively. Violaxanthin + antheraxanthin + zeaxanthin (V), neoxanthin (N), lutein (L) and β -carotene (β -Carot) are expressed as individual percentage (relative areas of the bars) to the total carotenoids content ($\text{mmol Carot mol}^{-1} \text{ Chl (a+b)}$); line with errors bars. Statistics of individual carotenoids not shown. ANOVA analysis for the different NH_4^+ doses ($P \leq 0.05$) and averages comparison of independent samples for the irradiance factor (with two levels; $P \leq 0.05$) were carried out. **(B)** Content of α -tocopherol (α -Toc; $\text{mmol } \alpha\text{-Toc mol}^{-1} \text{ Chl (a + b)}$). Data represent average values \pm S. E. ($n=5$). Letters show significant differences ($P \leq 0.05$) among NH_4^+ doses for HI (A, B, C and D) and LI (a, b, c and d). The asterisk (*) denotes significant differences ($P \leq 0.05$) for each NH_4^+ concentration between HI and LI.

Fig. 5. Relative contents of V cycle carotenoids cycle in pea leaves cv sugar-snap, grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM): **(A)** contents of V cycle carotenoids: violaxanthin (V) antheraxanthin (A), and zeaxanthin (Z) adapted to LI (left) and HI (right), respectively. Values are expressed in individual carotenoid percentage (relative bars areas) to the total V cycle carotenoids content ($\text{mmol Carot mol}^{-1} \text{ Chl (a + b)}$); line with errors bars). Data represent average values ($n=3-5$). ANOVA analysis for the different NH_4^+ doses ($P \leq 0.05$) and averages comparison of independent samples for the irradiance factor (with two levels; $P \leq 0.05$) were carried out. **(B)** Effect of NH_4^+ doses on the $(A + Z)/V+A+Z$ index. Data represent average values \pm S. E. ($n=3-5$). Letters show significant differences ($P \leq 0.05$) among NH_4^+ doses for HI (A, B, C and D) and LI (a, b, c and d). The asterisk (*) denotes significant differences ($P \leq 0.05$) for each NH_4^+ concentration between HI and LI.

Fig. 6. Rubisco contents in pea plants cv. sugar-snap grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM) and adapted to LI (●) or HI (○): **(A)** Rubisco contents in SDS-PAGE stained with Coomassie blue; 5 μg of protein were loaded into each line. **(B)** Relative Rubisco contents respect to reference treatment, 0.5 mM NH_4^+ and LI: (B1) relative percentage of Rubisco, protein (5 μg)-based (B2) relative percentage of Rubisco Chl (a+b) (μmol)-based, and (B3) relative percentage of Rubisco, leaf surface (cm^2)-based.

Fig. 7. Total superoxide dismutase (SOD) activity in gel in pea plants cv. sugar-snap grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM) and adapted to LI (●) or HI (○), as indicated in materials and methods. 30 μg of protein were loaded into each line. MnSOD, FeSOD, CuZnSOD-1(cytosolic) and CuZnSOD-2 (plastidial).

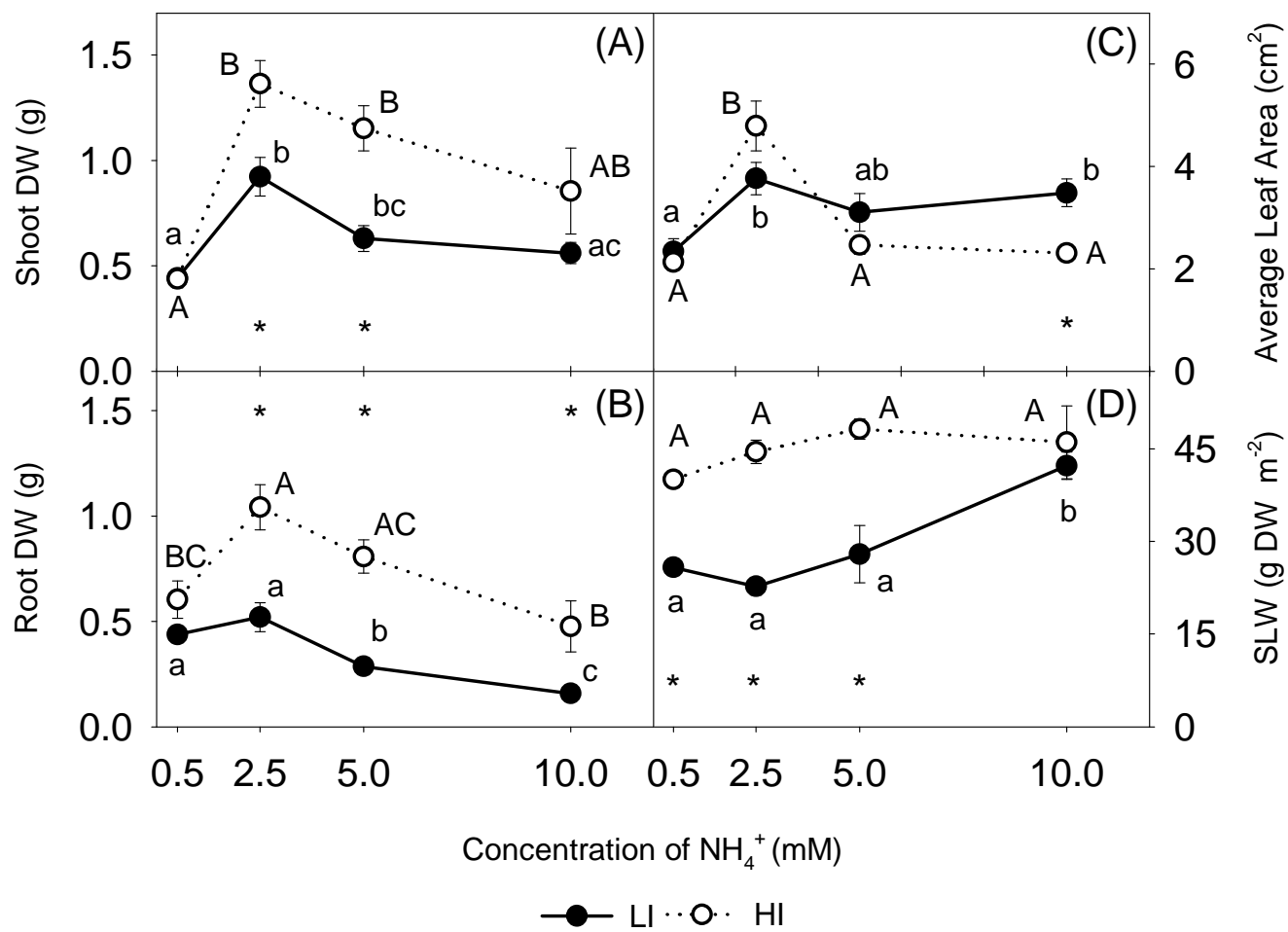


Fig.1

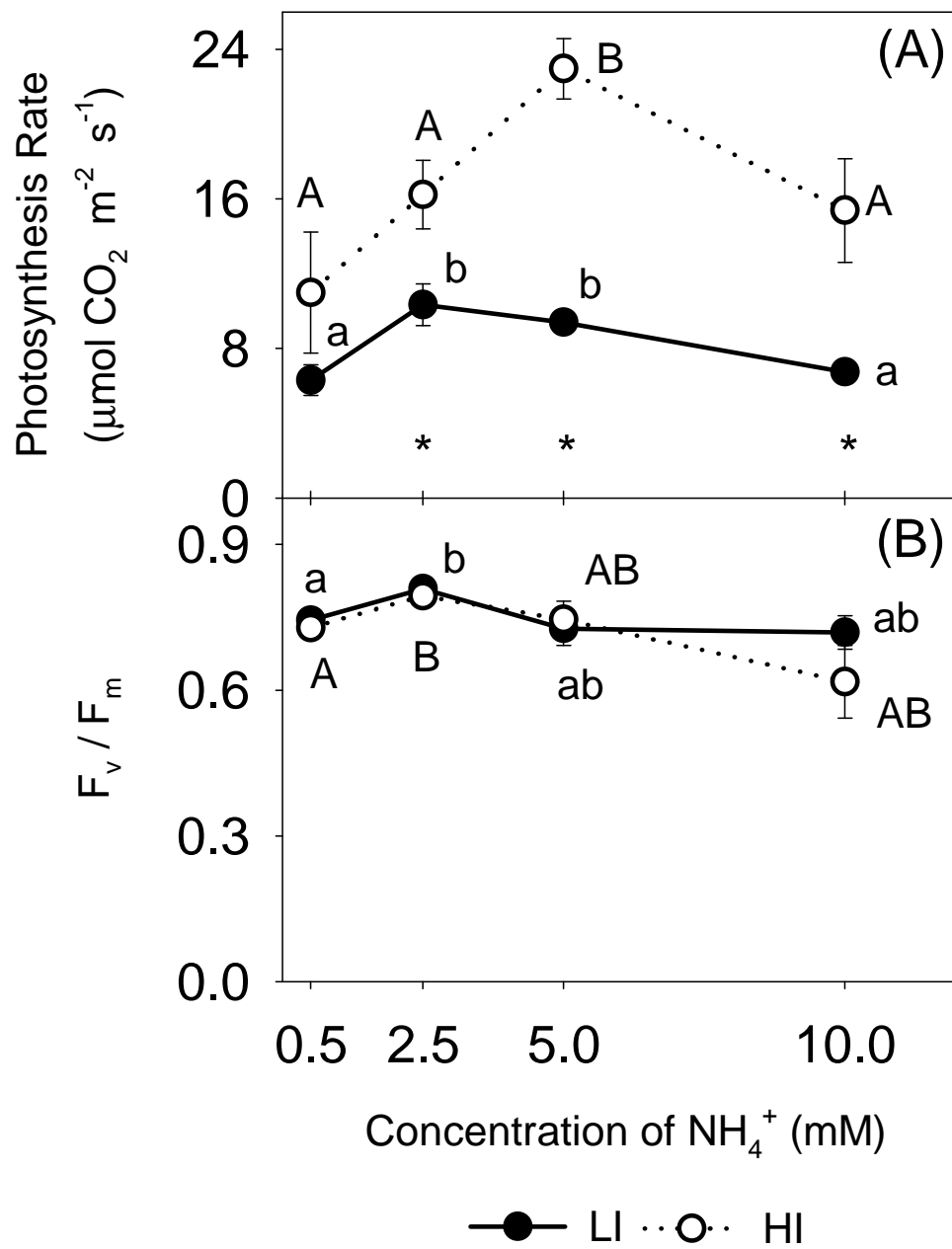


Fig.2

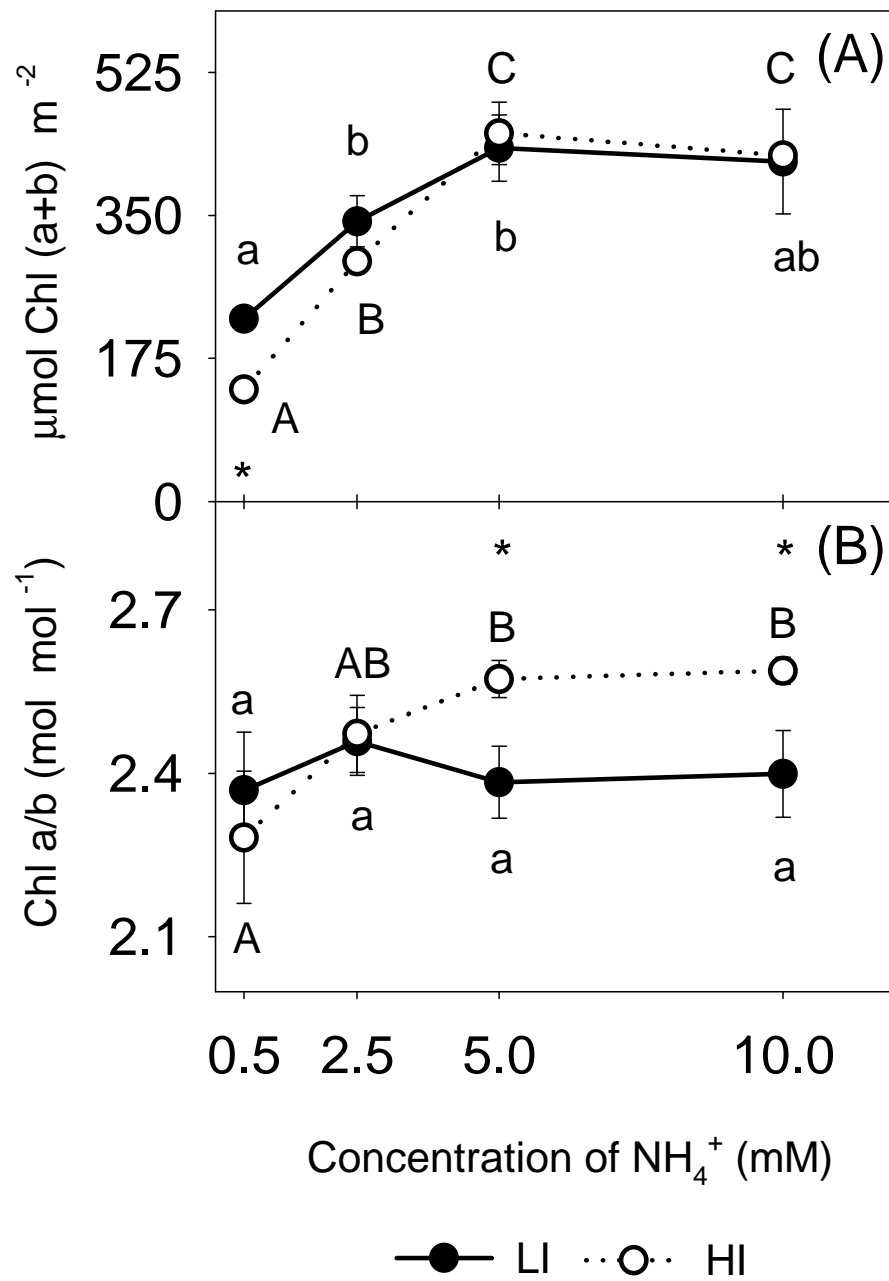


Fig.3

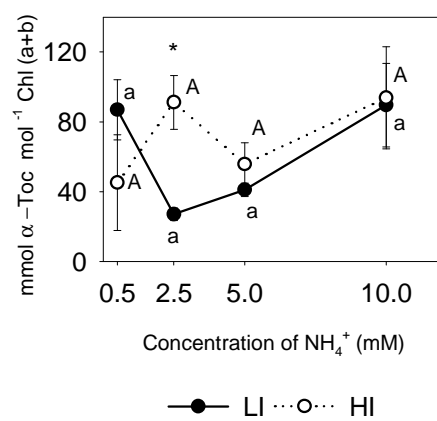
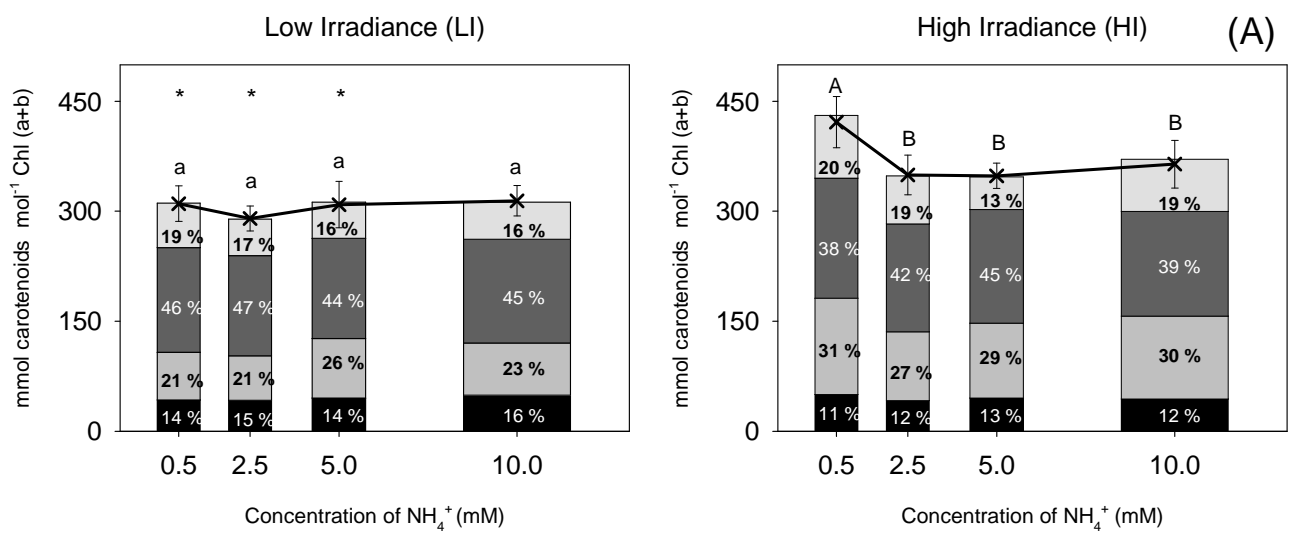


Fig.4

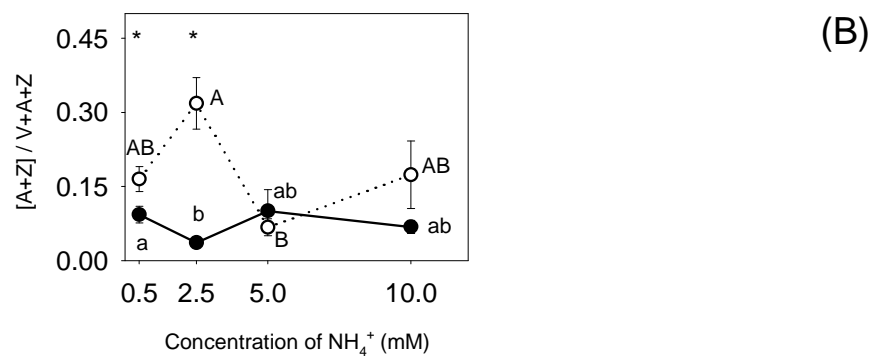
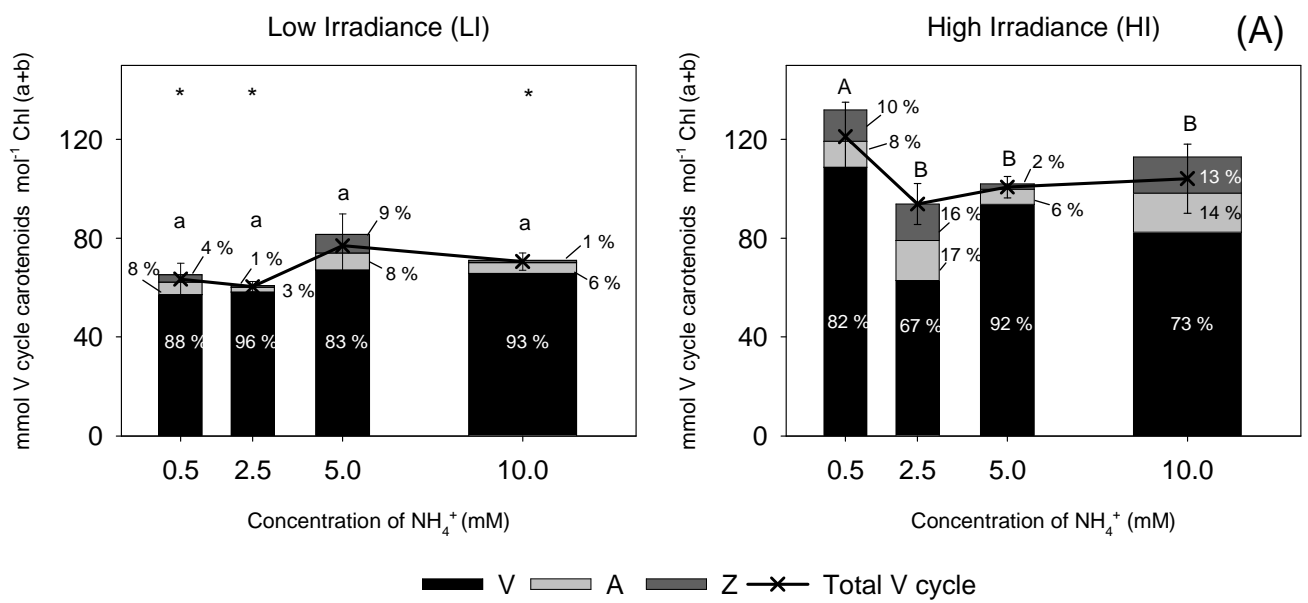


Fig.5

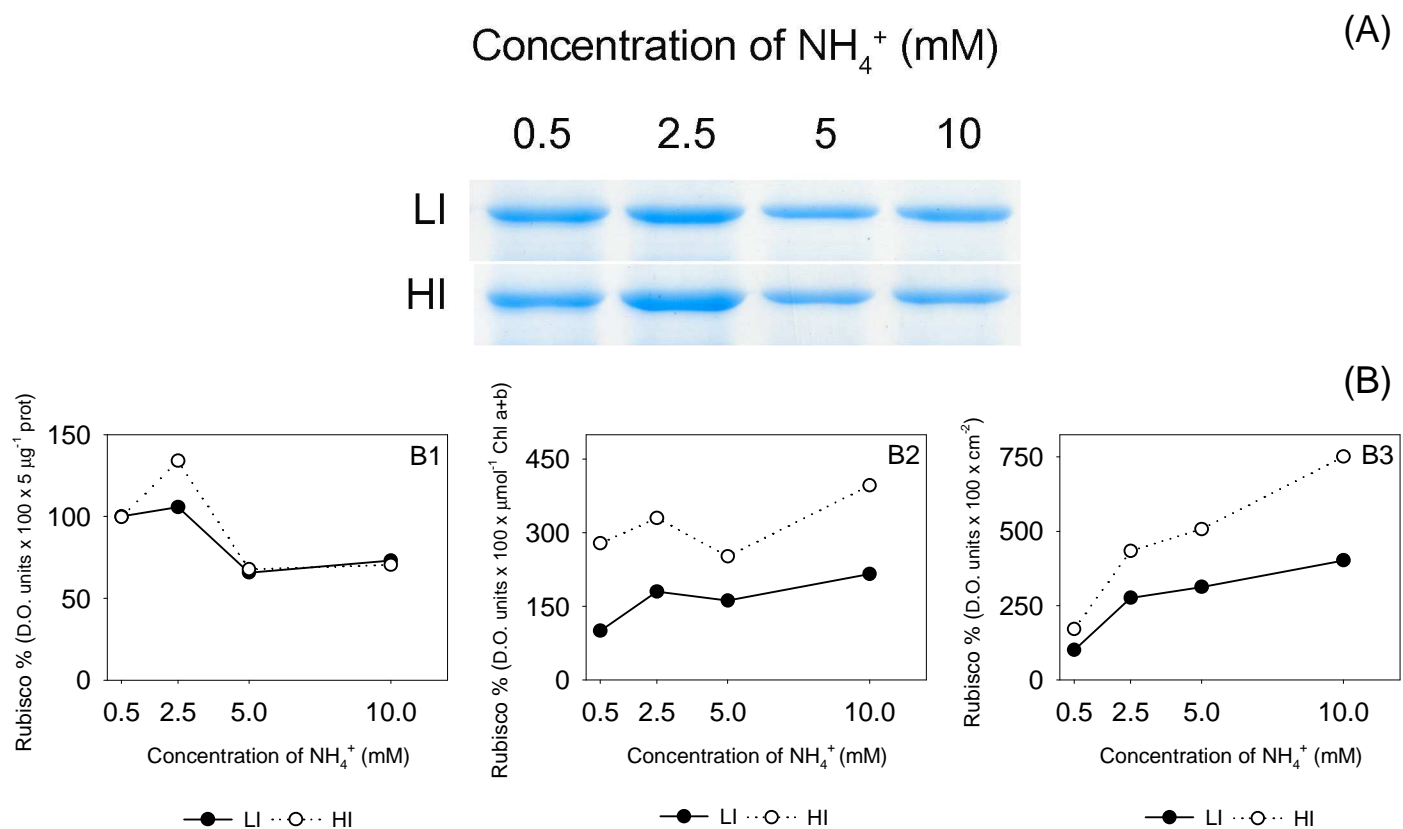


Fig.6

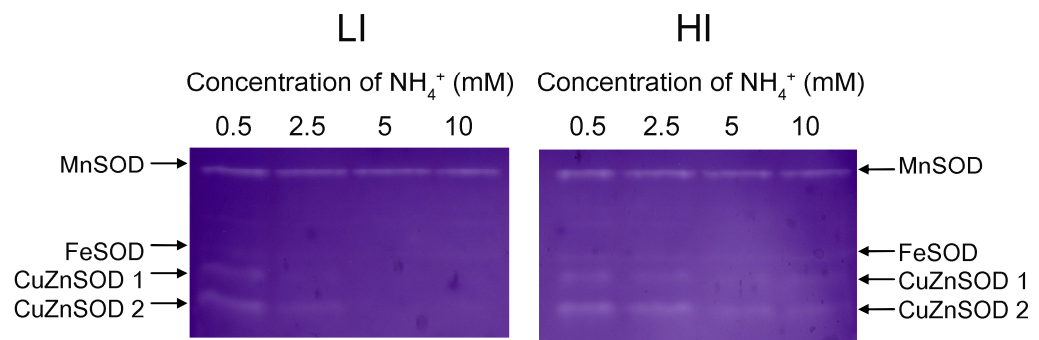


Fig.7