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ORIGINAL ARTICLE

Nitrogen fertilization increases ammonium accumulation during senescence of barley leaves

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Abstract Leaf senescence is a developmental process characterized by two events: (1) dismantling of the photosynthetic apparatus and, (2) nitrogen remobilization to other parts of the plant. Ammonium accumulation during senescence of barley leaves was produced by chloroplast proteins degradation. Although most of the ammonium is remobilized as amino acids from senescing leaves, a minor part is lost as NH_3 emitted across stomata. The amount of ammonia emitted depends on the amount of NH_3 accumulated in the substomatal cavity, which is continuously re-supplied with NH_4^{1+} from the cytoplasm of neighboring cells. Ammonia accumulation in tissues could increase the possibility of loss of N as NH_3 emitted. In this report we analyzed the effect of N fertilization on nitrogen metabolism during senescence of barley leaves during the vegetative and reproductive stages of development. During senescence of barley leaves protein degradation was accompanied by transient ammonium accumulation at both stages of development. The peak of ammonium occurred immediately after major protein degradation in all samples analyzed, thereafter levels of ammonium clearly decreased. N accumulated as ammonium during senescence of barley leaves represented a high percentage of protein-N, i.e., approximately 16 % in primary leaves and 23 % in flag leaves. A significant increase of ammonium peak concentration was observed when doses of N fertilizer increased, mainly at the reproductive stage, where the percentage of N accumulated as ammonium reached near 35 % of protein-N at that stage. Vascular cytosolic glutamine synthetase (Hv GS1_1) transcript levels were upregulated during senescence of the flag leaf, but they were downregulated by increases in N availability. These results suggest that the decreases of ammonia levels after its peak may be more closely related to NH_3 emission than to N re-assimilation by GS.

Keywords Barley · Senescence · Ammonia accumulation · N fertilization

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Introduction

Leaf senescence is a genetically controlled and highly regulated developmental process characterized by two events: (1) dismantling of the photosynthetic apparatus and, (2) nitrogen remobilization to other parts of the plant. High percentage of leaves proteins is localized in chloroplasts; most of them are enzymes involved in photosynthesis and nitrogen assimilation (Guiboileau et al. 2010). Ammonium accumulation during senescence of barley leaves was produced by chloroplast proteins degradation (Hörtensteiner and Feller 2002). Although most of the ammonium is remobilized as amino acids from older to younger leaves, a minor part is lost as NH_3 emitted from the leaves (Schjoerring and Mattsson 2001; Gregersen et al. 2008).

The amount of ammonia emitted depends on the amount of NH_4^{1+} accumulated in the leaf apoplast of mesophylls cells (Husted et al. 2002). The leaf apoplast is continuously supplied with NH_4^{1+} from the cytoplasm; therefore,

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increasing leaf ammonium level could increase NH_4^{1+} level in the apoplast and consequently increase NH_3 concentration in the substomatal cavity; this may increase the potential lost of N as NH3 emitted to the atmosphere (Husted and Schjoerring 1995). The amount of NH_3 lost from a canopy constitutes between 1 and 4 % of the amount of nitrogen present in the mature shoots (Schjoerring and Mattsson 2001).

In higher plants, glutamine synthetase (GS) catalyzes the following reaction:

$$\text{Glutamate} + \text{Ammonia} + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi} + H_2O$$

Glutamine provides organic nitrogen for the biosynthesis of nitrogenous compounds. According to their subcellular localization, cytosolic glutamine synthetase (GS1) and chloroplastic glutamine synthetase (GS2) can be distinguished. While a single gene encodes the plastidic isoform in plants, the cytosolic isoform is encoded by a multigene family (Bernard et al. 2008). There are several cytosolic GS1 isoforms which differ in your regulation and tissues localization; some of them are involved in ammonium recycling during leaf senescence while others are involved in glutamine synthesis for delivery across the phloem sap (reviewed by Bernard and Habash 2009; Lothier et al. 2011). Recently, 5 isoforms of GS1 have been described in barley, named HvGS1_1 to HvGS1_5 (Goodall et al. 2013; Avila-Ospina et al. 2015). These authors showed that in leaves there are two cytosolic GS1: HvGS1_1 was localized in vascular cells whereas HvGS1_2 was localized in mesophyll cells. HvGS1_1 and HvGS1_2 expression levels increased during senescence while HvGS1_4 and HvGS1_5 genes decreased with aging as does GS2 (Avila-Ospina et al. 2015). The chloroplastic GS2 is involved in two important functions: (1) ammonium assimilation coming from mineral nutrition after nitrate reduction and (2) ammonium re-assimilation produced by photorespiration in C3 plants (Masclaux et al. 2000).

The rate of senescence and the remobilization of leaf nitrogen are related to source/sink relations and to the nitrogen nutritional status of the plant (Masclaux et al. 2000). Throughout the development the leaves undergo a change with regard to the demand for nutrients. Young growing leaves import nitrogen to build photosynthetically active chloroplasts and, later during senescence, these organelles act as a source of nitrogen which is relocated to new sinks, such as growing leaves during the vegetative phase, or storage organs and seeds during the reproductive phase. The contribution of leaf N remobilization to grain N content is cultivar dependent. Barley, like other small-grained cereal, may mobilize up to 90 % of the nitrogen from the vegetative plant parts to the grain; since during senescence the capacity of uptake N from the soil decreases considerably. On the contrary, in maize approximately 40 % of the grain N comes from soil uptake after flowering because nitrogen reserve in leaves is low (Gregersen et al. 2008).

Soil nitrogen availability has a strong influence on senescence rate (Schildhauer et al. 2008). High N levels can delay senescence as sink demand may be satisfied to a higher extent by soil N uptake (Martre et al. 2006). On the contrary, low N levels accelerate senescence, and this is associated with earlier plastidial protein degradation (Gan and Amasino 1997). Additionally, it has been demonstrated that N remobilization is favored under limiting nitrate supply in *Arabidopsis thaliana* (Lemaitre et al. 2008). The interrelationships between nitrogen availability (fertilization) and development of leaf senescence is very important from an economic viewpoint.

As ammonium content increases, the chances of losing N as ammonia emitted from the leaves increases. The main objective of this paper was to evaluate the extent of ammonium accumulation during barley leaf senescence in relation to the age of the plant (vegetative *versus* reproductive phase) and the availability of N during growth.

Materials and methods

Plant material

Leaf senescence in young plants at the vegetative stage

Barley (*Hordeum vulgare* L.; cv MP1109; Maltería Pampa) plants were grown in a growth chamber with a 10 h photoperiod (350 µmol m^{-2} s^{-1} of photosynthetically active radiation provided by 160 W fluorescent lamps) at 20 °C temperature. Plants were grown in 500-mL pots filled with soil (4.1 % organic material; phosphorous 7.33 ppm; nitrogen 0.115 % and pH 5.92) and two levels of urea added: 0 (−N) or the equivalent to 60 kg ha^{-1} (+N) of N. Thirty pots were used for each level of urea and all pots were watered daily.

Flag leaf senescence

Barley plants were grown under semi-controlled greenhouse conditions from the beginning of April (beginning of Fall in the Southern Hemisphere, 2010 and 2011) in La Plata, Argentina (34°S, 57°W). Plants were grown in pots of 7 L with soil (4.1 % of organic material; Phosphorous 7.33 ppm; Nitrogen 0.115 % and pH 5.92) and two levels of urea: 0 (−N) or the equivalent to 30 kg ha^{-1} (+N) of N. Near tillering, a further application of urea was added to the +N treatment, to finally reach a level of N equivalent to 60 kg ha^{-1}. We used 60 kg N ha^{-1} because this is an N dose commonly used by farmers in our region (Prystupa

and Ferraris 2011). Thirty pots were used for each treatment and all pots were watered daily.

Chlorophyll content

For chlorophyll content determination Soil Plant Analysis Development analyser (SPAD-502 Chlorophyll Meter, Konica Minolta, Tokyo, Japan) was used. Each leaf was measured on three spots along the blade. Each data point is the average of twenty leaves.

Protein content

Protein content was determined according to Rolny et al. 2011. Three freshly cut leaf discs taken along the blade (0.5 cm diameter each disc) were homogenized with 500 µL of chilled buffer (50 mM Tris hydroxymethyl aminomethane–HCl, pH 7, 2 mM EDTA and 1 mM PMSF) and centrifuged at 10,000×*g* for 10 min at 4 °C. Content of protein in extract leaves was estimated according to the Bradford methods (Bradford 1976). Bovine Serum Albumin was used as standard. Four samples per date and treatment were analyzed.

Ammonium content

Ammonium content was determined according to Rolny et al. 2011. Eight freshly cut leaf discs taken along the blade (0.5 cm diameter each) were homogenized with 1 mL of 0.3 mM H_2SO_4. Samples were centrifuged at 10,000×*g* for 10 min at 4 °C and ammonium was determined with Indophenol blue according to Hung and Kao (2007). $(NH_4)_2SO_4$ was used as standard. Four samples per date and treatment were analyzed.

Glutamine synthetase (GS) activity

Total glutamine synthetase activity was analyzed similar to Rolny et al. 2011. The GS-containing extract was obtained from five freshly cut leaf discs taken along the blade (0.5 cm diameter each). Freshly discs were extracted in 1 ml of extraction buffer containing 100 mM HEPES pH 7.6, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM glutamate, 10 % v/v ethylene glycol, 10 µM leupeptin and 6 mM cysteine. The crude extract was centrifuged at 21,000×*g* for 30 min at 4 °C. GS activity was measured using the synthetase assay based on the method described by Lea et al. (1990). 100 µL of crude leaf extract was added to 380 µL of assay mix which consisted of 100 mM HEPES pH 7.6, 80 mM glutamate, 6 mM hydroxylamine–HCl, 20 mM $MgSO_4$, and 4 mM EDTA. The reaction was started by the addition of 20 µL of 0.2 M ATP at pH 7.6. After 0 and 10 min of incubation at 30 °C, the reaction was stopped by the addition of 500 µL of ferric chloride reagent (0.24 M TCA, 0.1 M ferric chloride, 1.0 M HCl). Samples were then centrifuged at 10,000×*g* for 5 min and absorbance was read at 505 nm. Enzyme activity was calculated from the molar extinction coefficient of gamma-glutamyl hydroxamate (850 L/mol cm) (Ronzio et al. 1969) and expressed as pmoles of gamma-glutamyl hydroxamate formed min^{-1} - cm^{-2}. Four samples per date and treatment were analyzed.

Quantitative real-time PCR

A quantitative RT-PCR analysis was performed to determine transcript levels of the barley HvGS1_1 (isoform associated to N remobilization during senescence) and GS2 genes (Avila-Ospina et al. 2015). Total RNA was isolated from mature flag leaves (M) and senescent flag leaves (S). Approximately 100 mg of barley leaves was homogenized in liquid N_2 and mixed with 600 µL of lysis buffer (2 % w/v SDS, 68 mM sodium citrate, 132 mM citric acid, 1 mM EDTA). Proteins and DNA were removed by precipitation with protein–DNA precipitation solution (4 M NaCl, 16 mM sodium citrate, 32 mM citric acid). After centrifugation, the supernatant was transferred to a new tube, added with 300 µL isopropanol, mixed by gently inverting the tube, and centrifuged again. After carefully pouring off the supernatant, the pellet was washed with 70 % ethanol, air-dried, and RNA was resuspended in 25 µL DEPC-water and incubated with DNase I (Invitrogen) during 30 min at 37 °C. After that, RNA was precipitated by addition of NH_4Ac 7.5 M (ammonium acetate) and 100 % ethanol. The tube was centrifuged for 20 min at 4 °C, the pellet was washed with 70 % ethanol, air-dried and RNA was again resuspended in DEPC-water. Approximately 1 µg of total RNA was taken for cDNA preparation using reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed in a BioRad i-cycler (BioRad, Munich, Germany) using the following primers:

Primer name	Sequence	Amplified fragment
GS1 forward	5′-TCGCTCGCTACATTCTTGA GAGG-3′	198
GS1 reverse	5′-CGATGTGCTCCTTGTG CTTC-3′	
GS2 forward	5′-CTTCGCCATGACTTGCA CATAG-3′	130
GS2 reverse	5′-CCACACGAATAGAGCAG CCA-3′	
GAPDH forward	5′-GTGAGGCTGGTGCTGATTA-3′	198
GAPDH reverse	5′-CGTGGTGCACTAGCAT TTGGAC-3′	

GAPDH was used as control gene (Bustin et al. 2009; Goodall et al. 2013). The thermal profile consisted of 1 cycle at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s, 57 °C for 15 s and 72 °C for 30 s; after this the melting curve was performed. Expression level was expressed following the formula of Pfaffl et al. (2001). We analyzed two stages of flag leaf development: M (approximately 37 and 43 SPAD units for –N and +N, respectively) and S (approximately 25 and 31 SPAD units for –N and +N, respectively).

Statistical analysis

Data were analyzed by ANOVA, and means were compared with the Tukey's test at a significance level of 0.05. For regression analysis, r^2 (linear correlation or exponential relation) values are reported if they are significant at $p < 0.05$.

Results

Effects of nitrogen fertilization on leaf senescence at the vegetative phase

Primary barley leaves reached full expansion on the 13th day after sowing. We used this stage as day 0 (D0) in our experiments. Chlorophyll content (SPAD units) declined sharply during senescence of primary barley leaves in both treatments (−N and +N, Fig. 1). In +N, a decrease in chlorophyll was observed after 13 days past D0, while in –N plants this decrease started earlier and was faster. Chlorophyll content declined continuously to almost zero within 38 and 44 days for –N and +N, respectively.

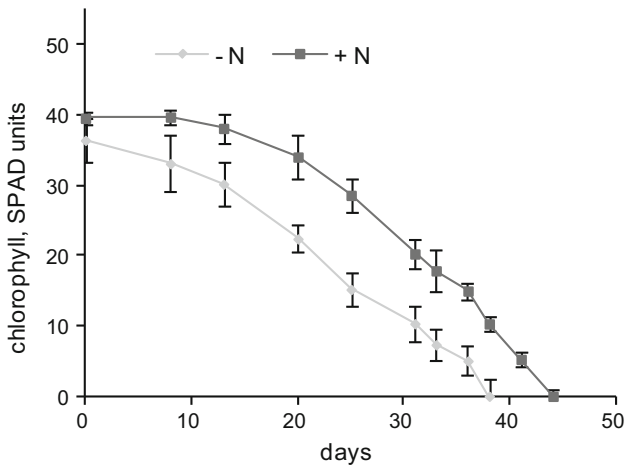

Fig. 1 Changes in chlorophyll content (SPAD units) during primary barley leaf senescence. Plants were grown without (−N) or with (+N) N fertilization. Each SPAD data point represents the mean of 10 independent measurements. *Bars* indicate standard deviation

Table 1 Definition of sampling dates for young, vegetative barley plants. Each SPAD values are means (± SD) of 10 replicates

		−N		+N	
		Day	SPAD	Day	SPAD
Mature fully expanded leaf	M	0	36 ± 4	0	40 ± 5
Early senescence	S1	13	30 ± 2	20	35 ± 2
Mid senescence	S2	25	16 ± 3	31	20 ± 1
Advanced senescence	S3	33	8 ± 1	38	12 ± 2

To study N metabolism during senescence in both treatments, sampling dates were defined based on the decrease in chlorophyll content (i.e., approximately 8 SPAD units between stages Fig. 1; Table 1). Each time selected represents mature leaves (M) and different degrees of senescence (S1, S2 and S3).

A significant decrease of protein content was observed during senescence of primary barley leaves in both treatments and there were generally no significant differences in protein levels between treatments (Fig. 2a), if the comparisons are made at similar stages of chlorophyll loss (Table 1). Approximately 70 % of the initial protein content was lost during senescence. We also observed that the main change in protein content occurred between M and S1 (approximately 50 % of protein was degraded in that period).

During senescence of barley primary leaves ammonium concentration increased dramatically between S1 and S2, and then decreased in both treatments (Fig. 2b). N fertilization increased ammonium accumulation compared to the –N treatment. It is noteworthy that peak ammonium concentration was observed after the most intense period of protein degradation. GS total activity decreased during barley senescence (Fig. 2c) and this decline was larger in –N plants (approximately 72 %) than in +N plants (approximately 62 %), i.e., at comparable stages of chlorophyll loss, GS activity was significantly lower in –N plants.

We analyzed the relationship between ammonium and protein levels on an N basis in both treatments and found a close negative exponential relation (Fig. 3). Thus, 77 and 62 % of ammonium accumulation variability is related to protein degradation in –N and +N plants, respectively.

Effect of Nitrogen fertilization on flag leaf senescence

To analyze flag leaf senescence in –N and +N plants, the time course of chlorophyll degradation was measured to determine sampling times. Again, D0 was the time in which flag leaf had reached full expansion (boot stage) and it was totally expanded. Plants fertilized with N showed higher chlorophyll content (20 %) in D0 and a slower rate

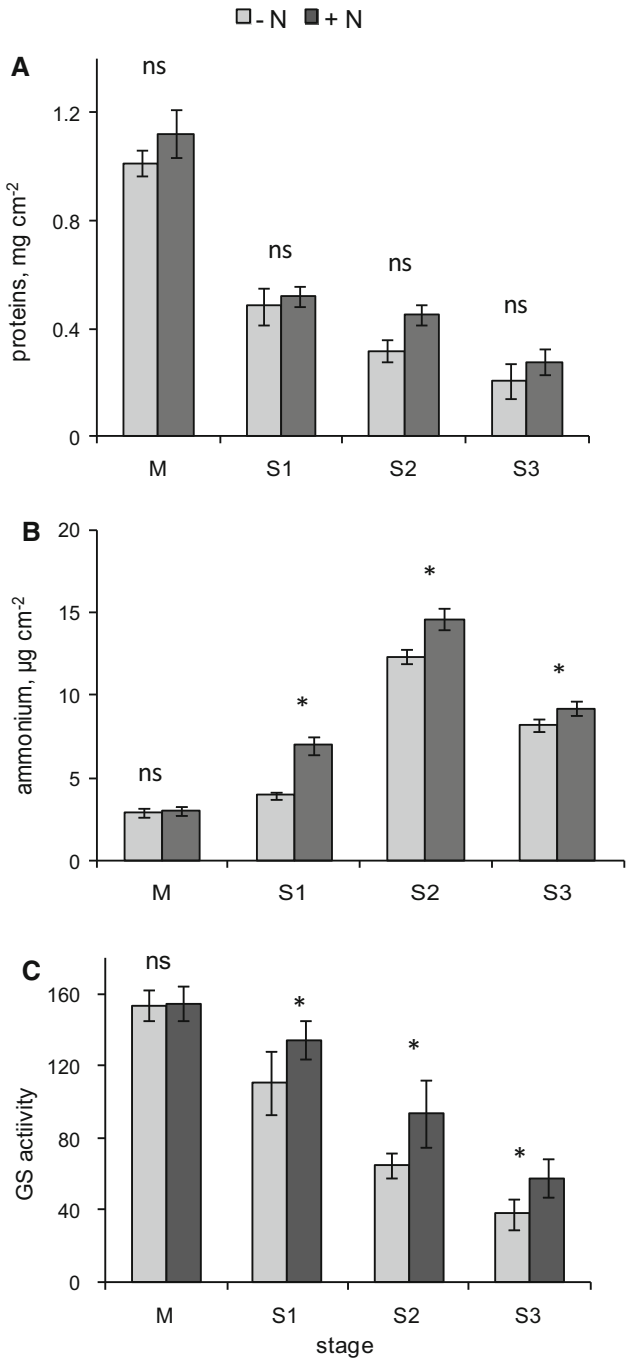

Fig. 2 Changes in protein (**a**), and ammonium (**b**) levels, and GS activity (**c**) in primary barley leaves without (−N) and with (+N) N fertilization. GS activity was expressed as picomoles of gamma-glutamyl hydroxamate formed min^{-1} cm^{-2}. *M*, *S1*, *S2* and *S3* represent different sampling dates during senescence (Table 1). Protein and ammonium content are expressed on the basis of leaf area. Four independent extracts were made for each sampling date and treatment. *Bars* indicate the standard deviation. On each sampling date the asterisk indicates significant differences between –N and +N treatments. There were no significant differences (ns) in all parameters between treatments at *M*

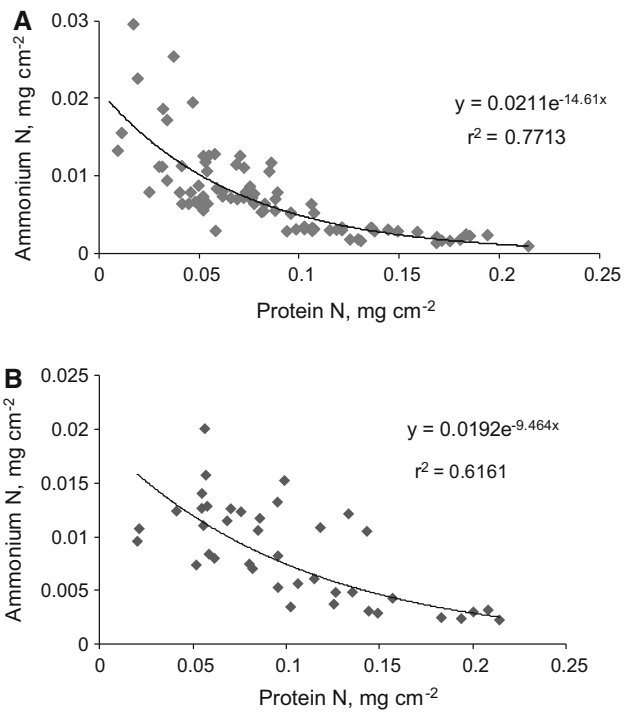

Fig. 3 Relationship between ammonium-N and leaf protein-N in primary barley leaves without, −N (panel **a**) or with, +N (panel **b**) N fertilization. There was a close negative exponential relation between both parameters

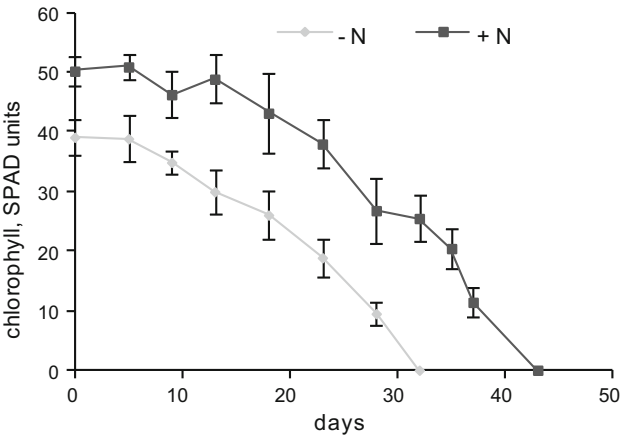

Fig. 4 Changes in chlorophyll content (SPAD units) during flag barley leaf senescence. Plants were grown without (−N) or with (+N) N fertilization. Each SPAD data point represents the mean of 10 independent measurements. *Bars* indicate standard deviation

of degradation during senescence than –N plants (Fig. 4). After 20 days, chlorophyll content in –N leaves showed a decrease of 35 % while +N leaves showed a decrease of only 8 % with respect to their initial chlorophyll levels. Chlorophyll declined continuously to almost zero after 33 days for –N and 43 days for +N plants.

Table 2 Definition of sampling dates for flag leaves

		−N		+ N	
		Day	SPAD	Day	SPAD
Mature fully expanded leaf	M	0	39 ± 3	0	50 ± 3
Early senescence	S1	18	27 ± 2	23	39 ± 3
Mid senescence	S2	23	20 ± 3	32	27 ± 2
Advanced senescence	S3	28	10 ± 2	35	20 ± 3

We used the decline in SPAD units (approximately 11 units) to define times for sampling: M (mature leaves) and S1, S2 and S3 (different degrees of senescence) (Table 2). The initial content of protein was higher in +N than –N flag leaves and protein declined continuously in both treatments, but the degradation rate was slower in fertilized plants (Fig. 5a). In the flag leaves of plants without fertilization, ammonium level increased twofold between M and S1, and decreased in S2 (Fig. 5b). This senescence-specific pattern of ammonium accumulation was delayed in +N flag leaves in which the peak occurred in S2 and the level of ammonium reached was higher than in –N leaves. Thereafter, ammonium level decreased dramatically reaching initial (M) levels in S3. The effect of N fertilization on ammonium accumulation was higher in the reproductive stage (flag leaf) than in the vegetative stage (primary leaf) (cf. Figs. 2b, 5b). Total GS activity of flag leaves decreased continuously in both treatments (Fig. 5c). Fertilization with N increased the initial activity of GS by approximately 43 %. GS activity decreased dramatically between M and S1 in +N, although thereafter there were no major differences between treatments in the rate of decrease of GS activity.

To relate the changes in GS activity during senescence in –N and +N leaves with expression of GS isoforms, we investigated the changes in transcript levels of HvGS2 (chloroplastic isoform) and HvGS1_1 (vascular cytosolic isoform associated with N remobilization during senescence) to determine their possible roles in ammonium assimilation during senescence of flag leaves. Transcript levels of HvGS2 (GS2) decreased 32 % between the mature and senescence flag leaf stages (Fig. 6). The senescence stage corresponds to a decrease of 10 units in SPAD values, compared to the mature stage. The dramatic decline in HvGS2 transcript levels was significantly higher in response to fertilization, reaching a decrease of 63 % at the S stage, compared to M leaves (Fig. 6a). Transcript levels of HvGS1_1 increased approximately threefold in senescing flag leaves with respect to mature leaves. The senescence-specific increase in HvGS1_1 was lower in +N compared with the –N treatment (Fig. 6b).

As with young leaves, protein and ammonium levels expressed on an N basis showed a close negative

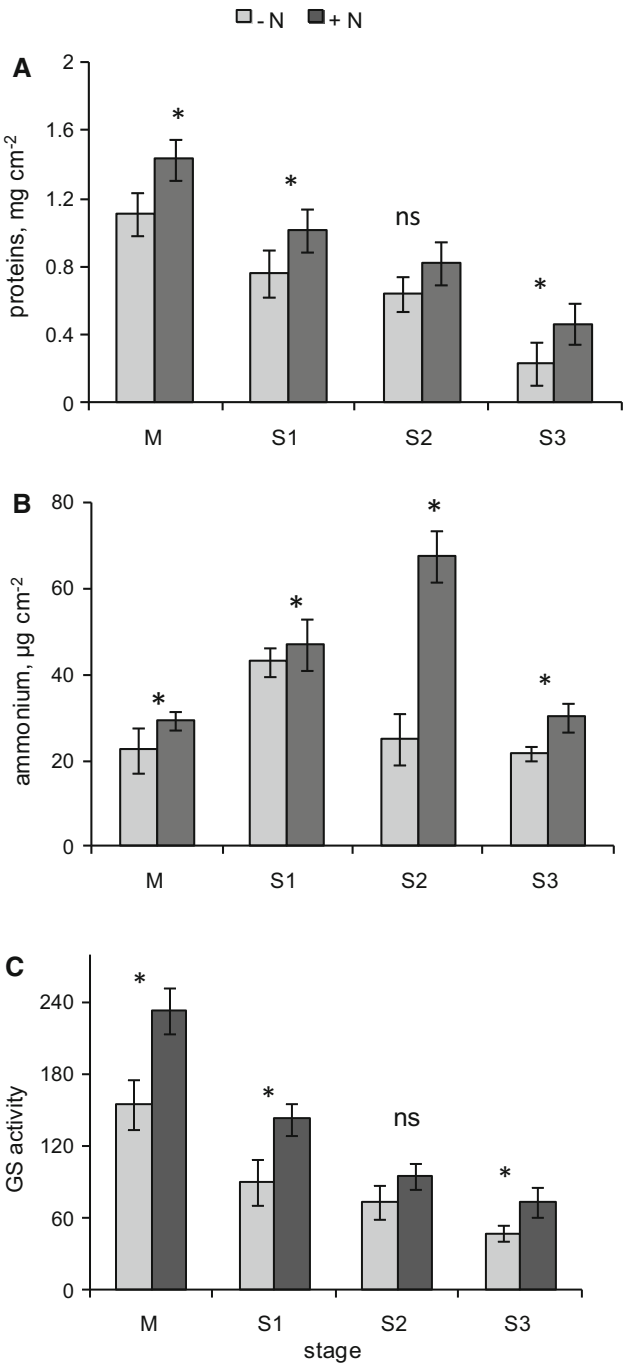

Fig. 5 Changes in protein (**a**) and ammonium (**b**) levels, and GS activity (**c**) levels in flag barley leaves without (−N) and with (+N) N fertilization. GS activity was expressed as picomoles of gamma-glutamyl hydroxamate formed. min^{-1} cm^{-2}. *M*, *S1*, *S2* and *S3* represent different sampling dates during senescence (Table 2). Protein and ammonium content are expressed on the basis of leaf area. Four independent extracts were made for each sampling date and treatment. *Bars* indicate the standard deviation. On each sampling date the asterisk representing significant differences at 0.05 level between –N and +N treatments. There were no significant differences (ns) in proteins and GS activity between treatments at S2

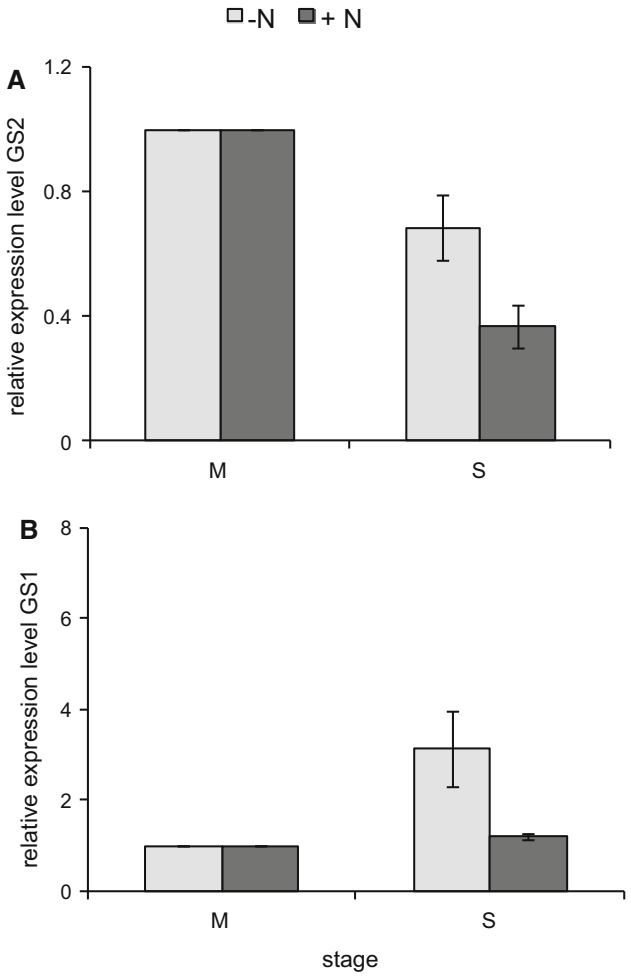

Fig. 6 Relative expression level of GS2 (**a**) and GS1 (**b**) of barley flag leaves without (−N) and with (+N) N fertilization. *M* mature leaves, *S* senescing leaves (S). There was a difference of 10 SPAD units between M and S samples used in RT-PCR. M (approximately 37 and 43 SPAD units for –N and +N, respectively) and S (approximately 25 and 31 for –N and +N, respectively). Transcript levels of each sample were normalized to their respective initial levels (M). *Bars* indicate standard deviation

exponential relationship (Fig. 7). This association was weaker in flag leaves ($r^2 = 0.50$) than in primary leaves ($r^2 = 0.77$) for plants under the low N treatment. In the reproductive stage, only 50 % of the variation in ammonium accumulation was accounted for by protein degradation in low N. The determination coefficient (r^2) was approximately 0.60 for both growth phases in the +N treatment (Fig. 7b).

Nitrogen fertilization increased the amount of ammonium-N during senescence of barley leaves

Under low- and high-nitrogen conditions, ammonium accumulated in senescing barley leaves, and this paralleled protein degradation (Figs. 3, 7). To analyze the extent of

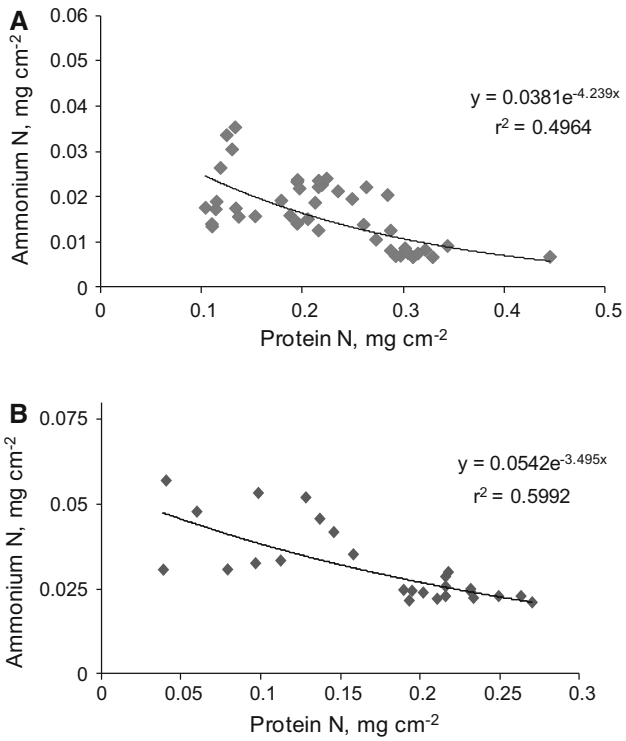

Fig. 7 Relationship between ammonium-N and leaf protein-N in flag leaves of barley without, −N (panel **a**) or with, +N (Panel **b**) N fertilization. There was a close negative exponential relation between both parameters

ammonium accumulation, we calculated the percentage of N accumulated as ammonium relative to protein-N at each senescence stage ($\%N_{NH4+}/N_{ProtSg}$, where ProtSg is protein level in M, S1, S2 or S3) using data from panels B and C of Figs. 2 and 5 (Table 3). The ammonium/protein ratio increased during senescence in both, the vegetative and reproductive phases of development, and reached high values. Without fertilization, peak $\%N_{NH4+}/N_{ProtSg}$ was 16.6 % in S2 and 23.6 % in S1 for primary and flag leaves, respectively. The peak $\%N_{NH4+}/N_{ProtSg}$ coincided with the peak of ammonium accumulation (Figs. 2b, 5b). N fertilization appreciably increased the $\%N_{NH4+}/N_{ProtSg}$ in both stages of development (vegetative and reproductive), reaching 19 and 35 % at S2 for primary and flag leaves, respectively. The effect of fertilization was larger during the reproductive stage.

Discussion

Substantial amounts of nitrogen applied as fertilizer are lost through ammonia volatilization from plant leaves (Schjoerring and Mattsson 2001). In spite of the importance of the relationship between NH_3 emitted and nitrogen metabolism in plants, the mechanisms involved in this relationship are

Table 3 Effect of N fertilization on ammonium-N accumulated at each senescence stage, expressed as a percentage of protein-N at the same stage of senescence

% Ammonium-N related to protein-N at each stage of senescence (mg N cm^{-2})				
	Leaf 1 −N	Leaf 1 +N	Flag leaf −N	Flag leaf +N
M	1.2	1.1	8.5	8.6
S1	3.4	2.9	**23.6**	19.3
S2	**16.6**	**19.1**	16.3	**34.7**
S3	16.8	14.7	39.2	27.1

Bold number means the maximun percentage reached during senescence for each treatment

poorly understood yet. Due to the fact that ammonia loss is linked with the level of ammonium in tissues, we analyzed ammonium accumulation and the effect of nitrogen fertilization on ammonium accumulation during senescence of barley leaves.

As leaves senesce photosynthetic proteins are extensively degraded, and the released nitrogen can be remobilized to growing leaves at the vegetative stage or seeds at the reproductive stage (Masclaux-Daubresse et al. 2010). Vegetative barley plants show a sequential senescence in which older leaves are developing senescence while new leaves are growing (Wiedemuth et al. 2005). Later, development of reproductive structures strongly induces senescence at the whole plant level. During the grain filling period the high demand of N increases N recycling from senescent leaves (Salon et al. 2001).

In this report, we analyzed the effect of N fertilization on ammonium accumulation during senescence of barley leaves in both the vegetative and reproductive stages of development. We found that during senescence of barley leaves protein degradation is accompanied by transient ammonium accumulation at both, the vegetative and reproductive stages (Figs. 2, 5). The peak ammonium content occurred immediately after the period of most intense protein degradation. Additionally, there was a close negative exponential relationship between ammonium accumulation and protein content (Figs. 3, 7). Therefore, our results strongly suggest that ammonium accumulation is closely related to protein degradation, and presumably amino acid de-amination, during senescence of barley leaves, irrespective of the developmental phase of the plant cycle. Ammonium accumulated during senescence of detached wheat, rice and barley leaves has been shown (Peeters and Van Laere, 1992; Thomas 1978; Rolny et al. 2011); however, ammonium accumulation during senescence has been rarely studied in attached leaves.

Adding nitrogen fertilizer to the soil delayed senescence in both, vegetative and reproductive plants, but increased ammonium accumulation (Figs. 1, 2, 4, 5). The amount of ammonium-N accumulated during senescence of barley leaves represented a high percentage of protein-N, i.e., at some senescent stages ammonium-N represented up to 16 and 23 % of protein-N in primary and flag leaves, respectively, of non-fertilized plants (Table 3). A significant increase of ammonium content was observed in plants fertilized with N, mainly at the reproductive stage (Table 3). Nitrogen is probably the most important nutrient recycled by plants during senescence, with up to 90 % recovered from leaves during this process (reviewed in (Liu et al. 2008). The contribution of leaf N remobilization to grain N content is species dependent, ranging from 50 % in maize up to 90 % in rice, wheat or barley (Masclaux-Daubresse et al. 2008). N remobilization increases under limiting nitrogen supply (Lemaitre et al. 2008). In this study, we found that a higher N availability increased the percentage of N accumulated as ammonium in barley leaves (Figs. 2b, 5b; Table 3). Although ammonium accumulated in both development stages, the effects of N fertilization were more marked at the reproductive stage, where as much as 35 % of protein-N may remain as ammonium (Table 3). During leaf senescence ammonium is probably generated mainly by protein degradation and amino acid de-amination. Ammonium thus generated is fixed to glutamine by GS with consumption of ATP. After that, glutamate synthase (GOGAT) catalyzes glutamate synthesis from glutamine by a reaction that requires carbon skeletons and reducing power (Foyer et al. 2002, 2001). Ammonium fixation needs carbon skeletons, ATP and reducing power which are provided through respiration of sugars or sugar–phosphates and by photosynthesis (Foyer et al. 2011). Clearly there is interdependency between nitrogen assimilation and respiratory carbon metabolism in terms of reducing power, ATP delivery and carbon metabolism. During senescence, photosynthetic activity decreases and this could generate an imbalance between N and C availability. In our experiments, nitrogen fertilization (+N) increased protein levels in leaves and it is possible that during senescence a large accumulation of ammonium exacerbated this imbalance.

Ammonia accumulation in tissues may increase the probability of N loss as NH_3 emitted from the leaves, since NH_4^{1+} is converted in NH_3 which is accumulated in substomatal cavity and easily emitted to surrounding ambient (Parton et al. 1988). We hypothesized that there were two possible ways to decrease ammonium accumulation: re-assimilation by GS or emission as NH_3.

Assimilation of NH_4^{1+} in leaf cells is catalyzed by the enzyme glutamine synthetase (GS). Hence, GS activity is important controlling the amount of NH_4^{1+} in leaf tissue, and this may subsequently affect NH_3 emission. In young and mature photosynthesizing leaves, both chloroplastic (GS2) and cytosolic GS (GS1) proteins have been detected (Habash et al. 2001; Tobin and Yamaya 2001). GS2 represents approximately 2/3 of total GS activity in annual C3 grasses such barley (Bernard el. Al. 2008). During the period of highly active photosynthesis, bulk GS activity is mainly due to the dominant GS2 form located in the chloroplast. It has been shown that GS activity in leaves decreases during senescence (Peeters and Van Laere 1992; Nagy et al. 2013; Avila-Ospina et al. 2015). Here, we showed that GS activity decreased during barley senescence, whether during the vegetative or reproductive phase of development (Figs. 2c, 5c). Decreased GS activity during leaf senescence may result, at least in part, in an accumulation of ammonium in leaves. Vascular cytosolic HvGS1_1 proteins accumulate in the cytoplasm of vascular cells in response to senescence suggesting a role for this isoform in the remobilization of nitrogen released as ammonium during the disassembly of the photosynthetic apparatus (Bernard and Habash 2009; Avila-Ospina et al. 2015). Generally, the senescence-associated GS1 is particularly important after anthesis and during grain development and filling in cereals, when nitrogen is remobilized to the reproductive sinks in rice and in maize (Tabuchi et al. 2005; Martin et al. 2006; Goodall et al. 2013; Avila-Ospina et al. 2015). Small-grain cereals, such as barley, may mobilize up to 90 % of the nitrogen from the vegetative plant parts to the grain (Gregersen et al. 2008). Because the relative proportions of the two GS isoforms change during senescence; it was assumed that GS1 could be important to decrease ammonium levels. We analyzed the changes in GS2 and HvGS1-1 in the flag leaf at the transcriptome level (Fig. 6a). Our data show that HvGS2 transcript level is downregulated during flag leaf senescence, and this decrease was exacerbated by N fertilization.

On the other hand, our results show that transcript levels of HvGS1_1 are upregulated during senescence of flag leaves, while they are downregulated by increased N availability (Fig. 6b). Our results are consistent with those of Avila-Ospina et al. (Avila-Ospina et al. 2015) who found that expression of HvGS1_1 was higher under low N compared to high N. These results suggest that the decreases of ammonia accumulation between S2 and S3 in +N plants may be more closely related to NH_3 emission than to GS assimilation or remobilization. Emission of NH_3 through the stomata may be a protection way from ammonium phytotoxicity in leaf without active nitrogen re-assimilation (Schjoerring et al. 1993).

It has been observed that N and C metabolites are involved in transcriptional regulation of GS, however our knowledge of the mode of action is limited. Expression of GS isoforms is induced by light and by the relative abundance of C skeletons available for amino acid synthesis (Oliveira and Coruzzi 1999). The substrates of the enzyme, NH_4^{1+} and glutamate increase GS1 expression (Masclaux-Daubresse et al. 2005; Goodall et al. 2013). Thomsen et al. (2014) suggest that a pivotal role of GS1 may be related to the maintenance of essential nitrogen (N) flows and internal N sensing during critical stages of plant development.

Protein degradation during senescence of flag leaves produce a significant amount of ammonium accumulation which could be re-assimilated and incorporated into grain; or nitrogen may be lost as volatile ammonia emitted from the canopy (Schjoerring et al. 1993). The influence of nitrogen source–sink relationships on the balance between NH_4^{1+} releasing and assimilating processes is still not known. In this report, we show that N accumulated as ammonium during barley senescence represents a high percentage of the N pool, particularly if it is expressed on the basis of protein-N, and that N fertilization increases ammonium accumulation. Additionally, we showed that transcript levels of senescence-associated GS1 are down-regulated by N fertilization, contributing to increased ammonium accumulation and potential NH_3 emission during leaf senescence.

Conclusion

During natural senescence of barley leaves, protein degradation was accompanied by transient ammonium accumulation, both during vegetative and reproductive growth. A significant increase of the ammonium peak content was observed when N fertilizer was supplied, and this was more marked at the reproductive stage. N accumulated as ammonium represented a substantial percentage of protein-N in the leaf. GS1 transcript levels were upregulated during senescence of the flag leaf, and down-regulated by N availability, contributing to increased ammonium accumulation and potential NH_3 emission during leaf senescence.

Author contribution statement Rolny and Costa did most experiments for this paper, Bayardo performed quantitative real-time PCR experiment, Guiamet and Costa designed experiments and wrote this paper.

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