Running Head: NH$_4^+$ tolerance in pea

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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 NO₃⁻ fertilization has had a major ecological impact. NH₄⁺ nutrition may help to reduce this impact, although high NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ content regulation (probably NH₄⁺ influx/efflux) and to an improvement of the cell ionic balance.

The limited C availability at lower irradiance (LI) and high NH₄⁺ 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 NH₄⁺ and LI is related to their inability to avoid the large-scale accumulation of NH₄⁺ 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$_4$NO$_3$. 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 NH₄⁺ influx, as measured by ¹³N radiotracing (Szczerba et al., 2008a; Balkos et al., 2010), thereby highlighting the pivotal role played by K⁺ in alleviating NH₄⁺ toxicity (Szczerba et al., 2008b; Balkos et al., 2010).

The importance of C-skeleton availability for NH₄⁺ assimilation is well known (Schortemeyer et al., 1997; Roosta and Schjoerring, 2008). Therefore the availability of carbohydrate may influence NH₄⁺ toxicity (Roosta and Schjoerring, 2008) by determining how fast the NH₄⁺ absorbed can be assimilated (Raab and Terry, 1994), thus alleviating the NH₄⁺ 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 NH₄⁺ conditions achieve an elevated tolerance to NH₄⁺ 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 NH₄⁺ nutrition (Magalhaes and Wilcox, 1983; Gerendás et al., 1997; Zhu et al., 2000) and reducing the cellular NH₄⁺ content. In previous studies (Domínguez-Valdivia et al., 2008; Cruz et al. 2010), we have established that pea (Pisum sativum L.) can grow on NH₄⁺ 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 NH₄⁺ nutrition.

Material and Methods

**Plant material and growth conditions.** Seeds of pea (Pisum sativum L., cv. sugar snap) were surfaced sterilized, germinated at 26 °C 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$_2$HPO$_4$; 2.68 mM KCl; 0.7 mM CaSO$_4$; 0.07 mM Na$_2$Fe-EDTA; 0.85 mM 
MgSO$_4$; 16.5 µM Na$_2$MoO$_4$; 3.7 µM FeCl$_3$; 3.4 µM ZnSO$_4$; 16 µM H$_3$BO$_3$; 0.5 µM MnSO$_4$; 
0.1 µM CuSO$_4$; 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$_4$)$_2$SO$_4$
 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$^{-2}$·s$^{-1}$; or low irradiance (LI), 350 µmol photons m$^{-2}$·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. Bioluminescence 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 N₂ 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 MgSO₄; 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 N₂ and CO₂ 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 (NO₃⁻, NO₂⁻ and NH₄⁺; see inorganic ion content determination below) of vegetal tissues and was expressed in percentage (g of organic N 100 g⁻¹ DW by subtraying 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⁺, Mg²⁺, Ca²⁺ and NH₄⁺) 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⁻, NO₃⁻, PO₄³⁻ and SO₄²⁻) determination (1:10 diluted extracts) was carried out by the gradient method as for organic acid determination (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 NH₄⁺). The Levene test was used, and LSD statistics
applied for variables with homogeneity of variance and the Dunnett T3 test for cases of non-homoscedasticity. For testing the irradiance effect on each N treatment, Student's t-tests were performed independently for each N concentration, and homoscedasticity condition determined by the Levene test. All statistical analyses were conducted at a significance level of 5 % (P ≤ 0.05). Plants in this study were grown at three different times. At least one sample from each series was used for all parameters measured. In post-hoc tests displayed in figures and table the letters represent significant differences between NH$_4^+$ concentrations for HI (A, B, C and D) and LI (a, b, c and d). An asterisk (*) in figures denotes a significant difference between HI and LI for each NH$_4^+$ concentration.

Results

Higher irradiance and carbon content

Higher PPFD (HI) was found to have a remarkable positive effect on the biomass accumulation (Ariz et al., 2010), showing a lower shoot/root ratio under HI relative to LI, except at 0.5 mM NH$_4^+$ (Table 1).

In general, the carbohydrate content (glucose, sucrose, and starch) was higher under HI than under LI (Fig. 1). The most notable change in C level as a function of NH$_4^+$ concentration was found for leaf starch content, with starch levels decreasing significantly at 2.5 mM NH$_4^+$ for LI (Fig. 1 A1) and 5 mM for HI (Fig. 1 B1). Glucose levels showed no significant trend, with the exception of an increase at 10 mM NH$_4^+$ under HI (Fig. 1 B1). Shoot sucrose levels at LI showed moderately significant decreases with increasing NH$_4^+$ (Fig. 1 A1), whereas the increase at 2.5 mM NH$_4^+$ under HI was followed by a decrease at higher NH$_4^+$ concentrations (Fig. 1 B1). Root sucrose levels decreased with increasing external NH$_4^+$ at LI and showed no significant changes at HI (Fig. 1 A2 and B2). Sucrose was the main root carbohydrate (Fig. 1 A2 and B2), with glucose and starch being below the detection level in roots, except for 10 mM
NH$_4^+$ at HI (Fig. 1 B2). Fructose was also below the detection limit in both leaves and roots. Other C molecules such as sugar alcohols (polyols) may be important molecules in the transport of C, however as they do not ionize themselves, and they do not modify charge balance, nor modify the pH value, they have not been considered on the background of ion balance.

Energetic capacity, N assimilation and ion content

Root respiration increased with NH$_4^+$ availability at both PPFDs, and respiratory activity was higher under HI than under LI at high NH$_4^+$ (5 and 10 mM; Fig. 2 A). Furthermore, root ATP content, as detected by chemiluminescence, was higher under HI than under LI at high NH$_4^+$ and it increased with NH$_4^+$ concentration at HI. In contrast, root ATP content under LI was invariable up to 5 mM and then decreased at 10 mM NH$_4^+$ (Fig. 2 B).

GS activity was higher in leaves than in roots (Fig 3 A). Foliar GS activity was higher under LI relative to HI at low NH$_4^+$ (0.5 and 2.5 mM; Fig. 3 A1). In contrast, root GS activity was higher under HI and at high NH$_4^+$ concentrations (5 and 10 mM; Fig. 3 A2). Root GS activity increased with NH$_4^+$ up to 2.5 mM NH$_4^+$ under LI, whereas it increased near-linearly up to 5 mM NH$_4^+$ under HI, remaining constant at higher N concentrations for both PPFDs (Fig. 3 A).

Two GS isoforms were detected in pea leaves (Fig. 3 B1). The chloroplastic isoform content (GS2; 44-kD (Tingey et al., 1987) was higher under LI than under HI for all NH$_4^+$ treatments, whereas cytosolic isoform content (GS1; 38-kD) was higher under LI only at high NH$_4^+$ (Fig. 3 B1). Levels of both GS isoforms increased with external NH$_4^+$ concentration irrespective of PPFD, except for HI and 0.5 mM NH$_4^+$, which showed a “peak” in GS1 content (Fig. 3 B1). The root GS content increased slightly under HI with respect to LI at high NH$_4^+$ (Fig. 3 B2).

Organic N content (%) increased with NH$_4^+$ concentration. Interestingly, organic N content (%) was higher under LI than under HI, except at 0.5 mM NH$_4^+$, in both organs (Fig. 4 A1 and A2), thus indicating a higher net N assimilation under LI. In contrast, the C/N ratio decreased with
increasing NH$_4^+$ concentration (Fig. 4 B1 and B2) and it was significantly lower under LI than under HI (Fig. 4 B).

NH$_4^+$ contents in leaves and roots increased with external NH$_4^+$ availability and, on average, was 30 times lower in leaf than in root (Fig. 5 A1 and A2). Unlike LI, where NH$_4^+$ contents in roots increased near-linearly with increasing NH$_4^+$ concentration, much lower NH$_4^+$ contents were found under HI (Fig. 5 A2). In contrast, leaf NH$_4^+$ content did not differ significantly between HI and LI (Fig. 5 A1). The K$^+$ content in root tissue was higher than in leaves and showed an opposite trend to the NH$_4^+$ content (Fig. 5 A2 and B2), thus indicating that the NH$_4^+$ and K$^+$ contents in roots are negatively correlated (Fig. 5 C). Under HI, the K$^+$ content was significantly higher than under LI for most of NH$_4^+$ treatments studied (2.5, 5 and 10 mM) (Fig. 5 B2).

Both total soluble anions and cations in root decreased with increasing NH$_4^+$ concentration (Figs. 6 A2 and A4, respectively), although pea plants adapted to HI showed higher anion content in roots (Fig. 6 A4). The anion content in leaves decreased with increasing external NH$_4^+$ under LI; no significant differences were observed under HI (Fig. 6 A3). Total cation content in leaves remained essentially unchanged (Fig. 6 A1). An important imbalance in anion content in pea roots was found upon comparing the two PPFDs (Fig. 6 A4 and B 2). The organic acid content under LI increased significantly with NH$_4^+$, whereas it decreased slightly with increasing NH$_4^+$ availability under HI (Fig. 6 B). Total organic acid content is presented as the sum of citrate, malate, 2-oxoglutarate and succinate. The pyruvate, isocitrate, cis-aconitate and trans-aconitate were also detected but in very small quantities and were not taken into consideration. Also, due to the extraction of the samples with TCA 5 % (w/v) in water, part of this acid masked the separation of fumarate and oxaloacetate preventing the observation of their peaks.
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 NH$_4^+$) 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.

<table>
<thead>
<tr>
<th>$[\text{NH}_4^+]$ (mM)</th>
<th>Shoot : Root ratio at LI</th>
<th>Shoot : Root ratio at HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>$1.02 \pm 0.04^a$</td>
<td>$0.85 \pm 0.10^A$</td>
</tr>
<tr>
<td>2.5</td>
<td>$2.17 \pm 0.32^b_*$</td>
<td>$1.40 \pm 0.10^B$</td>
</tr>
<tr>
<td>5</td>
<td>$2.29 \pm 0.11^b_*$</td>
<td>$1.45 \pm 0.06^B$</td>
</tr>
<tr>
<td>10</td>
<td>$3.75 \pm 0.16^c_*$</td>
<td>$2.33 \pm 0.31^B$</td>
</tr>
</tbody>
</table>

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 ($\mu$mol O$_2$ g$^{-1}$ DW·min$^{-1}$) and (B) root ATP content ($\mu$mol 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 ($\mu$mol 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 ($\mu$mol NH$_4^+$ g$^{-1}$ DW) of leaf (A1) and root (A2); (B) K$^+$ content ($\mu$mol 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 NH₄⁺ 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⁺, NH₄⁺, K⁺, Mg²⁺, Ca²⁺) and anions (∆; PO₄³⁻, SO₄²⁻, NO₃⁻, Cl⁻, malate, 2-oxoglutarate, citrate and succinate. Data represent average values ± SE (n=6-8).
Fig. 1
Fig. 2
Fig. 3

A

Concentration of NH$_4^+$ (mM)

![Graph showing GS (μmol GHM g$^{-1}$ DW min$^{-1}$) vs. Concentration of NH$_4^+$ (mM)]

B

Concentration of NH$_4^+$ (mM)

B1 LI

B2 LI

Legend:
- LI
- HI

B1 and B2 show protein bands with GS2 and GS1 labeled.
Fig. 4
Fig. 5
Fig. 6
Fig. S1