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ORIGINAL PAPER

Post-anthesis N and P dynamics and its impact on grain yield and quality in mycorrhizal barley plants

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Abstract An essential goal for modern agriculture is the simultaneous improvement of productivity efficiency and nutrient use efficiency. One way to achieve this goal in crops is to enhance nitrogen (N) and phosphorus (P) acquisition through the mycorrhizal association. This study examined the effect of mycorrhization on post-anthesis N and P dynamics and its impact on grain yield and quality in barley. In addition, the efficiency of both N and P utilization and remobilization was evaluated. With those purposes, barley plants inoculated or not with *Rhizophagus intraradices* were grown in a soil poor in N and P under greenhouse conditions. Inoculation with *R. intraradices* in barley enhanced both N and P content in grain and vegetative tissue and reduced phloem amino acid export rate. On the other hand, both N and P vegetative tissue content and phloem amino acid and P export rates decreased during grain filling, whereas N and P grain content increased in both treatments according to the senescence process. However, whereas N grain concentration decreased during grain filling, P grain concentration did not vary, thus suggesting a differential regulation on grain filling. Inoculation with *R. intraradices* improved the yield and grain quality, thus demonstrating that inoculation with *R. intraradices* in barley is beneficial, but mycorrhization caused a diminution in nutrient utilization efficiency. As the phloem remobilization rate of amino acids and P did not decrease during grain filling in *R. intraradices*-inoculated plants compared to non-inoculated ones, these results suggest that nutrient utilization efficiency is most probably regulated by sink strength rather by a mycorrhizal effect.

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Introduction

The use of fertilizers has been a decisive factor for the increase in crop grain yield. However, the excessive use of nitrogen (N) and phosphorus (P) fertilizers often results in harmful environmental effects, including leaching of nitrate into ground water, surface runoff of P and N, and eutrophication of aquatic ecosystems (Adesemoye and Kloepper 2009). In addition, the excessive use of commercial fertilizers also means an economic loss for producers. Therefore, one of the most important goals for modern agriculture is to improve nutrient use efficiency by crops in order to both preserve ecosystems and reduce production costs while maintaining high productivity and grain quality. Improving the efficiency of fertilizer use (yield of grain per unit of available nutrient in the soil) for crop growth requires enhanced nutrient acquisition by plants from the soil (nutrient uptake efficiency) and enhanced productivity per unit of nutrient taken up (nutrient utilization efficiency) (Vance 2001; Masclaux-Daubresse et al. 2008; Veneklaas et al. 2012). Inoculation with arbuscular mycorrhizal fungi (AMF) is one of the strategies that lead to an improvement in nutrient uptake efficiency (Vance 2001). Symbiotic association between plants and AMF is widespread and has been observed in various natural and agricultural ecosystems. It is well established that mycorrhizal symbiosis can increase nutrient uptake, particularly P but also N, and growth in numerous crops, especially in soils with low P availability (Jensen 1983; Vance 2001; Richardson et al. 2011). Mycorrhizal association may also reduce environmental pollution caused by excessive use of fertilizers by allowing a higher nutrient

acquisition to the plant and thus reducing nutrient runoff or leaching (Adesemoye and Kloepper 2009).

The commercial quality of malting barley (*Hordeum vulgare* L.) is defined as the set of characteristics related to grain behavior during malting and brewing, such as the germination rate, N concentration (between 1.6 and 1.8 %), and grain size (fraction retained in a sieve of 2.5 mm). Thus, adequate N management is essential because high N availability is required for high yields, but in excess, N concentration in the grain could exceed 1.8 % and grain size could decrease, reducing malt quality (Savin et al. 2006). Final N concentration depends on soil NO_3^- absorbed and reduced during grain filling and on the remobilization from source tissues of N accumulated during vegetative period. Under conditions of high N availability in the soil, remobilization efficiency is decreased and a significant amount of N remains in the straw after harvest (Dalling 1985; Fatta et al. 2000; Ercoli et al. 2008).

Barley, besides its importance as a crop, is an established model plant for agronomic, genetic, and physiological studies. However, knowledge of the biochemical and molecular mechanisms controlling N uptake, assimilation, and recycling is still fragmentary (Hirel et al. 2007). Moreover, the way in which AMF affect post-anthesis N and P dynamics has been subject to less extensive study. In this context, the present study was performed to evaluate the effect of *Rhizophagus intraradices* on N and P dynamics during grain filling and on grain yield and quality of barley. Barley plants were inoculated with the AMF and grown in a soil poor in N and P under greenhouse conditions. To study N and P dynamics during grain filling, we determined N and P concentrations and contents in vegetative tissues and developing grains, and phloem amino acid and P export rates during grain filling. Yield, grain quality, and the efficiency of N and P utilization and remobilization were evaluated as well.

Materials and methods

Barley seedlings (*H. vulgare* L. cv. Scarlett) were grown in plug trays with an autoclaved (100 °C for 1 h, three consecutive days) mixture of 1:1:1 soil/perlite/vermiculite to establish conditions of nutrient deficiency and to favor good mycorrhizal infection. Seedlings were inoculated by placing 1 cm^3 agar plugs, containing approximately 200 spores plus fragments of hyphae and colonized roots from a previous monoxenic culture of strain GA5 of *R. intraradices* provided by *Banco Glomeromycota* In Vitro (BGI, FCEyN, University of Buenos Aires, Argentina) on the roots of each barley seedling. Plants were grown in a greenhouse with natural light and temperature from June to November. After 60 days, plants were transferred into six liter pots (six plants per pot) with the same autoclaved mixture of 1:1:1 soil/perlite/vermiculite. Chemical properties of the substrate mixture were as follows: pH (H_2O) 7.8, NO_3–N 7.4 mg kg^{-1}, and extractable P 7.3 mg kg^{-1} (Bray-1). The plants were irrigated with tap water to keep substrate at field capacity. The experiment had two treatments, mycorrhizal (M+) and non-mycorrhizal plants (M −), and was carried out in four completely randomized blocks.

Aerial vegetative tissues, grains, and roots were sampled at anthesis (105 days after sowing, DAS) at two times during grain filling (112 and 117 DAS) and at physiological maturity (140 DAS). At 105, 112, and 117 DAS, phloem sap exudates were obtained with a modified EDTA-mediated exudation technique (Veliz et al. 2014) and the exudation rates of soluble sugars (by anthrone method according to Yemm and Willis 1954), amino acids (by ninhydrin method according to Yemm and Cocking 1955), and P (Kuo 1996) were determined.

The roots were removed from the soils, washed with sterile distilled water, and stored in 50 % (v/v) ethanol. Then, mycorrhiza formation was determined microscopically in root samples with the gridline intersection method at a magnification of ×20 after staining with trypan blue (Phillips and Hayman 1970).

Vegetative tissues and grains were oven-dried to determine dry weight. Then, the dried samples were milled and analyzed for total N (Baethgen and Alley 1989) and P (Kuo 1996) after Kjeldahl digestion. At maturity, barley grain yield, grain and spike number per pot, grain number per spike, individual grain weight, and grain fraction >2.5 mm were evaluated.

The efficiency of N and P remobilization was calculated as $(N_{anthesis}-N_{maturity})/(N_{anthesis})\times 100$ and $(P_{anthesis}-P_{maturity})/(P_{anthesis})\times 100$, respectively, where $N_{anthesis}$, $P_{anthesis}$, $N_{maturity}$, and $P_{maturity}$ are the N or P content in vegetative tissues at anthesis and physiological maturity, respectively. Finally, the efficiency of N and P utilization was calculated as grain yield/$N_{total}\times 100$ and grain yield/$P_{total}\times 100$, where N_{total} and P_{total} are the N or P content in vegetative tissue and grains at physiological maturity.

Results were statistically evaluated by the analysis of variance (ANOVA) using the Statistica software (R) after testing variables for normality and homogeneity of variance. When interaction between main factors (treatment and time) was significant, treatment effect was tested at each sampling time (t test, $p<0.05$).

Results

Mycorrhizal structures were detected in barley roots of all AMF-inoculated plants (M+). However, the percentage of mycorrhizal colonization was low and never exceeded 8 %. No mycorrhizal structures were detected in any control plants (M−).

Vegetative and grain dry weight (DW) were higher in M+ barley plants compared to M− plants (Fig. 1a, b). The

vegetative DW did not vary during the grain filling period in either treatment. In contrast, grain DW increased during grain filling, but the dynamics of DW deposition was different for each treatment: Grain DW in M+ plants increased linearly during grain filling, whereas grain DW in M− plants increased steadily until 117 DAS and then accumulation slowed down (Fig. 1a, b).

The M+ treatment produced an increase in P acquisition as shown by the increase in vegetative and grain P concentrations in M+ plants compared to M− plants (Fig. 2a, b). Given that vegetative and grain DW were also higher in M+ plants, vegetative and grain P content became larger (Fig. 2c, d). Vegetative P concentration decreased during grain filling, whereas it did not vary in grains (Fig. 2a, b). Therefore, vegetative P content decreased during grain filling, whereas grain P content increased as expected when taking into account the results of DW and P concentration (Fig. 2c, d).

Vegetative N concentration and content decreased during the grain filling period according to the progress of senescence (Fig. 3a–c). On the other hand, vegetative N concentration did not vary between inoculation treatments (Fig. 3a), whereas total vegetative N content was higher in M+ plants compared to M− plants (Fig. 3c), demonstrating that *R. intraradices* colonization improved the acquisition of N. The lack of increment in N concentration suggests that the extra N was employed to increase both plant biomass (Fig. 1) and the number of spikes (Table 1).

Grain N concentration was higher in M+ barley plants compared to M− plants (Fig. 3b). The values of grain N concentration in M− plants (1.38 %) were below those required by industry, whereas those of M+ plants (1.55 %) reached appropriate ones. In addition, grain N concentration decreased during grain filling in both inoculation treatments (Fig. 3c), whereas grain N content increased due to DW accumulation (Fig. 3d).

Phloem exudation rate of sugars did not vary neither in time nor with *R. intraradices* inoculation (Fig. 4a). On the contrary, effects of both time and inoculation treatment on phloem exudation rate of amino acids were observed. Phloem amino acid exudation rate was lower in M+ plants and decreased sharply after anthesis in both treatments, remaining constant thereafter (Fig. 4b). Phloem P exudation rate also decreased after anthesis, but no difference between inoculation treatment was observed (Fig. 4c).

Grain yield, grain and spike number per pot, and grain number per spike were significantly higher in M+ barley plants compared to M− plants (Table 1). Individual grain weight and grain fraction >2.5 mm did not vary between inoculation treatments (Table 1). Finally, the efficiency of both N and P utilization and N and P remobilization was lower in M+ plants compared to M− plants (Table 1).

Discussion

The development of sustainable agricultural systems will require new techniques to help minimize fertilizer application rates while maintaining adequate crop yields and grain qualities. In this sense, the present study provides knowledge about the use of *R. intraradices* as a mycorrhizal inoculant in barley through the evaluation of its effect on plant N and P utilization and remobilization efficiency and its impact on grain yield and quality.

AMF are known to improve plant nutrition through their hyphae increasing the surface area of roots available for soil exploration for nutrients, particularly P but also N, and they stimulate growth and yield of plants grown under low P and N conditions (Vance 2001). In the present study, although the percentage of mycorrhizal roots was low, inoculation with *R. intraradices* enhanced N and P content in grain and vegetative tissue and improved the yield and grain quality. Numerous studies have not only previously reported low mycorrhization in barley plants (Jensen 1982; Fay et al. 1996; Baon et al. 1993) but also high values (Zhu et al. 2003). In addition, mycorrhizal colonization was not always directly correlated with increased growth and nutrient uptake (Jensen 1982; Baon et al. 1993; Smith et al. 2004; Hildermann et al. 2010). It is well established that plants vary in their

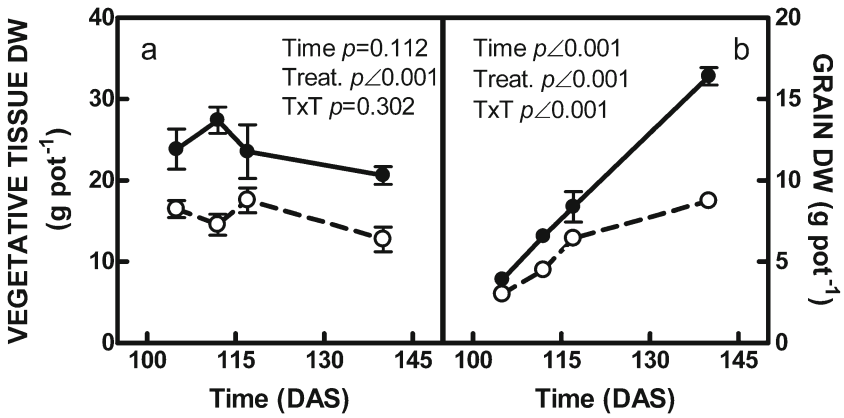

Fig. 1 Vegetative dry weight (**a**) and grain dry weight (**b**) of M+ (mycorrhizal, *closed circles*) and M− (non-mycorrhizal, *open circles*) barley plants during grain filling. Data are the means±SE (n=4). *Treat* Treatment, *TxT* Time×Treatment

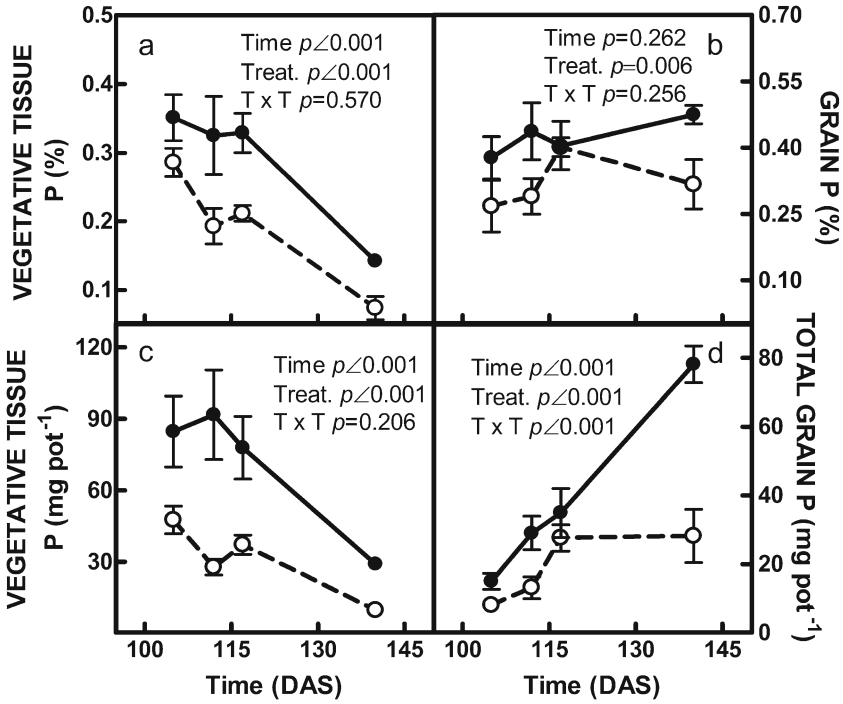

Fig. 2 Vegetative P concentration (**a**), grain P concentration (**b**), total vegetative P content (**c**), and total grain P content (**d**) of M+ (mycorrhizal, *closed circles*) and M− (non-mycorrhizal, *open circles*) barley plants during grain filling. Data are the means±SE (n=4). *Treat* Treatment, *TxT* Time×Treatment

responsiveness to AMF colonization, on the basis of whole plant P uptake and/or growth, and that many plant and environmental factors influence the magnitude of responses (Mohammad et al. 2003; Smith et al. 2004). In barley plants, while some authors found no differences between mycorrhizal and non-mycorrhizal barley plants (Fay et al. 1996 Jensen 1983), others reported that inoculation with *Glomus constrictus*, *Glomus fasciculatus* (Jensen 1982), or *Glomus mosseae* (Zaefarian et al. 2013) had a positive effect. In the present work, inoculation with *R. intraradices* produced a positive effect on barley biomass, as shown by the increase in vegetative and grain DW per pot. Since no difference was found in individual grain weight, grain size, or rate of sugar arriving to each spike between inoculation treatments, the

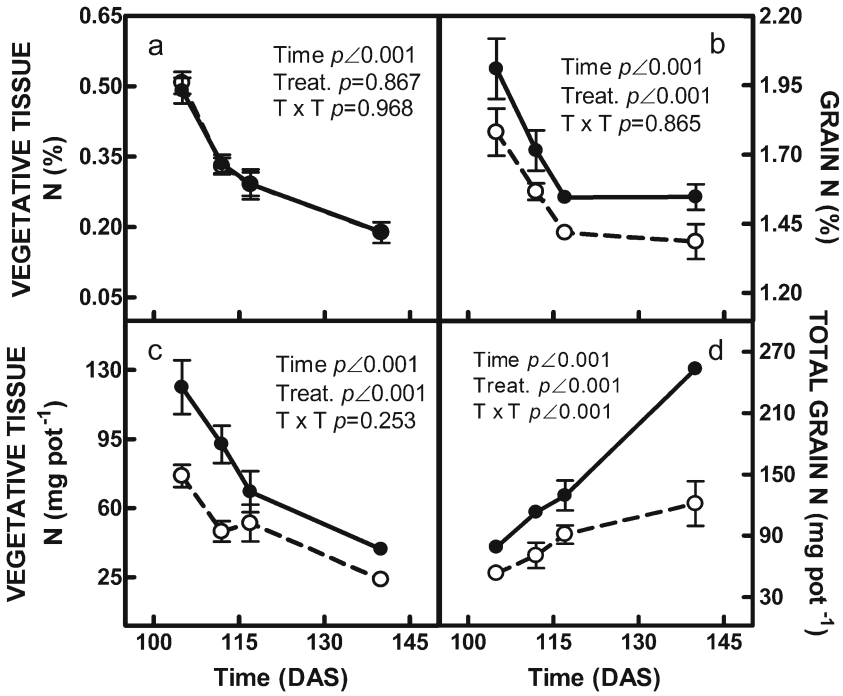

Fig. 3 Vegetative N concentration (**a**), grain N concentration (**b**), total vegetative N content (**c**), and total grain N content (**d**) of M+ (mycorrhizal, *closed circles*) and M− (non-mycorrhizal, *open circles*) barley plants during grain filling. Data are the means±SE (n=4). *Treat* Treatment, *TxT* Time×Treatment

Table 1 Grain yield, grain number per pot, spike number per pot, grain number per spike, individual grain weight grain size (fraction >2.5 mm), efficiency of N and P remobilization, and efficiency of N and P utilization of M+ and M− barley plants

	M+ plants	M− plants
Grain yield (g pot^{-1})	16.4±0.5**	8.7±1.5
Grain number (pot^{-1})	451.7±17.6**	257.9±32.7
Spike number (pot^{-1})	22.8±1.1*	17.2±1.1
Grain number ($spike^{-1}$)	20.0±0.5*	14.9±0.8
Individual grain weight (mg)	36.5±0.9	34.3±0.9
Grain size (fraction >2.5 mm) (%)	85.0±2.7	84.5±3.1
Efficiency of N remobilization (%)	63.3±1.9**	73.9±1.8
Efficiency of P remobilization (%)	57.5±5.5*	74.3±2.9
Efficiency of N utilization (%)	56.1±0.5*	62.6±0.8
Efficiency of P utilization (%)	153.6±1.88*	231.4±12.5

Data are the means±ES ($n=4$)

*$p<0.05$; **$p<0.01$ (significant difference of M+ plants compared to respective M− plants)

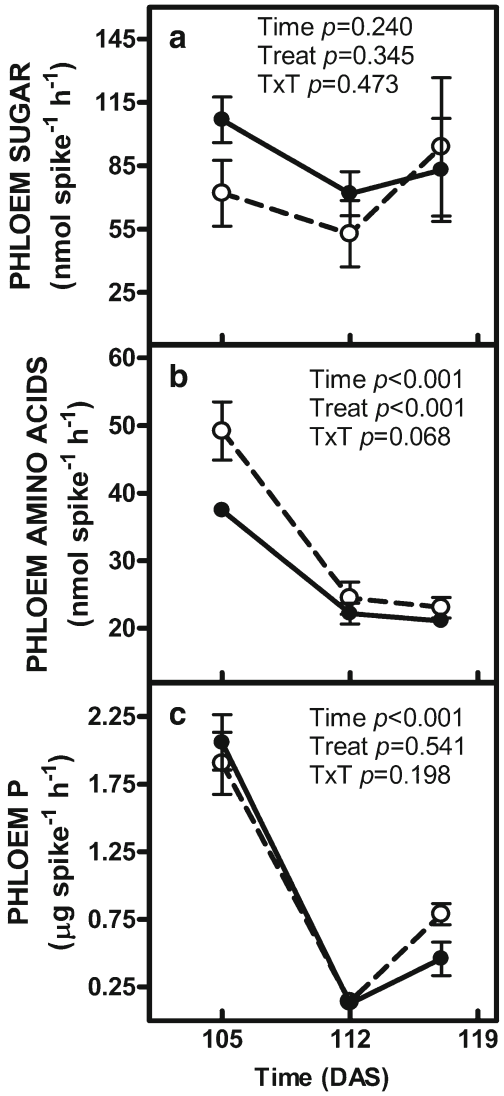

Fig. 4 Phloem exudation rate of sugars (**a**), amino acids (**b**), and P (**c**) obtained from four spikes of M+ (mycorrhizal, *closed circles*) and M− (non-mycorrhizal, *open circles*) barley plants during grain filling. Data are the means±SE ($n=4$). *Treat* Treatment, *TxT* Time×Treatment

difference in DW grain dynamics between M+ and M− plants could be attributed to a fewer number both of spikes per pot and of grains per spike in the M− plants.

As mentioned above, it is well documented that AMF can increase P uptake by host plants (Smith et al. 2004; Khaosaad et al. 2006; Bucher 2007). Accordingly, P concentration and content in vegetative tissues and developing grains were higher in the barley plants inoculated with *R. intraradices*. On the other hand, phloem P exudation rate of M+ plants did not differ from that of control plants, and in turn, the exudation rate decreased slowly in both treatments after anthesis. As a consequence, both P concentration and content in vegetative tissue decreased very slowly in both treatments as P remobilization progressed. This means that the rate of P remobilization does not vary in M+ plants and that the final rate of exudation is a consequence of a higher initial P concentration and content. Consequently, the present results indicate that P remobilization is not governed by P availability in vegetative tissues, and it may not be enhanced upon P deficiency, as was reported previously (Crafts-Brandner 1992; Snapp and Lynch 1996), but the remobilization process may be augmented over P absorption in the barley plants.

Increased N uptake caused by mycorrhization did not impact on vegetative tissue N concentration, but it did affect the number of spikes. On the other hand, vegetative tissue N concentration and content decreased during grain filling in both inoculation treatments as a consequence of senescence. During this phase, proteolytic enzymes hydrolyze leaf proteins stored in the leaves during vegetative growth, releasing amino acids (Roberts et al. 2011) that can be transported to the ears via the phloem. It has been observed that the efficiency of N remobilization is governed by the availability of N in the soil. That is, N remobilization efficiency decreases with the increase in N availability (Dalling 1985, Fatta et al. 2000, Ercoli et al. 2008). Furthermore, an increase in the phloem amino acid exudation rate at the beginning of the grain filling period has been observed in field-grown barley plants subject to N deficiency (Veliz et al. 2014). In this sense, the higher exudation rate of amino acids observed in M− barley plants at anthesis as compared to M+ plants suggests that *G. intraradices* is able to mitigate N− deficiency.

The difference between N content in vegetative tissues at anthesis and at physiological maturity was lower in M+ barley plants than in M− plants, as a consequence of a higher N content in the straw of M+ plants at the time of harvest. This greater residual N indicates a reduction in N remobilization efficiency, but given that the phloem exudation rate of amino acids was lower in M+ plant only at anthesis and after that it

was the same for both treatments, this diminution of N remobilization efficiency could not be assigned to a diminution in N remobilization during grain filling. Therefore, we postulate that the decrease in the P and N remobilization efficiency of M+ barley plants is associated with an increased capacity of M+ plants to absorb these nutrients that it are not accompanied by an increase in their remobilization capacity. However, although the presence of *G. intraradices* does not seem to be able to adjust P and N remobilization rates to the increased absorption capacity, the augmented yield and grain quality indicates a positive effect of the AMF.

With regard to the commercial quality of barley grains, inoculation with *G. intraradices* caused an increase in grain yield relative to control plants due to an increase in the number both of spikes per pot and of grains per spike. It has been observed that variations in the grain number of barley plants subjected to various levels of N or P availability are associated linearly with the biomass of spikes at anthesis (Prystupa et al. 2004). This suggests that the major effect of N deficiency on grain number is a reduced spike growth before anthesis. Inoculation of barley plants with *G. intraradices* caused no difference in grain weight or size compared to control plants, and both M+ and M− treatments were consistent with the requirements of the malting industry. In contrast, Prystupa et al. (2004) found in barley that individual grain weight was slightly reduced as grain number increased. The grain N concentration was always higher in M+ barley plants, and whereas M+ plants reached the grain N concentration required for the malting industry, M− plants were below those requirements probably due to the low N and P content in the soil used in this study. On the other hand, grain N concentration decreased during grain filling in both treatments. Whereas these results agree with some authors (Spiertz and Ellen 1978), others found that N concentration remained relatively constant throughout grain filling in wheat (Howarth et al. 2008; Roberts et al. 2011). It is interesting to note that grain N concentration decreased in the barley plants during grain filling, whereas grain P concentration did not vary. These results may reflect a differential regulation on grain filling for N and P in barley. It has been reported in wheat that the net dry matter deposition and P allocation to grains were not synchronous, indicating independent regulatory processes (Peng and Li 2005).

Improving N and P use efficiency has been considered essential in order to reduce the cost of fertilization in plant production and avoid environmental damage. This can be achieved by improving nutrient uptake and utilization efficiency (Vance 2001; Masclaux-Daubresse et al. 2008; Veneklaas et al. 2012). In the present study, mycorrhizal colonization increased N and P content in barley plants suggesting an improvement of nutrient uptake efficiency, but at the same time, more N and P remained in the straw at the time of harvest, indicating that mycorrhization can decrease utilization efficiency. In this sense, Baon et al. (1993) also reported that mycorrhizal colonization lowered the efficiency of P utilization in most of the assayed barley cultivars. In contrast, Hildermann et al. (2010) found no correlation between AMF symbiosis and nutrient uptake, so no difference in nutrient utilization efficiency parameters was reported. Increased N and P in the straw at harvest time may have different consequences. On the one hand, it could provide more N and P for the following crop when waste decomposes without risk of being lost by leaching during the period between crops (Adesemoye and Kloepper 2009), and on the other, it could mean a residue of better forage quality for grazing (Mehrvarz and Chaichi 2008). In the particular case of barley, a maximized partition of N to grains would be a desired goal because it is related to a more efficient use of the N absorbed (Hirel et al. 2007).

In relation to the use of a nutrient poor soil to perform the present experiment, in many developing countries, P fertilizer application is low because it is a relatively expensive input, and there is a clear need to develop farming systems that can maintain good productivity with limited P availability (Richardson et al. 2011). On the other hand, advantages in increased P uptake and growth of mycorrhizal compared to non-mycorrhizal plants at low P diminish with increasing soil P availability. There is often little mycorrhizal growth advantage at soil P levels adequate for maximum plant growth rates in intensive agricultural systems, and mycorrhizal fungi can even be considered to be parasitic on plants because the net cost of the symbiosis exceeds the net benefits (Bucher 2007). However, since this is a greenhouse study with imposed N and P deficiency conditions, field experiments are needed to do draw extensive conclusions on the value of *R. intraradices* inoculation.

In summary, the present results demonstrate that inoculation with *R. intraradices* in barley is beneficial because it not only increases grain yield and quality but it also decreases the efficiency of N and P utilization and remobilization. The phloem remobilization rate of amino acids and P did not decrease in M+ plants compared to M− ones during grain filling suggesting that nutrient utilization efficiency is most probably regulated by sink strength rather than by a mycorrhizal effect. Results also suggest a differential regulation on grain filling for N and P.

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