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High irradiance increases NH_4^+ tolerance in *Pisum sativum*: Higher carbon and energy availability improve ion balance but not N assimilation

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Abstract

The widespread use of NO_3^- fertilization has had a major ecological impact. NH_4^+ nutrition may help to reduce this impact, although high NH_4^+ concentrations are toxic for most plants. The underlying tolerance mechanisms are not yet fully understood, although they are thought to include the limitation of C, the disruption of ion homeostasis, and a wasteful NH_4^+ influx/efflux cycle which has an extra energetic cost for root cells.

In this study, high irradiance (HI) was found to induce a notable tolerance to NH_4^+ in the range 2.5 to 10 mM in pea plants by inducing higher C availability, as shown by carbohydrate content. This capacity was accompanied by a general lower relative N content, thus indicating that tolerance is not achieved through higher net N assimilation on C-skeletons, and it was neither attributable to increased GS content nor activity in roots or leaves. Moreover, HI plants showed higher ATP content and respiration rates. This *extra* energy availability is related to the internal NH_4^+ content regulation (probably NH_4^+ influx/efflux) and to an improvement of the cell ionic balance.

The limited C availability at lower irradiance (LI) and high NH_4^+ resulted in a series of metabolic imbalances, as reflected in a much higher organic acid content, thereby suggesting that the origin of the toxicity in plants cultured at high NH_4^+ and LI is related to their inability to avoid the large-scale accumulation of NH_4^+ ion.

Keywords: Ammonium nutrition, ammonium tolerance, ammonium toxicity, C-N metabolism, high irradiance, ion balance, *Pisum sativum* L.

Abbreviations: HI, high irradiance; LI, low irradiance; PPFD, Photosynthetic photon flux density;

Introduction

Intensive agriculture requires the use of N compounds to supplement the natural supply in the soil. This N is usually added in the form of urea, NO_3^-, or combined as NH_4NO_3. NH_4^+ as the sole source of N (NH_4^+ nutrition) may, however, represent a valid alternative because, unlike NO_3^-, it does not need to be reduced in order to be assimilated and can be obtained from organic manure or derived from urea. Although NH_4^+ is less mobile, thus reducing NO_3^- leaching and the ecological impact of N fertilization, it is toxic to many plants. The symptoms observed upon NH_4^+ application are variable but often include altered mineral inorganic ion content and organic acid levels (Gerendás et al., 1997; Miller and Cramer, 2004). A reduction in plant growth with increasing external NH_4^+ concentrations, as compared with NO_3^- nutrition is a classical effect of NH_4^+ nutrition (Bennett et al., 1964). Nowadays, plant growth is probably yet the best indicator of stress as it is a comprehensive measure of the physiology of the plant as a whole (Cruz et al., 2006; Domínguez-Valdivia et al., 2008).

Substantial variations in NH_4^+ tolerance can be seen amongst closely related species (Monselise and Kost, 1993) or even within species (Li et al., 2010; Cruz et al., 2010). However, there is no consensus regarding which traits confer NH_4^+ tolerance to plants, as this tolerance seems to arise from a physiologically complex process, or may even be reached by convergent mechanisms. The biochemical mechanism of NH_4^+ toxicity is not fully understood, although several hypotheses have been proposed. The higher C consumption in root caused by excess NH_4^+ (Krupa, 2003) and the high C demand for NH_4^+ detoxification under moderate NH_4^+ supply conditions (Gerendás et al., 1997) could partially explain the NH_4^+-induced growth inhibition. These authors (Gerendás et al., 1997) also pointed to a change in the osmoregulation observed under these conditions as a possible source of NH_4^+ toxicity. A futile plasma transmembrane cycle of NH_4^+ uptake and efflux through cell roots, the energetic cost of which may well explain the different tolerances exhibited by different plant species, has subsequently been suggested to be a critical factor in NH_4^+ toxicity (Britto

et al., 2001; Britto and Kronzucker, 2002). More recently, it has been reported that increasing K^+ supply also results in a significant reduction in NH_4^+ influx, as measured by ^{13}N radiotracing (Szczerba et al., 2008a; Balkos et al., 2010), thereby highlighting the pivotal role played by K^+ in alleviating NH_4^+ toxicity (Szczerba et al., 2008b; Balkos et al., 2010).

The importance of C-skeleton availability for NH_4^+ assimilation is well known (Schortemeyer et al., 1997; Roosta and Schjoerring, 2008). Therefore the availability of carbohydrate may influence NH_4^+ toxicity (Roosta and Schjoerring, 2008) by determining how fast the NH_4^+ absorbed can be assimilated (Raab and Terry, 1994), thus alleviating the NH_4^+ toxicity effects (Roosta and Schjoerring, 2008). On the other hand, it has been shown that the enhancement of glutamine synthetase (GS) activity in crop plants has the potential of increasing nitrogen utilization efficiency (Miflin and Habash, 2002). Moreover, plant species with higher GS activities in high NH_4^+ conditions achieve an elevated tolerance to NH_4^+ nutrition (Cruz et al., 2006; Fei et al., 2006; Li et al., 2010). Coordination between C and N assimilation is controlled by C/N status (Foyer et al., 2003), with C metabolites assimilated by photosynthesis being involved in the regulatory cycles which control N usage. In light of this, it has been proposed that a higher photosynthetic photon flux density (PPFD) could increase the amount of carbohydrate translocated to the root, thereby improving the tolerance to NH_4^+ nutrition (Magalhaes and Wilcox, 1983; Gerendás et al., 1997; Zhu et al., 2000) and reducing the cellular NH_4^+ content. In previous studies (Domínguez-Valdivia et al., 2008; Cruz et al. 2010), we have established that pea (*Pisum sativum* L.) can grow on NH_4^+ ions as the sole N source. In order to examine the effect of C availability, we have studied the effect of two PPFDs, as a means of varying the supply of C to the plant, thereby allowing us to clarify important aspects of the mechanism of tolerance of pea plants to NH_4^+ nutrition.

Material and Methods

Plant material and growth conditions. Seeds of pea (*Pisum sativum* L., cv. sugar snap) were surfaced sterilized, germinated at 26 ºC in for 96 h (dark) in perlite:vermiculite (1:2) and

grown hydroponically as in (Ariz et al., 2010). N-free modified Rigaud and Puppo's solution (1.15 mM K_2HPO_4; 2.68 mM KCl; 0.7 mM $CaSO_4$; 0.07 mM Na_2Fe-EDTA; 0.85 mM $MgSO_4$; 16.5 µM Na_2MoO_4; 3.7 µM $FeCl_3$; 3.4 µM $ZnSO_4$; 16 µM H_3BO_3; 0.5 µM $MnSO_4$; 0.1 µM CuSO4; 0.2 µM $AlCl_3$; 0.1 µM $NiCl_2$; 0.06 µM KI) was exchanged weekly. The solution was buffered with $CaCO_3$ (5mM) to pH 7–7.5 and NH_4^+ was supplied as $(NH_4)_2SO_4$ during the treatment period, as the only N source, at different concentrations (0.5; 2.5; 5 and 10 mM). Given the high nutrient solution volume (8 L) relative to the small pea plants size, the concentration of solution was not significantly modified along each week (not shown). Two PPFDs were applied for three weeks of treatment: high irradiance (HI), 750 µmol photons $m^{-2} \cdot s^{-1}$; or low irradiance (LI), 350 µmol photons $m^{-2} \cdot s^{-1}$ (Ariz et al., 2010). Samples were collected 4-6 h after the beginning of the light period and frozen in liquid N and stored at -80 ºC for further analysis.

Carbohydrate extraction and determination. Soluble carbohydrates (fructose, glucose, and sucrose) were extracted from roots and leaves (0.2 g of fresh weight) in boiling ethanol (80 % v/v) as described by (Zabalza et al., 2004). The ethanol-insoluble residue was dried and extracted for starch, and the glucose produced by amyloglucosidase enzyme was analyzed as for soluble carbohydrates (Zabalza et al., 2004). Soluble sugars were expressed as µmol per gram of dry weight (DW), and starch was expressed as µmol of glucose per gram of DW. Fucose 0.5 mM was used as internal standard in the extracts.

Respiratory capacity in roots. Root respiration measures were taken on 0.05 g– fresh weight and 0.5-1 cm-long root cuttings using a Clarke type oxygen electrode at 25 ºC in a total volume of 1 mL of nutrient solution (Frechilla et al., 2002).

ATP content in roots. Root ATP contents were measured from 0.2 g of tissues. Tissues were homogenized to a fine powder in liquid N with a mortar and pestle. A 1.5 mL aliquot of 5 % (w/v) Trichloroacetic (TCA) acid in water was added. The homogenate was analyzed using the ATP Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the

manufacturer's instructions. Biolumiscence was measured using a Synergy HT Multi-Mode Microplate Reader (Biotek Instruments). ATP concentration interval for the standard curve determination was from 0.1 to 200 µM of ATP.

Activity and protein gel blot analysis of glutamine synthetase (GS; EC 6.3.1.2) enzyme. GS activity was determined as described by a glutamyl hydroxamate synthesis-based biosynthetic assay (O'Neal and Joy, 1973). Plant samples were ground with liquid N_2 and then homogenized with 1.5:1 (v/w) extraction buffer (50 mM Tris-HCl pH 8; 1 mM EDTA; 10 mM 2-mercaptoethanol; 5 mM dithiothreitol; 10 mM $MgSO_4$; 1 mM cystein; 0.6 % polyvinylpolypyrrolidone). Phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO, USA) were added to a final concentration of 2.5 µL mL^{-1} each. Extracts were centrifuged at 20,000 x *g* and 4 ºC for 30 min. SDS-PAGE was performed with the following antibody: anti-GS IgG, which was raised in rabbit against a specific peptide from pea GS (Acc. # CAJ87510.1; (Cruz et al., 2010)) and used at a 1:2,000 dilution overnight at 4 ºC. A peroxidase conjugated goat anti-rabbit IgG, followed by luminescence detection with the ECL™ Plus kit (Amersham Biosciences, Buckinghamshire, UK) was used in foliar tissues and an alkaline phosphatase labeled goat-anti-rabbit IgG was visualized with NBT-BCIP (Sigma-Aldrich) in roots samples.

N percentage and C/N ratio. N content (%) was calculated from dry material. Leaves and roots, were ground in a mixer mill (MM200, Retsch, Haan, Germany). 2-3 mg of DW were placed into tin capsules and analyzed by Dumas combustion in an elemental analyzer CNS 2500 (CE Instruments, Milan, Italy). The N_2 and CO_2 produced were detected by thermal conductivity. Acetanilide was used as standard in total N content parameter. Organic N content was calculated from total N content minus internal inorganic N content (NO_3^-, NO_2^- and NH_4^+; see inorganic ion content determination below) of vegetal tissues and was expressed in percentage (g of organic N 100 g^{-1} DW by substraying C accumulation from starch).

The C/N ratio has been calculated from N percentage data (g N 100 g^{-1} DW) and C percentage data (g C 100 g^{-1} DW).

Organic acids determination. Frozen (-80 ºC) pea leaves or roots (0.2 g) samples were extracted as described in paragraph "ATP content in roots". The extracts were kept frozen at -20 ºC until use. Succinate, malate, 2-oxoglutarate and citrate contents were determined by ion chromatography in a DX-500 system (Dionex Corporation, CA, USA) by gradient separation with a Dionex IonPac AS11 (4x250 mm) column and suppressor column Dionex ASRS Ultra II (4 mm) with the ion trap Dionex Ion-Pac ATC-3 (9x24 mm), and a pre-column Dionex Ion-Pac AG11 (4x50 mm). Samples were injected with an AS40 autosampler (Dionex) at a 1:20 dilution in milli-Q distilled water. A 2 mL min^{-1} flow of solvent (methanol 18 % / NaOH 0.2 mM) was applied, and organic acids separation was carried out using a gradient of NaOH (from 0.2 mM to 35 mM) for 16 min. Detection was carried out by a conductivity method in the electrochemical detector ED 40 (Dionex).

Determination of inorganic soluble ion content: extraction and analysis. Cellular soluble ionic content was obtained by centrifugation (20,000 x *g*, 30 min) of tissues (0.2 g) incubated in 1 mL of milli-Q water at 80 ºC in a bath for 5 min. The supernatants (leaves and roots) were stored at -20 ºC until analysis by ion chromatography. Soluble cation content (Na^+, K^+, Mg^{2+}, Ca^{2+} and NH_4^+) was determined using a isocratic method with 20 mM metanosulphonic acid solution as eluent in a Dionex-DX500 ion chromatograph (Dionex) with Ion Pac CG12A and Ion Pac CS12A columns. Detection was carried out by conductivity as above. The extracts were diluted (1:10) for analysis. Anion soluble content (Cl^-, NO_3^-, PO_4^{3-} and SO_4^{2-}) determination (1:10 diluted extracts) was carried out by the gradient method as for organic acid determinantion (see above).

Statistical treatment. All statistic analyses were performed with Statistical Product and Service Solutions (SPSS) for Windows, version 15.0, using unifactorial analyses of variance (ANOVAS; factor: concentration of NH_4^+). The Levene test was used, and LSD statistics

153 applied for variables with homogeneity of variance and the Dunnett T3 test for cases of non-
154 homoscedasticity. For testing the irradiance effect on each N treatment, Student's *t*-tests were
155 performed independently for each N concentration, and homoscedasticity condition determined
156 by the Levene test. All statistical analyses were conducted at a significance level of 5 % ($P \leq$
157 0.05). Plants in this study were grown at three different times. At least one sample from each
158 series was used for all parameters measured. In post-hoc tests displayed in figures and table the
159 letters represent significant differences between NH_4^+ concentrations for HI (A, B, C and D)
160 and LI (a, b, c and d). An asterisk (*) in figures denotes a significant difference between HI and
161 LI for each NH_4^+ concentration.

162

163 **Results**

164 *Higher irradiance and carbon content*

165 Higher PPFD (HI) was found to have a remarkable positive effect on the biomass accumulation
166 (Ariz et al., 2010), showing a lower shoot/root ratio under HI relative to LI, except at 0.5 mM
167 NH_4^+ (Table 1).

168 In general, the carbohydrate content (glucose, sucrose, and starch) was higher under HI than
169 under LI (Fig. 1). The most notable change in C level as a function of NH_4^+ concentration was
170 found for leaf starch content, with starch levels decreasing significantly at 2.5 mM NH_4^+ for LI
171 (Fig. 1 A1) and 5 mM for HI (Fig. 1 B1). Glucose levels showed no significant trend, with the
172 exception of an increase at 10 mM NH_4^+ under HI (Fig. 1 B1). Shoot sucrose levels at LI
173 showed moderately significant decreases with increasing NH_4^+ (Fig. 1 A1), whereas the
174 increase at 2.5 mM NH_4^+ under HI was followed by a decrease at higher NH_4^+ concentrations
175 (Fig. 1 B1). Root sucrose levels decreased with increasing external NH_4^+ at LI and showed no
176 significant changes at HI (Fig. 1 A2 and B2). Sucrose was the main root carbohydrate (Fig. 1
177 A2 and B2), with glucose and starch being below the detection level in roots, except for 10 mM

178 NH_4^+ at HI (Fig. 1 B2). Fructose was also below the detection limit in both leaves and roots.
179 Other C molecules such as sugar alcohols (polyols) may be important molecules in the
180 transport of C, however as they do not ionize themselves, and they do not modify charge
181 balance, nor modify the pH value, they have not been considered on the background of ion
182 balance.

183 *Energetic capacity, N assimilation and ion content*

184 Root respiration increased with NH_4^+ availability at both PPFDs, and respiratory activity was
185 higher under HI than under LI at high NH_4^+ (5 and 10 mM; Fig. 2 A). Furthermore, root ATP
186 content, as detected by chemiluminescence, was higher under HI than under LI at high NH_4^+
187 and it increased with NH_4^+ concentration at HI. In contrast, root ATP content under LI was
188 invariable up to 5 mM and then decreased at 10 mM NH_4^+ (Fig. 2 B).

189 GS activity was higher in leaves than in roots (Fig 3 A). Foliar GS activity was higher under LI
190 relative to HI at low NH_4^+ (0.5 and 2.5 mM; Fig. 3 A1). In contrast, root GS activity was higher
191 under HI and at high NH_4^+ concentrations (5 and 10 mM; Fig. 3 A2). Root GS activity
192 increased with NH_4^+ up to 2.5 mM NH_4^+ under LI, whereas it increased near-linearly up to 5
193 mM NH_4^+ under HI, remaining constant at higher N concentrations for both PPFDs (Fig. 3 A).

194 Two GS isoforms were detected in pea leaves (Fig. 3 B1). The chloroplastic isoform content
195 (GS2; 44-kD (Tingey et al., 1987) was higher under LI than under HI for all NH_4^+ treatments,
196 whereas cytosolic isoform content (GS1; 38-kD) was higher under LI only at high NH_4^+ (Fig. 3
197 B1). Levels of both GS isoforms increased with external NH_4^+ concentration irrespective of
198 PPFD, except for HI and 0.5 mM NH_4^+, which showed a "peak" in GS1 content (Fig. 3 B1).
199 The root GS content increased slightly under HI with respect to LI at high NH_4^+ (Fig. 3 B2).

200 Organic N content (%) increased with NH_4^+ concentration. Interestingly, organic N content (%)
201 was higher under LI than under HI, except at 0.5 mM NH_4^+, in both organs (Fig. 4 A1 and A2),
202 thus indicating a higher net N assimilation under LI. In contrast, the C/N ratio decreased with

203 increasing NH_4^+ concentration (Fig. 4 B1 and B2) and it was significantly lower under LI than
204 under HI (Fig. 4 B).

205 NH_4^+ contents in leaves and roots increased with external NH_4^+ availability and, on average,
206 was 30 times lower in leaf than in root (Fig. 5 A1 and A2). Unlike LI, where NH_4^+ contents in
207 roots increased near-linearly with increasing NH_4^+ concentration, much lower NH_4^+ contents
208 were found under HI (Fig. 5 A2). In contrast, leaf NH_4^+ content did not differ significantly
209 between HI and LI (Fig. 5 A1). The K^+ content in root tissue was higher than in leaves and
210 showed an opposite trend to the NH_4^+ content (Fig. 5 A2 and B2), thus indicating that the NH_4^+
211 and K^+ contents in roots are negatively correlated (Fig. 5 C). Under HI, the K^+ content was
212 significantly higher than under LI for most of NH_4^+ treatments studied (2.5, 5 and 10 mM) (Fig.
213 5 B2).

214 Both total soluble anions and cations in root decreased with increasing NH_4^+ concentration
215 (Figs. 6 A2 and A4, respectively), although pea plants adapted to HI showed higher anion
216 content in roots (Fig. 6 A4). The anion content in leaves decreased with increasing external
217 NH_4^+ under LI; no significant differences were observed under HI (Fig. 6 A3). Total cation
218 content in leaves remained essentially unchanged (Fig. 6 A1). An important imbalance in anion
219 content in pea roots was found upon comparing the two PPFDs (Fig. 6 A4 and B 2). The
220 organic acid content under LI increased significantly with NH_4^+, whereas it decreased slightly
221 with increasing NH_4^+ availability under HI (Fig. 6 B). Total organic acid content is presented as
222 the sum of citrate, malate, 2-oxoglutarate and succinate. The pyruvate, isocitrate, cis-aconitate
223 and trans-aconitate were also detected but in very small quantities and were not taken into
224 consideration. Also, due to the extraction of the samples with TCA 5 % (w/v) in water, part of
225 this acid masked the separation of fumarate and oxaloacetate preventing the observation of
226 their peaks.

227

Discussion

Irradiance and NH_4^+ nutrition tolerance

In this study we have compared two different PPFDs (350 and 750 µmol photons m^{-2} s^{-1}) as a means of increasing C availability and characterizing some aspects of the NH_4^+ tolerance mechanisms. No photo-inhibitory symptoms were detected at HI (Ariz et al., 2010). The effect of NH_4^+ nutrition on plant growth is widely used as an important marker of NH_4^+ toxicity (Bennett et al., 1964; Cruz et al., 2006; Domínguez-Valdivia et al., 2008) as it covers all the metabolic processes involved. In contrast to other studies with high irradiance and NH_4^+ nutrition (Magalhaes and Wilcox, 1983; Gerendás et al., 1997; Zhu et al., 2000), (Ariz et al., 2010) clearly showed that HI significantly improves the biomass accumulation in pea plants, thereby leading to an improved tolerance to NH_4^+ nutrition. The best positive effect of HI on NH_4^+ tolerance was observed at 5 mM of N, where NH_4^+ toxicity in LI respect to NO_3^- nutrition occurs (data not shown). In fact, at 5 mM NH_4^+ and HI showed the highest increase of dry biomass (+113.8 %) with respect to LI treatment (Ariz et al., 2010). One of the typical symptoms of "ammoniacal syndrome" is the increased shoot:root ratio (Miller and Cramer, 2004). In this study under HI, the shoot/root ratios were significantly lower relative to LI conditions, suggesting also the lower toxic effect under HI (Table 1). Moreover, we have shown in a previous report that the photosynthetic rates increased under HI on varying the NH_4^+ concentration from 2.5 to 10 mM, reaching a maximum at 5 mM NH_4^+ (Ariz et al., 2010). This induced in a higher accumulation of carbohydrates, mainly starch in shoots and sucrose in roots (Fig. 1) at low NH_4^+ under HI. In contrast, the reduced accumulation of starch at HI and 5 mM NH_4^+ (Fig. 1 B1), and the peak in respiration rate at the same concentration (Fig. 2 A), indicate that under HI, at high NH_4^+ concentrations, photosynthate availability and its utilization became reduced. This was probably a result of either NH_4^+ toxicity or insufficient photosynthate to meet the demand for NH_4^+ assimilation at 10 mM NH_4^+ and HI. The accumulation of starch in leaves at 0.5 (HI and LI) and 2.5 mM NH_4^+ (HI) (Fig. 1 A1 and

B1), along with an increase in the proportion of photosynthate translocated to the root observed at 0.5 mM NH_4^+ (Table 1), have been described as indicators of N-deficiency conditions (Rufty et al., 1988; Miller and Cramer, 2004). This idea was supported by the lower soluble protein content in leaves (data not shown) and the higher C/N ratio at 0.5 mM NH_4^+ under HI and LI, and at 2.5 mM NH_4^+ under HI, in comparison to the other treatments (Fig. 4 B1). The C/N ratio was also significantly higher under HI than in LI in the tolerance range 2.5 to 5 mM NH_4^+ (Fig. 4 B). This resulted in an increase in photoassimilates translocated to the roots, as indicated by the lower shoot/root ratio under HI in comparison to LI (Table 1; (Miller and Cramer, 2004)

N assimilation, energy and ion-content regulation

The increased root respiration rate and the greater root ATP content (Fig. 2 A and B respectively) are associated with higher C availability (Fig. 1 C2; Fig. S1) and with the key fact that pea plants under HI were able to control internal NH_4^+ root levels more efficiently than those grown at LI (Fig. 5 A2). This supports the proposal of a need for additional energy to control internal NH_4^+ content in NH_4^+-sensitive plants (Britto et al., 2001). The plants grown under HI have an increased ability to reduce internal NH_4^+ content, thus avoiding the negative effects of high NH_4^+ content. Thus, root respiratory activity (Fig. 2 A) may provide the energy required for both NH_4^+ influx/efflux (Britto et al., 2001) and NH_4^+ assimilation (Bloom et al., 1992; Plaxton and Podestá, 2006).

Interestingly, despite plants grown under HI and at high NH_4^+ (5 and 10 mM NH4+) showed higher root GS activities and contents (Fig. 3 A2 and B2) than those under LI conditions, they contained less organic N per biomass unit in both organs (Fig. 4 A). Thus, we evidence that the increased C availability achieved under HI does not result in higher N net assimilation. However, we cannot rule out that the increase in N assimilation observed in LI plants is related to an NH_4^+ tolerance mechanism, as suggested previously (Miflin and Habash, 2002; Cruz et al., 2006; Fei et al., 2006; Li et al., 2010). A complementary and detailed study on

amino acids has been undertaken. The results observed for amino acids contents point out in the same direction that the organic acids results (Fig. 6B), as the amino-acid content increased with NH_4^+ concentration under both PPFDs, with a dramatic effect observed in leaves under LI (Data not shown). Hence, the improved NH_4^+ tolerance in pea plants grown under HI is not due exclusively to increased C-skeleton availability (Fig. 1) for N assimilation, and the higher tolerance in HI plants may arise from the use of C to increase the energy availability (Fig. 2) required for NH_4^+ uptake regulation (NH_4^+ influx/efflux) by the plant (Britto et al., 2001). Furthermore, the change in C/N leaf ratio with NH_4^+ availability (Fig. 4 B1) is related to the starch profile (Fig. 1 A1 and B1). Thus, at LI the starch and C/N ratio decline at 2.5 mM NH_4^+, whereas this does not occur at HI until 5 mM. It would therefore appear that C is a major limiting factor when NH_4^+ is applied as the sole N source.

As regards the soluble ion content in tissues, the higher root K^+ levels detected in HI (Fig. 5 B2) are associated with low root NH_4^+ contents under HI (up to seven times lower than LI; Fig. 5 A2). Our experiments (working K^+ concentration: 5 mM) indicated a strong correlation between the decrease in internal root K^+ content and the toxicity of high internal NH_4^+ content under LI (Fig. 5 C). Hence, the greater tolerance towards NH_4^+ nutrition observed under HI conditions (Fig. 5) is clearly related to a better K^+ cytosolic homeostasis in plants, as proposed by (Szczerba et al., 2008a; Szczerba et al., 2008b), who observed that NH_4^+ influx is rapidly suppressed when a low-K^+ condition is suddenly altered to a high-K^+ condition, thus substantially reducing the amount of futile cycling of NH_4^+ ion. Our results indicate that pea plants grown under improved energetic conditions are able to balance their internal K^+ contents independently of external NH_4^+ concentration, thereby contributing to overall increased growth (Ariz et al., 2010). According to our findings, the positive effect of HI application exerted through higher C and energy availability allows lower NH_4^+ levels and higher K^+ content to be maintained within the plant under NH_4^+ nutrition (Fig. 5 A and B).

The decrease of root positive charges (Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}) observed under HI at high NH_4^+ availability (Fig. 6 A2) is frequently associated with an increase in inorganic anion levels (Cl^-, SO_4^{2-} and PO_4^{3-}) (Miller and Cramer, 2004). However, the opposite effect was noted in our experiments, with negative charges also decreasing with increasing NH_4^+ concentration in both tissues and at both irradiances (Fig. 6 A3 and A4). In contrast, the light intensity had a marked effect on the main organic acids in the tricarboxylic acid cycle (Fig. 6 B). Hence, the organic acid content was much higher under LI than under HI at high NH_4^+, probably due to a mechanism that compensates for the drastic decrease of inorganic anions detected under LI at high NH_4^+; (Fig. 6 A3 and A4). In fact, negative charges exceeded positive charges at lower external NH_4^+ under HI conditions (Fig. S1 B). However, this net negative charge is very low under LI conditions (Fig. S1 A2). Taking into consideration plasma membrane electrical potentials, this effect could be related to the regulation of cellular membrane potential (always negative), which would mean that under LI, a strong energetic limitation could lead to a worse regulation of the membrane potential and severe modifications in the electrical charge balance (Fig. 6 and S1). Additional experimentation, such as membrane potential measures, is needed to prove this point.

Concluding remarks

Our results on growing pea plants using a combination of NH_4^+ and PPFDs have led us to emphasize several biochemical mechanisms that allow the plants to cope with the NH_4^+ stress. Under LI conditions, the C-deficiency observed at high external NH_4^+ is associated with a disruption of ionic homeostasis. This means that the plants are unable to adequately regulate internal NH_4^+ levels, organic acid content, or the cell charge associated with NH_4^+ uptake under these conditions. Plants adapted to HI have been shown to have higher C availability, which moderates the negative effects induced by high concentrations of external NH_4^+. This *extra* C is not utilized to increase N net assimilation or to increase the synthesis of organic acids to compensate for the ionic imbalance; in contrast, it appears that pea plants use this additional C

as an energy support to maintain a low NH_4^+ content inside their tissues, especially in roots. This results in a better C/N ratio and better control of the electrolytic homeostasis, and finally in improved growth and development. Thus, under LI and high NH_4^+, we can more strictly refer to "*energy deficiency*" rather than "*C-deficiency*".

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Table 1. Effect of external NH_4^+ on shoot/root ratio in pea plants grown under LI or HI.

$[NH_4^+]$ (mM)	Shoot : Root ratio at LI	Shoot : Root ratio at HI
0.5	1.02 ± 0.04 ^{a}	0.85 ± 0.10 ^{A}
2.5	2.17 ± 0.32 ^{b*}	1.40 ± 0.10 ^{B}
5	2.29 ± 0.11 ^{b*}	1.45 ± 0.06 ^{B}
10	3.75 ± 0.16 ^{c*}	2.33 ± 0.31 ^{B}

Data represent average values ± SE (n=12-14).

Figure legends

Fig. 1 Relative and total carbohydrate contents in shoots (1) and roots (2) of pea plants adapted to LI **(A)** and HI **(B)**, grown on increasing doses of NH_4^+ (0.5; 2.5; 5 and 10 mM). The relative bars areas represent the individual carbohydrate percentage relative to the summation of carbohydrate contents (mmol g^{-1} DW; Black line). Data represent average values ± SE (n=3-5).

Fig. 2 Effect of external NH_4^+ availability on **(A)** root respiratory rate (µmol O_2 g^{-1} DW·min^{-1}) and **(B)** root ATP content (µmol g^{-1} DW) in pea plants grown under LI (●) or HI (○). Data represent average values ± SE (A: n=3; B: n=16).

Fig. 3 Effect of external NH_4^+ availability on **(A)** GS activity (µmol GHM g^{-1} DW·min^{-1}) in leaf (A1) and root (A2) and **(B)** GS expression in leaf (B1) and root (B2) for pea plants grown under LI (●) or HI (○). Data represent average values ± SE (A: n=6-8).

Fig. 4 Effect of external NH_4^+ availability on: **(A)** organic N percentage (%; g of organic N 100 g^{-1} DW) of leaf (A1) and root (A2); **(B)** C/N ratio of leaf (B1) and root (B2) in pea plants grown under LI (●) or HI (○). Data represent average values ± SE (n=3).

Fig. 5 Effect of external NH_4^+ availability on the following internal inorganic ion contents: **(A)** NH_4^+ content (µmol NH_4^+ g^{-1} DW) of leaf (A1) and root (A2); **(B)** K^+ content (µmol K^+ g^{-1} DW) of leaf (B1) and root (B2); **(C)** correlation of root internal NH_4^+ content and root inorganic soluble K^+ content in pea plants grown under LI (●) or HI (○). Data represent average values ± SE (n=6-8).

Fig. 6 Effect of external NH_4^+ availability on: **(A)** internal inorganic ion content: cations (Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) in leaf (A1) and root (A2); anions (PO_4^{3-}, SO_4^{2-}, NO_3^-, Cl^-) in leaf (A3) and root (A4); **(B)** internal organic acid content (malate, 2-oxoglutarate, citrate and succinate) in leaf (B1) and root (B2), in pea plants grown under LI (●) or HI (○). Ion contents are expressed in meq g^{-1} DW. Data represent average values ± SE (A: n=6-8 and B: n=7-8).

Fig. S1. Effect of external NH_4^+ availability on internal ion contents (meq g^{-1} DW) of leaves (1) and roots (2) of in pea plants grown on LI (**A**; filled symbols) or HI (**B**; unfilled symbols): cations (○; Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}) and anions (△; PO_4^{3-}, SO_4^{2-}, NO_3^-, Cl^-, malate, 2-oxoglutarate, citrate and succinate. Data represent average values ± SE (n=6-8).

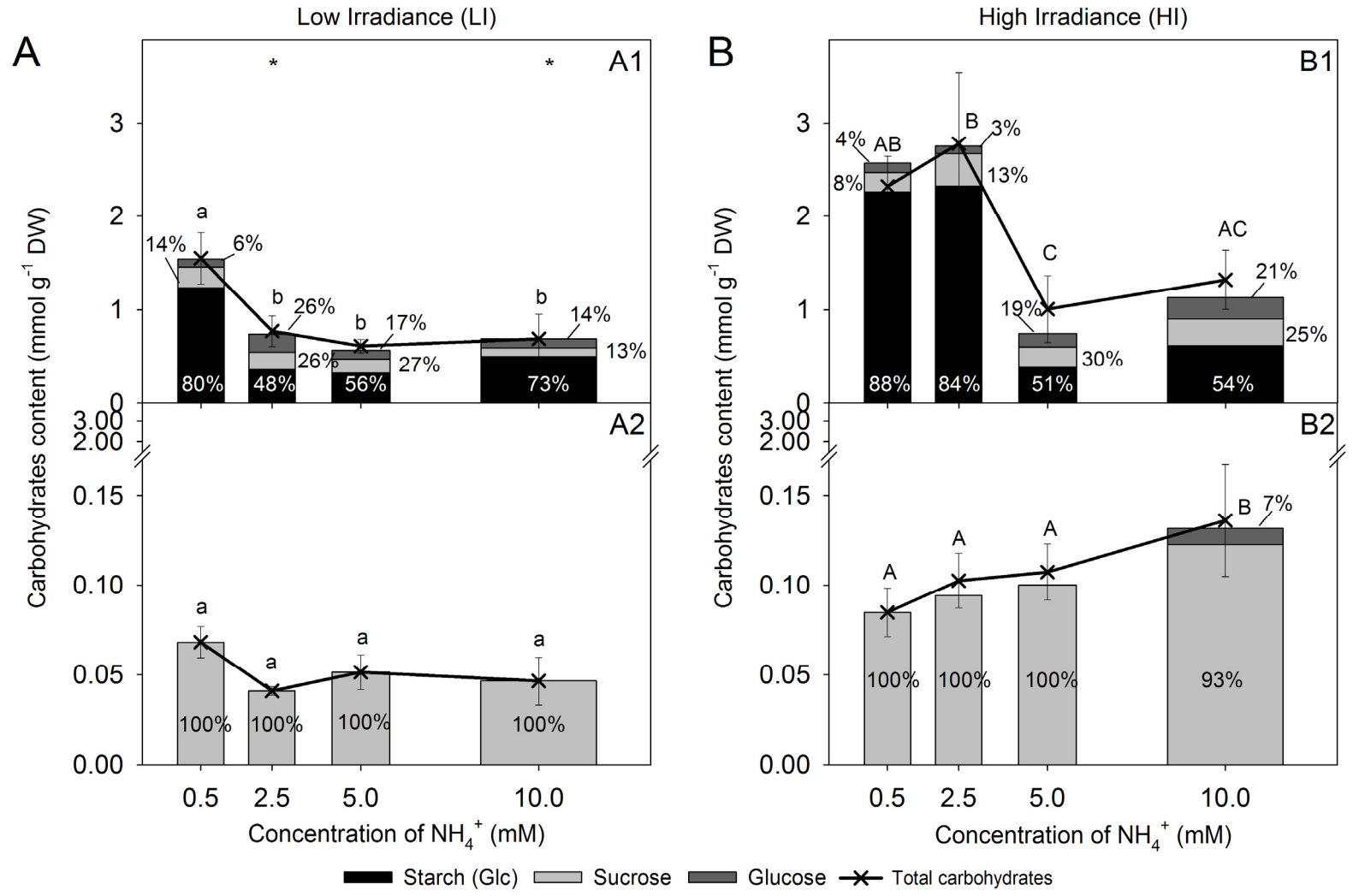

Low Irradiance (LI)
High Irradiance (HI)
A
B
A1
A2
B1
B2
Carbohydrates content (mmol g-1 DW)
Concentration of NH4+ (mM)
0.5
2.5
5.0
10.0
Starch (Glc)
Sucrose
Glucose
Total carbohydrates

Fig. 1

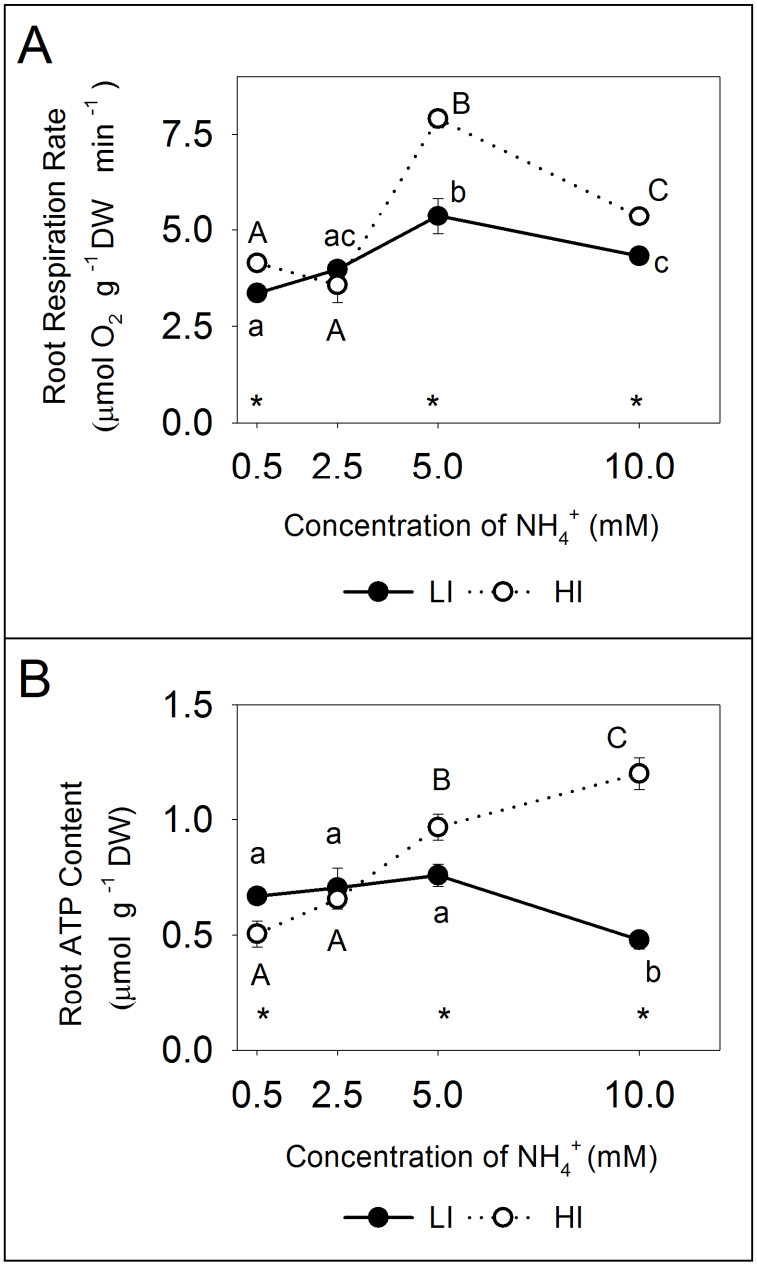
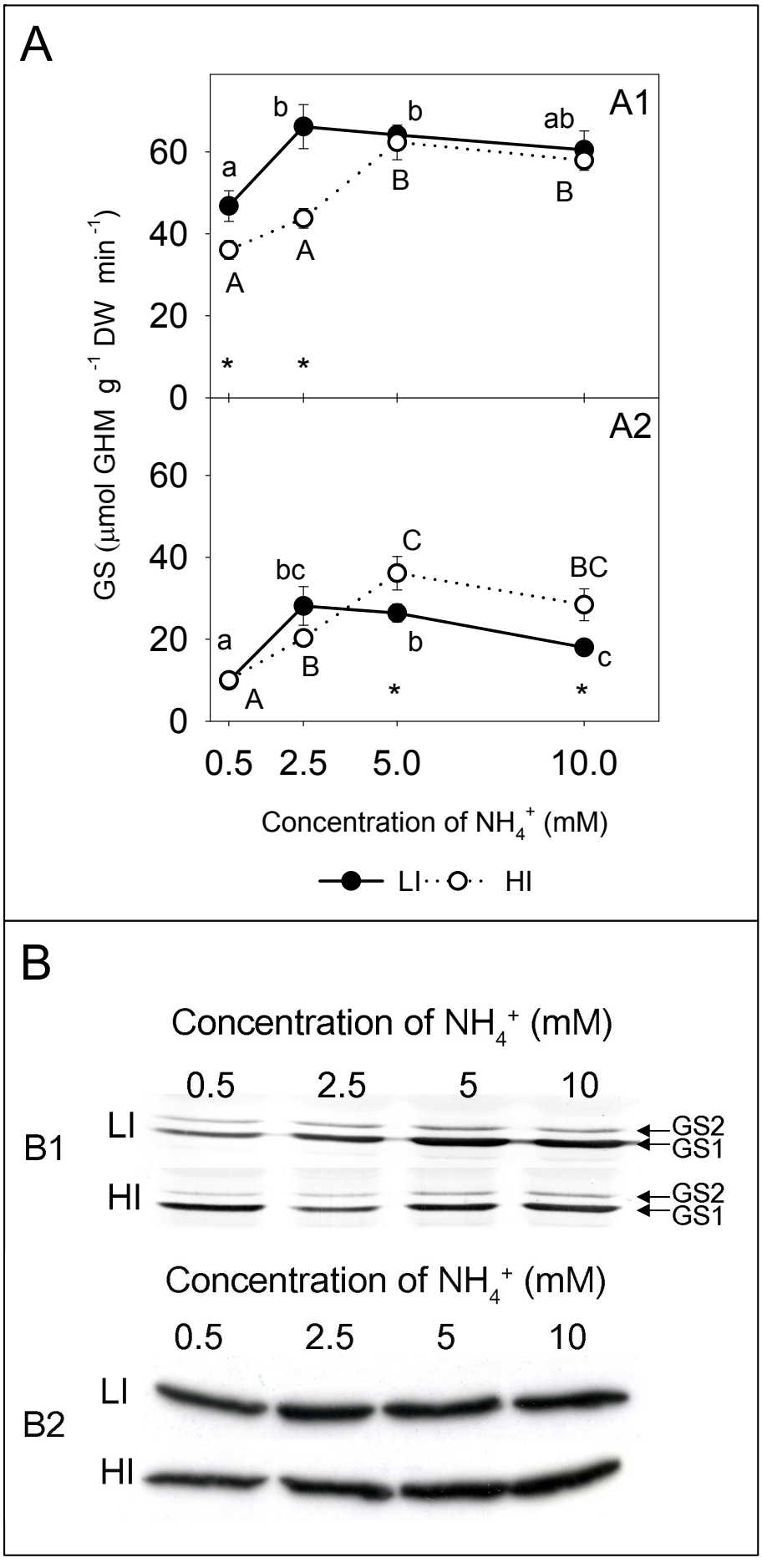

A
Root Respiration Rate
(μmol O2 g-1 DW min-1)
7.5
5.0
2.5
0.0
B
C
b
c
A
ac
a
A
*
*
*
0.5
2.5
5.0
10.0
Concentration of NH4+ (mM)
LI
HI
B
Root ATP Content
(μmol g-1 DW)
1.5
1.0
0.5
0.0
C
B
a
a
a
A
A
b
*
*
*
0.5
2.5
5.0
10.0
Concentration of NH4+ (mM)
LI
HI

Fig. 2

Fig. 3


A
A1
A2
GS (μmol GHM g-1 DW min-1)
0
20
40
60
a
b
b
ab
A
A
B
B
*
*
bc
C
BC
a
b
c
A
B
*
*
0.5
2.5
5.0
10.0
Concentration of NH4+ (mM)
LI
HI
B
Concentration of NH4+ (mM)
0.5
2.5
5
10
B1
LI
HI
GS2
GS1
GS2
GS1
Concentration of NH4+ (mM)
0.5
2.5
5
10
B2
LI
HI

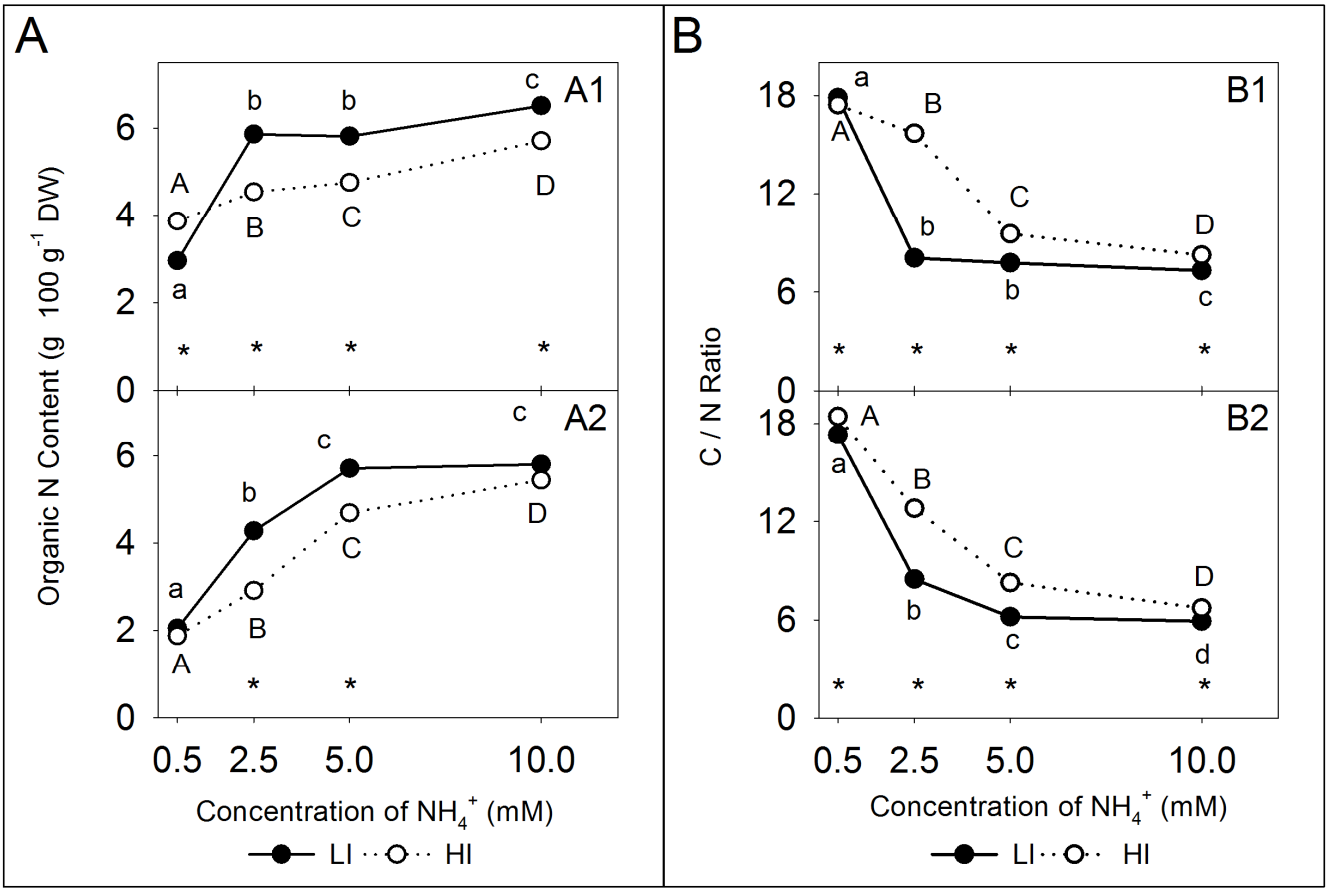

A
A1
A2
Organic N Content (g 100 g-1 DW)
0
2
4
6
a
b
b
c
A
B
C
D
*
0.5
2.5
5.0
10.0
Concentration of NH4+ (mM)
LI
HI
B
B1
B2
C / N Ratio
0
6
12
18
a
b
c
d
A
B
C
D

Fig. 4

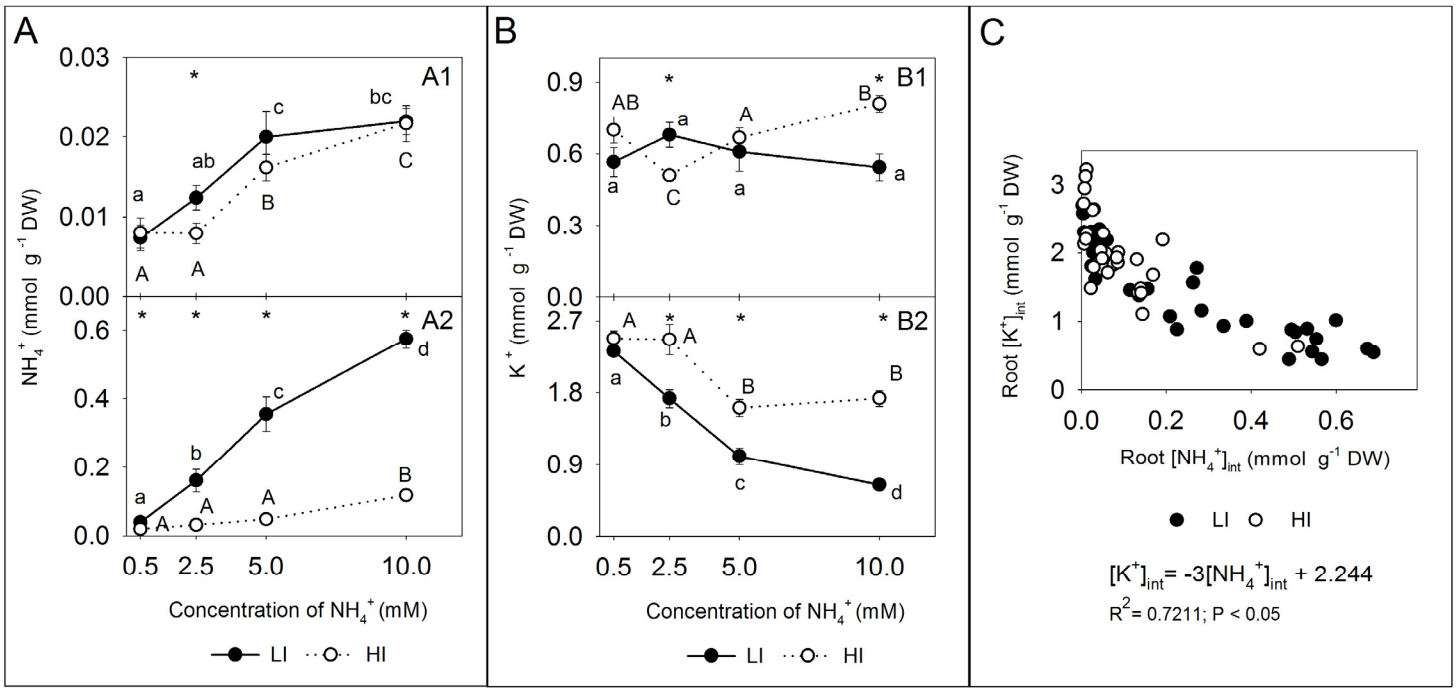

A
A1
A2
B
B1
B2
C
NH_4^+ (mmol g^{-1} DW)
K^+ (mmol g^{-1} DW)
Concentration of NH_4^+ (mM)
0.5 2.5 5.0 10.0
LI HI
Root $[K^+]_{int}$ (mmol g^{-1} DW)
Root $[NH_4^+]_{int}$ (mmol g^{-1} DW)
$[K^+]_{int}$= -3$[NH_4^+]_{int}$ + 2.244
R^2= 0.7211; P < 0.05

Fig. 5

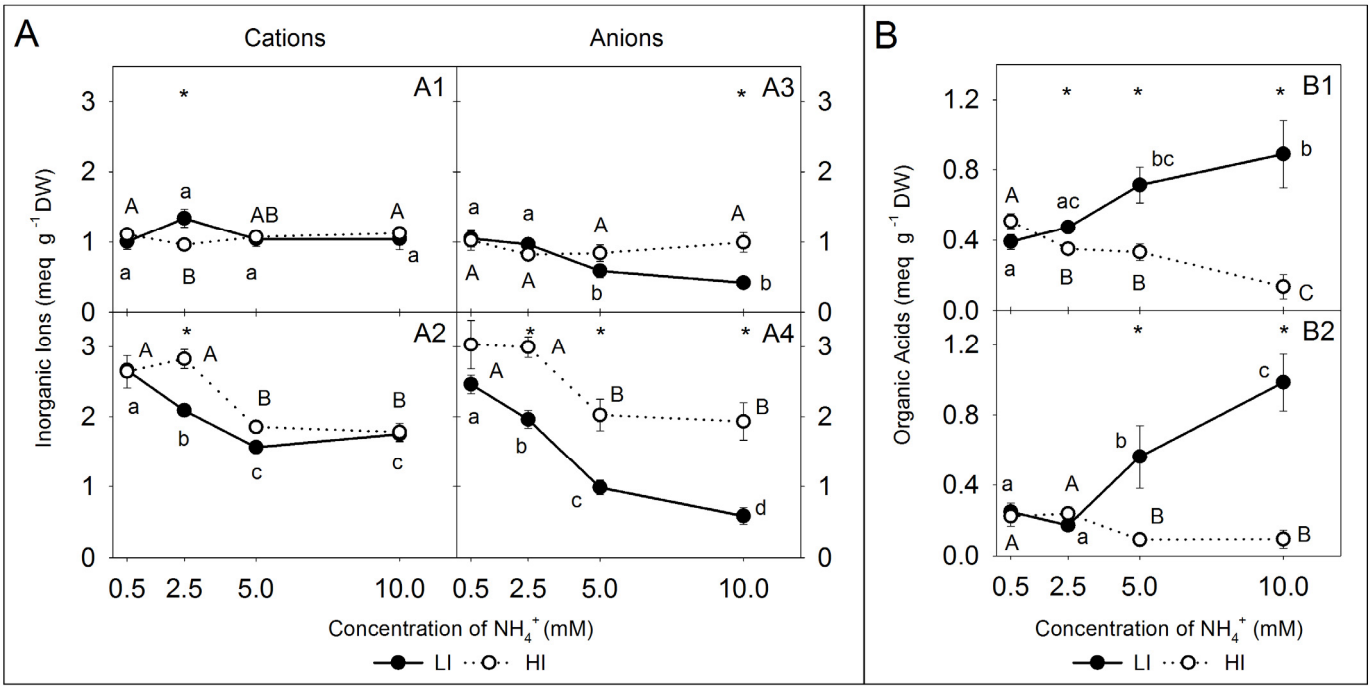

A
Cations
Anions
A1
A2
A3
A4
B
B1
B2
Inorganic Ions (meq g-1 DW)
Organic Acids (meq g-1 DW)
Concentration of NH4+ (mM)
0.5 2.5 5.0 10.0
LI HI

Fig. 6

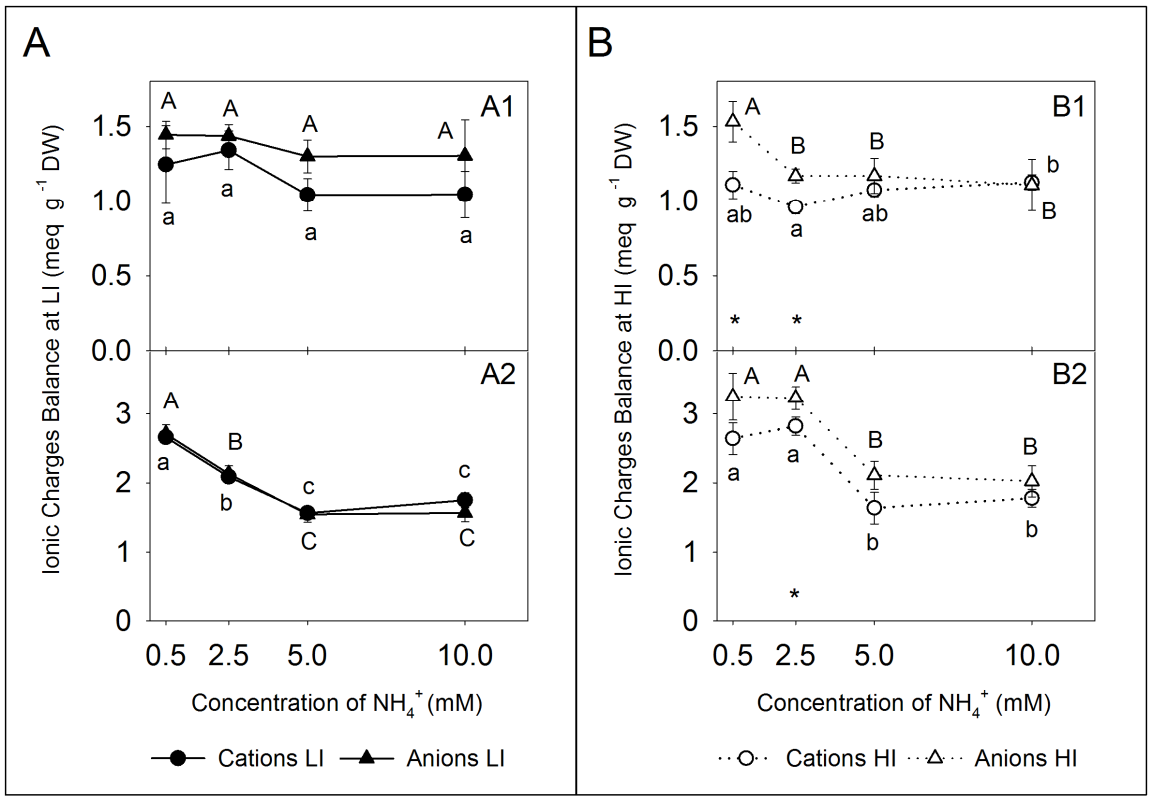

A
B
A1
A2
B1
B2
Ionic Charges Balance at LI (meq g-1 DW)
Ionic Charges Balance at HI (meq g-1 DW)
Concentration of NH4+ (mM)
Cations LI
Anions LI
Cations HI
Anions HI

Fig. S1