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ARTICLE

Phloem transport of assimilates in relation to flowering time and senescence in barley grown with different availabilities of nitrogen and phosphorus

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ABSTRACT

Understanding the way in which N and P availability affects the transport of sugar and amino acids is crucial to improve grain quality and yield. Thus, in the present study, two greenhouse and field experiments were conducted with barley plants grown with different N and P availabilities to assess the dynamics of the phloem transport of assimilates in relation to the beginning of flowering and senescence. The phloem transport of assimilates decreased before the beginning of protein degradation in all treatments, but the onset of flowering and senescence varied according to the N and P availability, as evidenced by the concentrations of proteins, amino acids, and sugar and the gene expression of senescence-related proteases and all glutamine synthetase isoforms. In N-deficient plants, the phloem transport decreased before flowering, but only when P was not limiting; in N- and P-sufficient plants it decreased at flowering; and in P-deficient plants it decreased after flowering. Therefore, only N-deficient but P-sufficient plants have a post-anthesis period with high export rate of assimilates. This alteration of phenology in relation to phloem leads to important consequences in assimilate utilization, as shown by the higher yield and N content of the former compared to P-deficient plants.

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Flowering time; phloem transport; grain quality; grain yield; *Hordeum vulgare* L

Introduction

Barley (*Hordeum vulgare* L.) is the fourth cereal crop by production volume in the world after wheat, maize and rice. Barley is used not only for human food and animal feed but also for industrial purposes, such as malting and brewing. In the malting industry, the demand for barley is highly specific in regard to quality parameters, and aspects such as grain size and grain nitrogen (N) concentration are carefully taken into account when receiving a batch of barley. Thus, for this crop, achieving high yields is as important as obtaining an optimal commercial quality of the grain. However, in the new barley varieties developed, which show higher yields than before, the minimum protein content demanded by the brewing industry is not always achieved.

Grain yield and grain N concentration can be increased by N and phosphorus (P) fertilizations. N fertilization can increase both factors, but usually affects grain N accumulation more than total weight, thus determining an increase in grain N concentration (Magliano et al. 2014), whereas P

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fertilization usually increases yield but has little effects on grain N accumulation, thus determining a decrease or not in N concentration (Gately 1968; Therrien et al. 1994).

Grain yield and grain quality are also determined by the accumulation and redistribution via phloem of carbon and N. It is widely understood that the N accumulated before anthesis provides the main source of grain N. In wheat, around 60–95% of the grain N at harvest comes from the remobilization of N stored in shoots and roots before anthesis (Hirel et al. 2007), being the leaves and stems the most important sources of grain N (Gaju et al. 2014). A less important fraction of grain N comes from post-flowering N uptake and translocation to the grain (Hirel et al. 2007). On the other hand, the main sources of sugar available to crops for grain filling are in decreasing order of importance: photosynthesis in the leaves, remobilization of reserves stored in the stems, and photosynthesis in the spikes (Esmaeilpour-Jahromi et al. 2012). We have recently assessed the interaction between N and P availabilities in the establishment of phloem transport of assimilates in young barley plants, and found that an optimum availability of N and P is required for the plant to maintain high level of N remobilization (Criado et al. 2017).

Therefore, the main aim of the present study was to establish the effects of N, P and their interaction on the dynamics of phloem transport of amino acids and sugars at flowering and during grain filling. Besides, as N deficiency accelerates flowering of annual plants and P deficiency often delays it, we also aimed to correlate the dynamics of the phloem transport of assimilates with the onset of flowering. The knowledge of the specific phloem export rate established since flowering in each N and P availability condition will allow us to better understand the relative contribution of the remobilization process in the determination of grain yield and grain quality. This, in turn, will provide new information for the search of genotypes with high efficiency in the use of nutrients to improve the quality of grains through a sustainable agriculture.

Materials and methods

Experimental design and analysis of the growth and development of plants

Field experiments

Field experiments were conducted in a commercial barley (*H. vulgare* L. cv. Scarlett) crop located in the Pampean region (Argentina, 34°38′S, 60°56′W) in two consecutive years (2010 and 2011). Since the results obtained were similar, the results of only one of the two experiments are presented here. The content of nitrate-N in the soil at 0–20, 20–40 and 40–60 cm immediately before sowing (Markus et al. 1985) was 22, 12, and 9 mg kg^{-1} respectively, whereas that of extractable P (0–20 cm) (Bray-1) was 12.7 mg kg^{-1} (Kuo et al. 1996).

Four treatments resulting from the factorial combination of two rates of N (0 and 60 kg ha^{-1} of N) and two rates of P (0 and 30 kg ha^{-1} of P) fertilization at sowing were carried out. Treatments were organized in four completely randomized blocks, and defined as: N0P0 (without N and P fertilization), N0P1 (fertilized with P), N1P0 (fertilized with N) and N1P1 (fertilized with both N and P). In addition, all plots (20 m^2 each) were fertilized with 15 kg ha^{-1} of sulfur (S) to avoid S deficiency. One linear meter of shoots per plot were sampled during the grain filling period at 118, 127, 136 and 145 days after sowing (DAS) and at final maturity harvest at 157 DAS, and dried at 60ºC for 48 h for biomass determination. At physiological maturity, grains were dried at 60°C for 48 h for yield and N content determination. Grain N content was quantified by colorimetry of Kjeldahl digest (Baethgen and Alley 1989). The last two expanded leaves and the phloem exudates were sampled at 118, 127, 136 and 145 DAS and frozen in liquid nitrogen and stored at −80°C for further biochemical analysis. Phloem exudates were obtained from eight spikes from each treatment with the EDTA-facilitated method in a 3-h incubation period, as described in Veliz et al. (2014). Afterward, the exudation solution (5 ml) was stored at −80°C for further analysis.

Greenhouse experiments

Greenhouse experiments were conducted at the School of Agriculture of the University of Buenos Aires (Argentina, 34°59′S, 58°48′W) with natural light and temperature. Seeds of barley (*H. vulgare* L. cv. Scarlett) were grown in 6-L pots (five plants per pot), using vermiculite:perlite (1:1) as substrate, and watered every two days with nutrient solution (Hoagland and Arnon 1950) modified so as to obtain the combinatorial low and good availability of P and N: N0P0 (50μM KH_2PO_4, 2 mM KNO_3), N0P1 (200μM KH_2PO_4, 2 mM KNO_3), N1P0 (50μM KH_2PO_4, 10mM KNO_3) and N1P1 (200μM KH_2PO_4, 10 mM KNO_3), and then watered periodically. Treatments were arranged in four completely randomized blocks and each pot was considered as a sampling unit.

The number of spikes was counted in the sampling during heading stage, and their development stage was determined according to Zadoks et al. (1974). The flowering date was first established by spike dissection and occurred, on average, when the upper third of the spikes had emerged from the flag leaf sheaths. Thereafter, the flowering dates were estimated without dissection according to this criterion.

Shoots, leaves and spikes were sampled at 104, 112, 121 and 133 DAS and at final maturity harvest at 143 DAS, and dried at 60°C for 48 h for biomass determination. At physiological maturity, grains were dried at 60°C for 48 h to determine yield (g per pot), grain size fraction >2.5 mm (by size fractionation with a screening machine; Sortimat), and N content (by the Kjeldahl method). The last two expanded leaves and the phloem exudates were sampled at 104, 112, 121 and 133 DAS and frozen in liquid nitrogen and stored at −80°C for further biochemical analysis. Finally, the expanded frozen leaves sampled at 112 DAS were used for gene expression analysis.

Biochemical analysis

Frozen leaves were homogenized (2.5 ml buffer g^{-1} fresh weight) with 50 mM Tris–HCl buffer pH 7.5 containing 1% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant obtained was used for the determination of soluble proteins (Bradford 1976), free amino acids by the ninhydrin method (Yemm et al. 1955) after protein precipitation with trichloroacetic acid 5% (w/v), and soluble sugars by the anthrone method (Yemm and Willis 1954). In phloem exudates, free amino acids and soluble sugars were also determined by the ninhydrin and anthrone methods.

Total RNA extraction, cdna synthesis and quantitative PCR (qrt-PCR)

Total RNA was extracted from 100 mg of ground leaf tissue by using PureLink® Plant RNA Reagent according to the manufacturer's instructions (Invitrogen, USA). One microgram of DNAse-treated total RNA was used for cDNA synthesis by reverse transcription using M-MLV Reverse Transcriptase (Promega Corporation, USA) and Oligo(dT), following the protocol provided by the manufacturer.

cDNA samples were used as templates to analyze the expression level of the genes of interest. Quantitative PCR analysis (qRT-PCR) was performed in the Stratagene Mx3000P QPCR thermocycler (Agilent Technologies) by using FastStart Universal SYBR Green Master (ROX) from Roche. Samples were denatured at 95°C for 10 min, followed by 40 cycles (95°C for 15 s, 62°C for 30 s, and 60°C for 1 min) and 1 cycle of dissociation (95°C for 1 min, 55°C for 30 s and 95°C for 30 s). Senescence-associated genes (*SAG12, SUBTILASE* and *PAPAIN*) (Parrott et al. 2010), Glutamine synthetase genes (*GS1_1, GS1_2* and *GS2*) (Goodall et al. 2013) and the reference genes Actin (*Actin*) (AY145451, forward primer 5′-GTATGGAAACATCGTGCTCAGTGG −3′ and reverse primer 5′- CTTGATCTTCATGCTGCTCGGA −3′) and Translation Elongation Factor 1-alpha (*TEF*) (Z50789, forward primer 5′- AGGTCCACCAACCTTGACTG −3′ and reverse primer 5′- CAACAGGCACAGTTCCAATG-3′) were amplified using gene-specific primers manufactured by Eurofins (Tecnolab). As the relative expression pattern of the genes of interest was similar for both reference genes, only the results obtained against Actin are shown. The comparative Ct (threshold cycle) method (ΔΔCt) was applied for relative quantification of gene expression using the Stratagene Mx3000P thermocycler software.

Statistical analysis

Analysis of variance (ANOVA) and multiple comparison analysis of the data were performed. The *p*-values showing the level of significance of N, P and their interactions calculated from a two-way ANOVA of Figures 1, 4 and 6 are shown in the same figures, whereas the *p*-values showing the level of significance of N, P, time (T) and their interactions calculated from a three-way ANOVA of Figures 2, 3 and 5 are shown in their respective table.

Results

Field experiments

Plant growth

Fertilization with P increased barley biomass; this effect was significantly higher in the treatment also fertilized with N (N1P1) compared to the non-N-fertilized one (N0P1) (Figure 1(a)), since the final crop dry weight (DW) was increased by 54 % in N1P1 in comparison to N1P0 and by 27% in N0P1 in comparison to N0P0. In turn, the non-P-fertilized treatments (N0P0 and N1P0) presented the same lower biomass (Figure 1(a)). Clearly, these results indicate that even though crop was deficient of both P and N, the P was the most growth-limiting nutrient. Besides, the crop response to P fertilization varied according to the N status, evidencing an N × P interaction supported by statistical analysis.

In addition, P fertilization resulted in higher grain yield (Figure 1(b)), whereas N fertilization resulted in higher grain N content (Figure 1(c)), in agreement with previous reports (Gately 1968; Magliano et al. 2014). Therefore, the percentage of N in grains varied with the availability of N and P, being higher in N-fertilized treatments and lower in P-fertilized ones (Figure 1(d)).

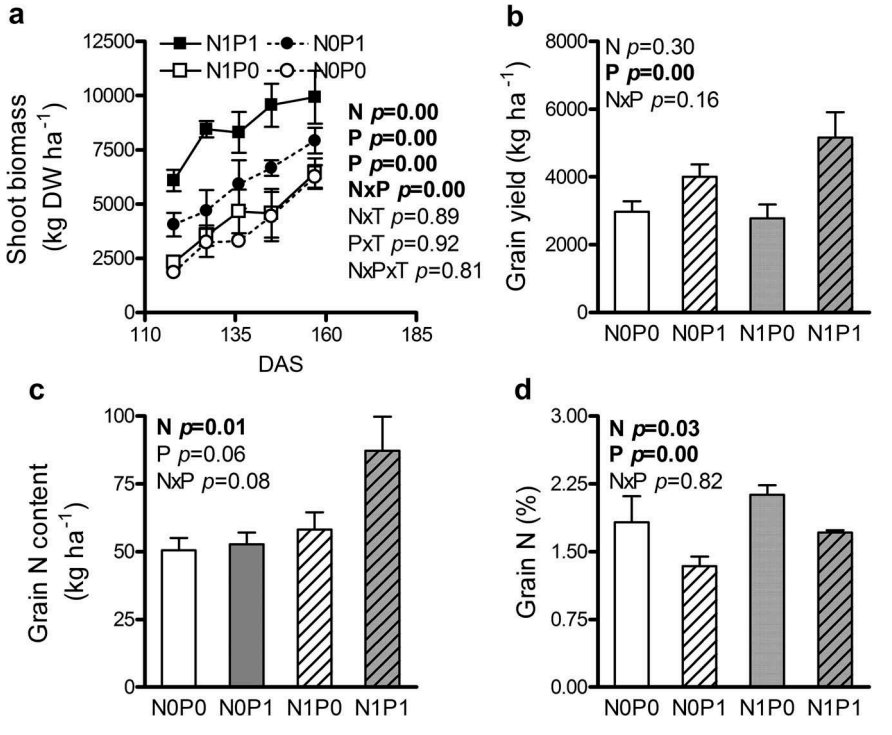
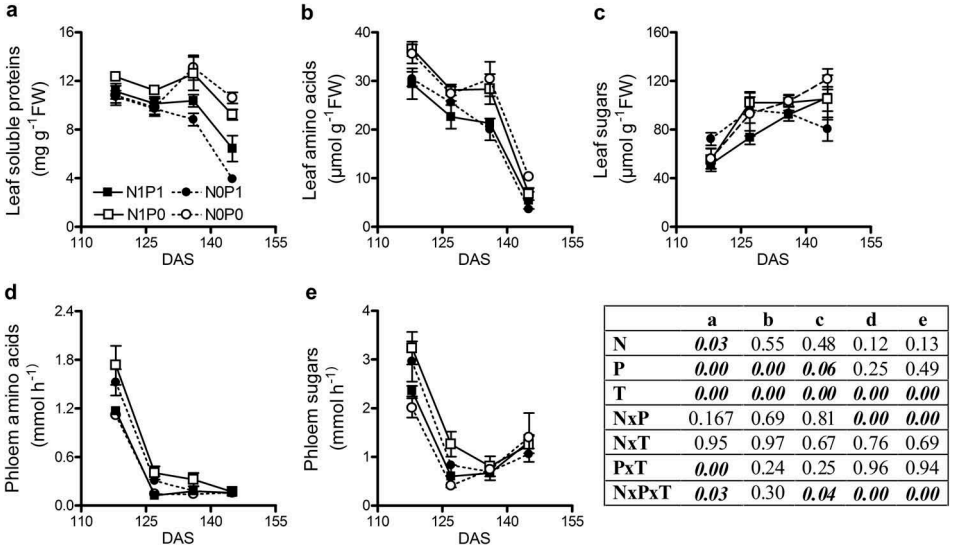
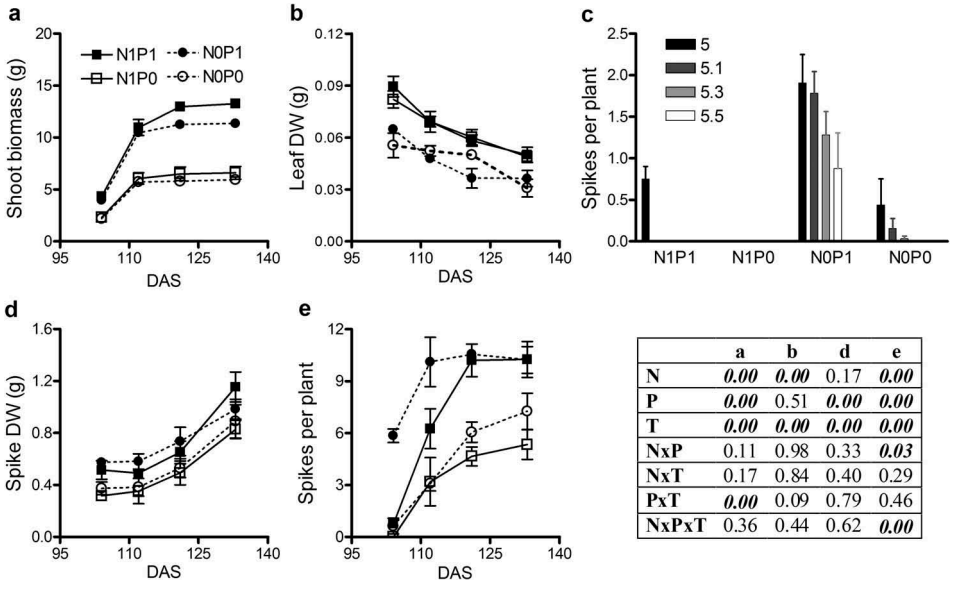


Figure 1. Shoot biomass (a), grain yield (b), grain N content (c) and grain N percentage (d) of N0P0, N0P1, N1P0 and N1P1 plants in the field experiment. Data are the means ± SE ($n = 4$).



	a	b	c	d	e
N	***0.03***	0.55	0.48	0.12	0.13
P	***0.00***	***0.00***	***0.06***	0.25	0.49
T	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
NxP	0.167	0.69	0.81	***0.00***	***0.00***
NxT	0.95	0.97	0.67	0.76	0.69
PxT	***0.00***	0.24	0.25	0.96	0.94
NxPxT	***0.03***	0.30	***0.04***	***0.00***	***0.00***

Figure 2. Changes in leaf soluble proteins (a), leaf amino acids (b), leaf sugars (c), phloem amino acids (d) and phloem sugars (e) of N0P0, N0P1, N1P0 and N1P1 plants during grain filling in the field experiment. Data are the means ± SE ($n = 4$). The table shows the *p*-values indicating the level of significance of N, P, time (T) and their interactions calculated from a three-way ANOVA of each figure. $p <$ or = 0.05 are shown in bold.



	a	b	d	e
N	***0.00***	***0.00***	0.17	***0.00***
P	***0.00***	0.51	***0.00***	***0.00***
T	***0.00***	***0.00***	***0.00***	***0.00***
NxP	0.11	0.98	0.33	***0.03***
NxT	0.17	0.84	0.40	0.29
PxT	***0.00***	0.09	0.79	0.46
NxPxT	0.36	0.44	0.62	***0.00***

Figure 3. Changes in shoot biomass (a), leaf dry weight (b), spike dry weight (d) and the number of spikes per plant (e) of N0P0, N0P1, N1P0 and N1P1 plants in the greenhouse experiment. Number of spikes with different development stages according to Zadoks et al. (1974) at 108 DAS (c). Data are the means ± SE ($n = 4$). The table shows the *p*-values indicating the level of significance of N, P, time (T) and their interactions calculated from a three-way ANOVA of each figure. $p <$ or = 0.05 are shown in bold.

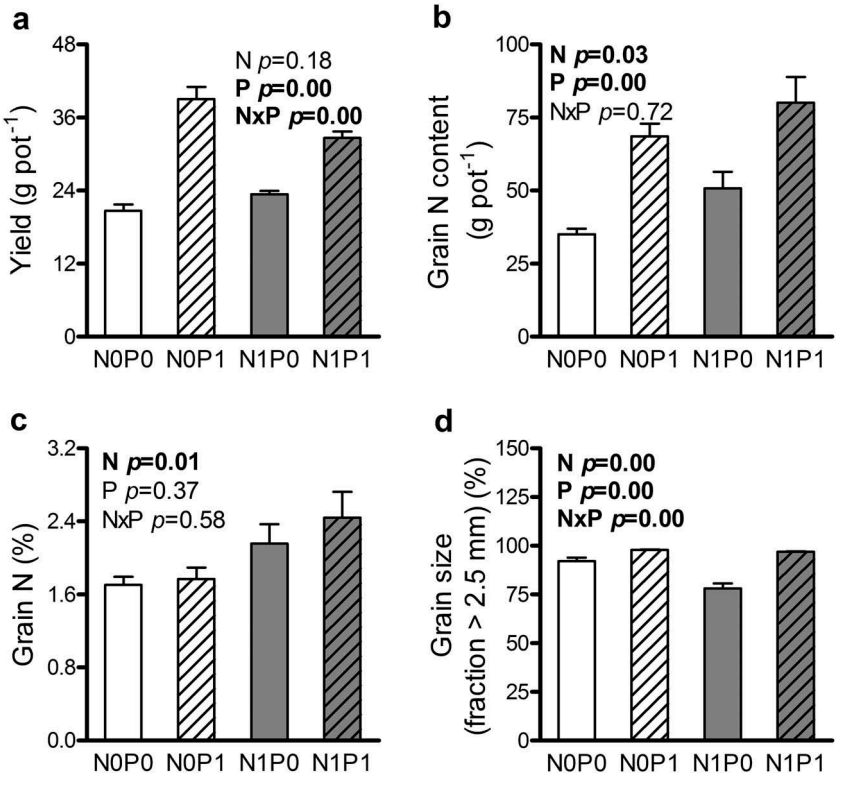
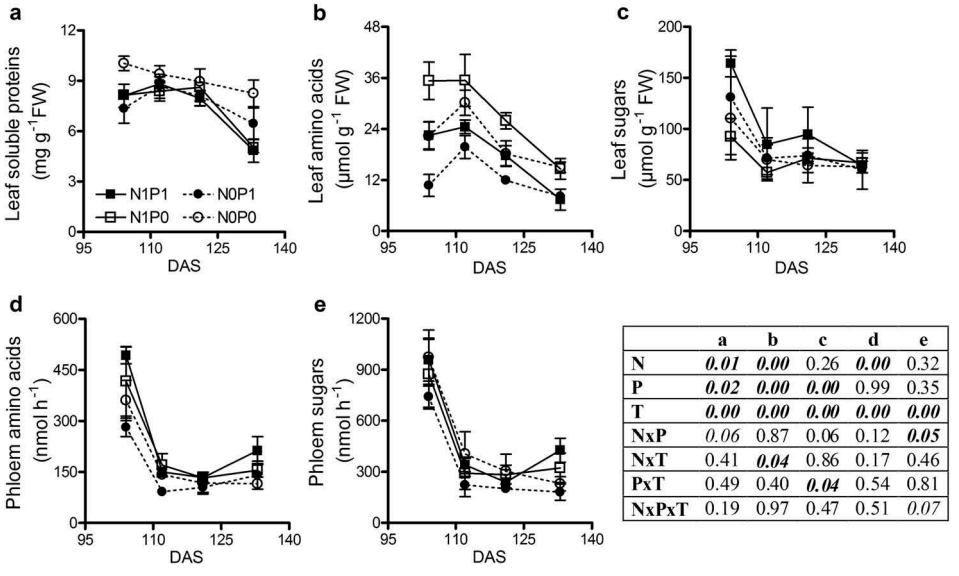


Figure 4. Grain yield (a), grain N content (b), grain N percentage (c) and grain size (d) of N0P0, N0P1, N1P0 and N1P1 plants in the greenhouse experiment. Data are the means ± SE ($n = 4$).

Biochemical determination in leaves and phloem exudates

The dynamics of protein concentration in leaves was different for each treatment, showing an N × P × T interaction. Initially, protein concentration was similar for all treatments, but after the third sampling date, soluble proteins decreased faster in the N0P1 treatment, followed by the N1P1, N1P0 and N0P0 treatments (Figure 2(a)), suggesting that senescence began earlier in N0P1 and was delayed in non-P-fertilized treatments. Regarding amino acids, concentrations were higher in non-P-fertilized treatments than in P-fertilized ones, and no differences were found due to N fertilization (Figure 2(b)). In all cases, amino acid concentrations decreased along the experiment according to the progress of senescence (Figure 2(b)). On the other hand, sugar concentrations increased showing an N × P × T interaction. Sugar concentration increased steadily with time in N0P0 and N1P1 (Figure 2(c)). Instead, in treatments in which one of the two nutrients were deficient (N1P0 and N0P1), sugar concentration increased only until the second sampling date, and then remained constant in N1P0, or decreased in N0P1 (Figure 2(c)). These results suggest that when the available concentrations of N and P are unbalanced (N0P1 and N1P0), the late increase in sugar concentration is restricted.

Interestingly, the exudation rate of amino acids and sugars decreased sharply between the first and second sampling dates (Figure 2(d,e)) before the decrease in proteins (Figure 2(a)) and, therefore, before the onset of senescence. At the beginning of the experiment (118–127 DAS), plants fertilized only with one nutrient (N1P0 and N0P1) showed higher exudation rate of amino acids and sugars compared to unfertilized (N0P0) or double fertilized (N1P1) plants. After that, no significant differences between treatments were observed (Figure 2(d,e)).



	a	b	c	d	e
N	***0.01***	***0.00***	0.26	***0.00***	0.32
P	***0.02***	***0.00***	***0.00***	0.99	0.35
T	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
NxP	*0.06*	0.87	0.06	0.12	***0.05***
NxT	0.41	***0.04***	0.86	0.17	0.46
PxT	0.49	0.40	***0.04***	0.54	0.81
NxPxT	0.19	0.97	0.47	0.51	*0.07*

Figure 5. Changes in leaf soluble proteins (a), leaf amino acids (b), leaf sugars (c), phloem amino acids (d) and phloem sugars (e) of N0P0, N0P1, N1P0 and N1P1 plants during grain filling in the greenhouse experiment. Data are the means ± SE ($n = 4$). The table shows the p-values indicating the level of significance of N, P, time (T) and their interactions calculated from a three-way ANOVA of each figure. $p <$ or = 0.05 are shown in bold.

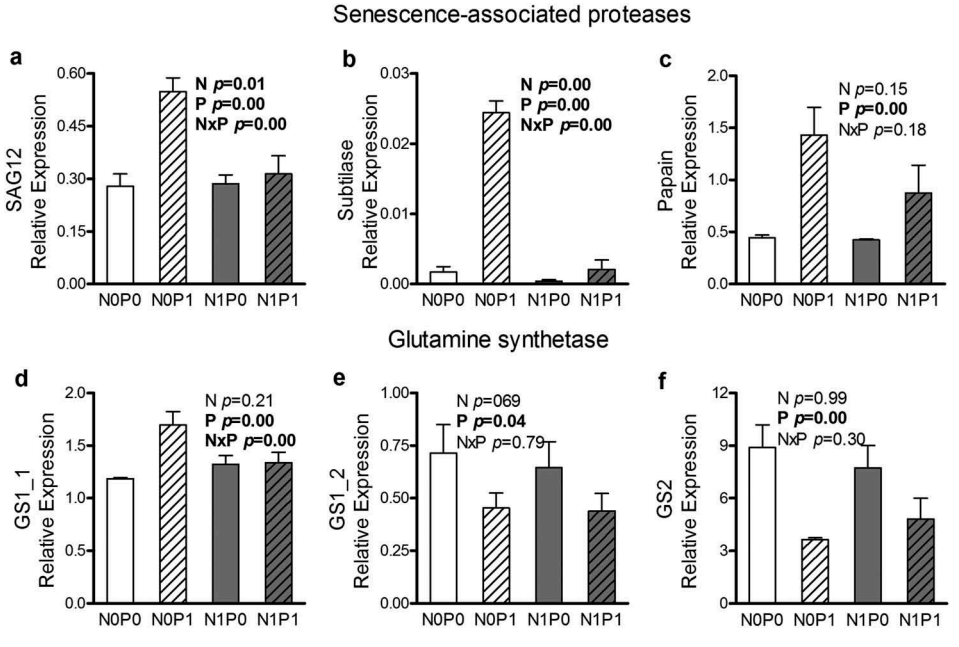


Figure 6. Relative expression of barley senescence-associated proteases: SAG12 (a), SUBTILASE (b) and PAPAIN (c) and different isoforms of GS: GS1_1 (d), GS1_2 (e) and GS2 (f) of N0P0, N0P1, N1P0 and N1P1 plants in the greenhouse experiment. Data are the means ± SE ($n = 4$).

Greenhouse experiments

Plant growth

In greenhouse conditions, the low availability of P also led to lower biomass of plants (Figure 3(a)), similar to the previous results obtained in field conditions (Figure 1(a)), mainly due to a lower number of spikes (Figure 3(e)) and DW of each spike (Figure 3(d)) and not to leaf DW (Figure 3(b)). In turn, this decrease in both the number (Figure 3(e)) and weight of spikes (Figure 3(d)), associated with the formation of smaller grains (Figure 4(d)), led to lower yield (Figure 4(a)). On the other hand, the low availability of N also led to lower biomass of plants (Figure 3(a)), mainly due to leaf DW (Figure 3(b)). Finally, leaf DW decreased with time, whereas spike DW increased according to the processes of senescence and grain filling (Figure 3(b–d)).

Flowering and number of spikes per plant

Initiation of flowering (50% of the spikes with visible edges) was detected first in N0P1 plants at 100 DAS, then in N1P1 plants at 108 DAS, then in N0P0 plants at 114 DAS and finally in N1P0 plants at 116 DAS. To better assess the changes in flowering time, the number of spikes at the different inflorescence emergence stages (stages 5.1–5.9; Zadoks et al. (1974)) per plant was recorded at 108 DAS. N0P1 plants had an average of six spikes distributed between stages 5 and 5.5, N0P0 plants had one spike between stages 5 and 5.3, N1P1 plants had one spike in stage 5, and N1P0 plants had no spikes (Figure 3(c)).

The number of spikes per pot also supported the correlation between nutrient availability and flowering. N0P1 plants were the only ones that had a higher number of spikes on the first sampling, reaching the highest value on the second sampling date. N1P1 plants increased the number of spikes on the second sampling, reaching the same number of spikes as the N0P1 plants on the third sampling date. P-deficient plants (N1P0 and N0P0) showed later emergence of spikes and smaller number of spikes per pot (Figure 3(e)). This different dynamics of the emergence of spikes evidences a triple interaction between N, P and time. On the last sampling date (133 DAS), only P-sufficient plants showed a higher number of spikes than P-deficient ones (ANOVA, fourth sampling N $p = 0.32$, P $p = 0.00$, N × P $p = 0.32$).

Grain quality (size and N concentration)

In agreement with field results, the high availability of P caused an increase in yield, being greatest the effect on plants with low status of N (Figure 4(a)). The high availability of P also increased the N accumulated in grains (Figure 4(b)), thus percentages of N were not affected in these plants (Figure 4(c)). As in field experiments and consistent with previous reports, the low availability of N did not affect grain yield directly (Figure 4(a)), but decreased grain N content per pot (Figure 4(b)), and therefore, the percentage of N in grains (Figure 4(c)).

Regarding grain size, a nutrient interaction was observed (Figure 4(d)). Although P deficiency caused a decrease in the percentage of grains retained on a sieve of 2.5 mm, this decrease was higher in plants with good availability of N than in plants with low availability of N, being only the grain size values of N1P0 plants below commercial requirements (Figure 4(d)).

Biochemical determination in leaves and phloem exudates

Soluble protein concentration was higher in plants grown with low availability of N or P (Figure 5(a)). In contrast, leaf amino acid concentration was higher in P-deficient plants and lower in N-deficient plants (Figure 5(b)), in agreement with results obtained under field conditions (Figure 2(b)). Finally, sugar concentration was lower in plants cultivated with low availability of P and did not change as a consequence of N availability (Figure 5(c)). Regarding its dynamics over time, protein concentration decreased throughout the experiment according to the progress of senescence (Figure 5(a)), whereas the dynamics of amino acid and sugar concentrations varied according to the availability of N and P respectively (Figure 5(b,c)). That is, amino acid concentration did not vary between the first and

second sampling dates in N-sufficient plants, whereas it increased in N-deficient plants, and then decreased in all treatments (Figure 5(b)). Regarding the concentration of sugars, the difference was also between the first and second sampling, when plants with sufficient P showed a more pronounced decrease than those grown with low availability of P (Figure 5(c)).

Regarding the phloem transport of amino acids, an N-dependent effect was observed. That is, plants grown with good availability of N had higher exudation rates than those grown with low availability (Figure 5(d)). Differently, the phloem transport of sugars was higher in N-sufficient plants only when there was a high availability of P. (Figure 5(e)).

Interestingly, the sharp decrease in the exudation rate of amino acids and sugars between the first and second sampling dates for all treatments was also observed under greenhouse conditions (Figure 5(d,e)), as described for the field-grown plants (Figure 2(d,e)). These results, together with those of flowering time, indicate that the phloem transport of assimilates decreased after anthesis in N0P1 plants, at anthesis in N1P1, and before anthesis in P-deficient plants (N1P0 and N0P0).

Proteases and N assimilation

At 112 DAS, the expression of the senescence-related proteases SAG12 and subtilase was induced only in N0P1 plants, whereas that of papain was also induced in N1P1 plants (Figure 6(a–c)), confirming that, when P availability is optimal, N deficiency accelerates senescence. The lack of effect of P deficiency observed in this work over the expression of the proteases was expected and strongly supports the fact that P deficiency delayed senescence. On the other hand, GS1_1 expression was higher in N0P1 (Figure 6(d)), whereas both GS1_2 and GS2 expressions were repressed in P-deficient plants (Figure 6(e,f)).

Discussion

In the malting industry, achieving good yields is as important as obtaining a proper N concentration in barley grains, two parameters that can be affected by the availability of N or P. As carbon and N reach the grain mainly through phloem transport, understanding the way in which N and P availability affects phloem transports of sugars and amino acids is central to improve yield and grain quality. Our results showed a decrease in phloem transport of amino acids and sugars just after the beginning of the decrease in proteins in source leaves, regardless of the availability of N and P in the soil in both field and greenhouse experiments (Figure 2(d,e), 5(d,e)). However, although high and low nutrient availability conditions were implanted in both experiments, a different nutrient availability along time was established in each, which may have led to some of the variance in the leaf biochemical determination and phloem exudation data obtained between them. In the field experiments, the lack of N or P was sensed at the end of the plant growth cycle, resulting in an adjustment of the remobilization process, whereas in the greenhouse experiments, both high and low nutrient availabilities were constant throughout the experiment. Thus, the plant metabolism was already set to a lower growth rate, without the need for an adjustment in remobilization. Therefore, the decrease in the rate of assimilate export would not be conditioned by N or P availability along time. Our results also showed that the decrease in phloem transport of amino acids and sugars does not correlate with the concentration of amino acids and sugars in source leaves (Figures 2 and 5). In this sense, we have previously reported that in young barley plants grown in culture chamber, P deficiency causes higher concentration of N compounds in source leaves without an increase in the export of amino acids through the phloem, pointing to a lack of correlation of N concentrations between leaves and phloem (Criado et al. 2017). Thus, the present work highlights that the decrease in phloem exudation not only is independent of N and P availability, but also seems not to be directly affected by the availability of N or P along time or by their concentrations in source leaves. However, the reason for the abrupt decrease in phloem transport of assimilates remains an open question. Jongebloed et al. (2004) reported that callose is deposited in the sieve tubes of *Ricinus communis* from the time of maximum leaf expansion,

causing a blockage in the phloem prior to the onset of senescence and the decrease in chlorophyll, N and C concentrations. Therefore, it would be interesting in future research to assess whether the decrease in amino acids and sugars in the phloem in barley plants is related to a possible blockage of the phloem by accumulation of callose.

The time of flowering is known to be regulated by various environmental signals, but with the exceptions of photoperiod and vernalization, the roles of other environmental signals are less understood (Kolář and Seňková 2008; Kazan and Lyons 2016). Abiotic stresses have been found to affect flowering in some plants, but these effects are often species-specific. Such differences among species might be related to their particular life-history strategies (Kolář and Seňková 2008). The results of the present study showed an acceleration of the time of flowering only for the N0P1 treatment, and a delay in P-deficient plants (Figure 3(c,e)). That is, in barley, the alteration of phenology depends on the availabilities of each particular nutrient. The response to each nutrient has an adaptive effect which could impact on the acquisition of resources for the plant. N availability often decreases throughout the growing season; therefore, there is little advantage for plants to delay maturity in conditions of N deficiency. Consequently, like most abiotic stresses, accelerated flowering and senescence of leaves are typical symptoms of N deficiency (Marschner 2011) which allow better utilization of N and ensure production of seeds. The impact of low P on the time of flowering and maturity has received less attention, but a delay in flowering and maturity has been reported as a response to low P in annual plants (Nord and Lynch 2008). It has been shown that delayed flowering and maturity in *Arabidopsis* is mainly a result of a lengthening of the vegetative phase and that the length of the reproductive phase is less affected. In addition, it has been suggested that the phenological delay may simply be caused by the inability of plants to flower until they have attained some threshold of tissue P or size, which would be achieved by a slower growth rate (Nord and Lynch 2008; Marschner 2011). In this sense, it has been reported that P deficiency in barley decreases the rate of appearance of leaves and increases both the duration of leaf appearance and the duration of the period from emergence to flowering (Prystupa et al. 2003), whereas that in *Arabidopsis* increases root length duration, which allows greater P acquisition (Nord and Lynch 2009).

The results observed in flowering and phloem transport of assimilates indicate that the decrease in the transport occurs before flowering in N0P1, at flowering in N1P1 and after flowering in N0P0 and N1P0 plants. Therefore, N0P1 plants may have a post-anthesis period with a high export rate of assimilates from vegetative tissues to the grain compared to the others (Figure 7), having important effects on resource acquisition for the grains (Figures 1 and 4). So, the high yield obtained in both experiments and the high N content in greenhouse conditions of the N0P1 plants compared to N1P0 and N0P0 plants could be attributed to the high rates of post-anthesis phloem transport of the former

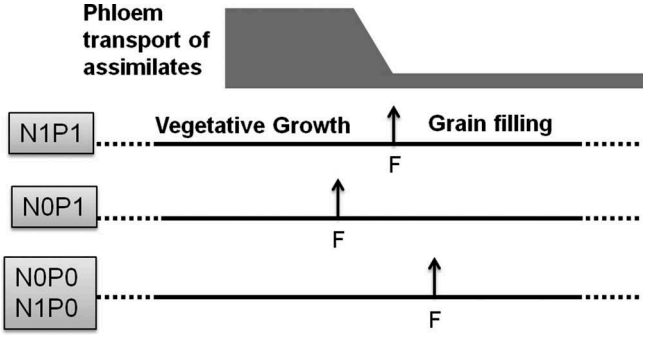


Figure 7. Theoretic scheme representing the decrease in phloem transport of assimilates in relation to flowering time in barley plants. Since the decrease in phloem transport of assimilates in N0P1 plants occurred after anthesis, it could be assumed that there is a post-anthesis period with high export rate of assimilates from vegetative tissues to the grain. In contrast, in P-deficient plants, the decrease in phloem transport of assimilates occurred before anthesis; therefore, the phloem transport of assimilates would take place only at low rates. This would have important effects on resource acquisition of grains, which, in turn, would impact grain yield and N content. F = flowering.

(Figures 1 and 4). This fact supports the known high efficiency of N remobilization in N-deficient plants compared to those well supplemented. So, high rates of post-anthesis phloem transport could be expected for N0P0 plants. However, this was not observed in the present study, thus indicating that the high efficiency of remobilization known for N deficiency occurs only when there is no deficiency of another nutrient, like P (Figure 7) or sulphur (Veliz et al. 2014). Finally, the higher grain N content but not yield of N1P0 compared to N0P0 may be due to a greater post-flowering N absorption owing to a greater availability of N in the soil of the N1P0 plants.

The remobilization process initiated after flowering is mainly supported by the beginning of senescence, a nutrient redistribution program, involving the catabolism of macromolecules in source tissues and phloem transport of the newly generated mobile products to developing grains, is initiated (Gan and Amasino 1997). In this sense, several authors have observed that the concentration of N compounds in leaves decreases during grain filling (Delogu et al. 1998; Veliz et al. 2014), whereas others have shown an increase in sugars due to the preferential export of N from leaves (Schaffer et al. 1991; Ono and Watanabe 1997; Wingler et al. 1998), which partly explains the results shown here (Figures 2 and 5). On the other hand, nutrient deficiencies are known to influence the onset of senescence, but the effects of N and P availabilities on the onset of senescence have been studied mainly as isolated factors, although deficiencies in field conditions of N and P are often combined. Our results evidence that senescence starts earlier in N-deficient plants only when P is not limiting, as indicated by the decrease in protein concentration in source leaves (Figure 2(a) and 5(a)) and the higher gene expression of senescence-related proteases and GS1_1 (Figure 6(a-d)). GS is known to play a central role in N metabolism and this complex role varies according to the context in which N metabolism is taking place (Miflin and Habash 2002). Particularly, mRNA expression and enzymatic activity of GS1_1 have been observed to increase under N starvation and natural senescence in barley and wheat plants (Bernard and Habash 2009; Caputo et al. 2009; Goodall et al. 2013; Avila-Ospina et al. 2015). In previous works, we have also observed an acceleration of the senescence process due to N deficiency in wheat plants (Criado et al. 2007) and a requirement of S for the induction of senescence triggered by N deficiency in barley plants (Veliz et al. 2014). Instead, in the present work, P deficiency delayed senescence regardless of the presence of N, as indicated by the concentration of proteins and amino acids (Figure 2(a) and 5(a)) and the lower gene expression of senescence-related proteases (Figure 6(a-c)), GS1_2 and GS2 (Figure 6(e-f)). GS1_2 has been associated with primary assimilation of N (Goodall et al. 2013) and the fact that GS1_2 expression varied only with P availability, being lower in P-deficient plants than in P-sufficient ones (Figure 6(e)), suggests that ammonium assimilation is increased in P-deficient plants. Thus, these results support that P deficiency expands the assimilative period of leaves and retards the onset of senescence. Finally, it is well known that the main function of chloroplastic GS2 is the reassimilation of ammonium released during photorespiration and that its expression is repressed during senescence (Masclaux-Daubresse et al. 2010). Therefore, the increased GS2 mRNA expression as a consequence of P deficiency (Figure 7(f)) adds evidences in favor of a delayed transition from nutrient assimilation to nutrient remobilization in P-deficient plants. Therefore, it can be assumed that the decreasing rate of assimilate export would not be conditioned by the onset of senescence either. The main environmental factors that significantly modify the development of crops are temperature, photoperiod and vernalization. Therefore, whether one or more of these signals could be the one that triggers the decrease in phloem transport of assimilates remains to be answered.

Conclusions

The results of the present study show an abrupt decrease in the phloem transport of assimilates taking place at the same time in all treatments but coinciding with anthesis only for N- and P-sufficient plants. Since the onset of flowering and senescence was accelerated in N-deficient but P-sufficient plants, the decrease in the phloem transport occurred after flowering in N0P1 plants. On the contrary, flowering and senescence were delayed in P-deficient plants, making that the decrease in phloem

transport occurs before flowering in N0P0 and N1P0 plants. Therefore, only the N0P1 plants showed a post-anthesis period with high export rate of assimilates, which may explain the known high efficiency of N remobilization of N-deficient plants. Here, we demonstrated that such high efficiency could only be achieved when P is not limiting. This alteration of phenology in relation to the phloem transport has important consequences in assimilate utilization, and provides valuable information for the development of new barley genotypes to improve grain quality through a sustainable agriculture.

Disclosure statement

No potential conflict of interest was reported by the authors.

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