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ORIGINAL PAPER

Mycorrhizal colonisation and P-supplement effects on N uptake and N assimilation in perennial ryegrass under well-watered and drought-stressed conditions

Bok-Rye Lee · Sowbiya Muneer · Jean-Christophe Avice · Woo-Jin Jung · Tae-Hwan Kim

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Abstract To compare the effect of arbuscular mycorrhiza (AM) and P-supplement on N uptake and N assimilation under well-watered or drought-stressed conditions, *Glomus intraradices*-colonised, P-supplemented non-mycorrhizal (P) and non-mycorrhizal (control) plants of *Lolium perenne* were exposed to 12 days of water treatment. Leaf water potential (Ψ_w), photosynthetic ability, and N and P nutritional status were measured at the beginning (day 0) and end (day 12) of water treatment. N absorption, amino acid and protein synthesis were quantified using the isotopic tracer ^{15}N at day 12. Under well-watered conditions, growth response and physiological parameters were similar in AM and P plants, as compared to controls. Drought (10% water) significantly decreased these parameters in all three treatments. As compared to control plants, the negative impact of water deficit on the Ψ_w, photosynthesis, biomass, and N and P content was highly alleviated in AM plants, while only slightly improved or remained the same level in P plants. The effect of AM symbiosis on N absorption and N assimilation was greater than that of the P supplement under well-watered and drought-stressed conditions, and this effect was highly enhanced under drought-stressed conditions. At terminal drought stress on day 12, the effect of AM colonisation on de novo synthesis of amino acids and proteins was 4.4- and 4.8-fold higher than that of the P supplement. These results indicate that the AM symbiosis plays an integrative role in N nutrition by alleviating the negative impacts of drought on N or P uptake and N assimilation, whereas the efficiency of a direct P supplement is very limited under drought-stressed conditions.

Keywords Arbuscular mycorrhiza · *Lolium perenne* · Drought · N uptake · N assimilation · P nutrition

Introduction

Drought is considered as one of the most widespread agricultural problems limiting plant growth, productivity and survival. Root colonisation by arbuscular mycorrhizal (AM) fungi can increase the efficiency of nutrient absorption and, in turn, enhance growth of the host plant, particularly at low soil water potential (Subramanian and Charest 1998, 1999; Rains and Bledsoe 2007). For these reasons, eco-physiological studies have often suggested that the physical, nutritional, physiological and cellular effects of AM symbiosis during drought could be exploited as a strategy for increasing drought tolerance (Azcón et al. 1996;

B.-R. Lee · S. Muneer · T.-H. Kim (✉)
Environment-Friendly Agriculture Research Center (EFARC),
Department of Animal Science, Institute of Agricultural Science
and Technology, College of Agriculture & Life Science,
Chonnam National University,
Buk-Gwangju, P.O. Box 205, Gwangju 500-600, South Korea
e-mail: grassl@chonnam.ac.kr

J.-C. Avice
INRA, UMR, INRA–UCBN, Écophysiologie Végétale,
Agronomie et nutritions NCS, Institut de Biologie
Fondamentale et Appliquée, Université de Caen,
14032 Caen Cedex, France

W.-J. Jung
Environment-Friendly Agriculture Research Center (EFARC),
Division of Applied Bioscience and Biotechnology,
Institute of Agriculture Science Technology, College
of Agriculture & Life Science, Chonnam National University,
Gwangju 500-757, South Korea

Present Address:
B.-R. Lee
Department of Biochemistry and Molecular Biology,
College of Natural Science, Michigan State University,
East Lansing, MI 48824, USA

Porcel and Ruiz-Lozano 2004). Indeed, AM symbiosis may alter plant–water relations (Kothari et al. 1990; Khalvati et al. 2005; Maiquetía et al. 2009): higher transpiration rates have been found in mycorrhizal rangeland grass (*Bouteloua gracilis*) (Allen 1982), maize (Kothari et al. 1990) and barley (Khalvati et al. 2005) than in non-mycorrhizal controls.

While the importance of mycorrhiza for P nutrition of plants is firmly established, few studies have been carried out on the role of AM symbiosis in N nutrition, particularly under water-stressed conditions. It has been established that external hyphae take up ammonium (Tobar et al. 1994; Rains and Bledsoe 2007) and that the external mycelium plays a direct role in provision of NO_3^- to mycorrhizal roots, especially under drought conditions (Subramanian and Charest 1999). The increased N uptake may in turn lead to increased activities of key N-assimilating enzymes (Cliquet and Stewart 1993; Subramanian and Charest 1998) as well as protein and amino acid concentrations (Arines et al. 1993; Subramanian and Charest 1995). Increased nitrate reductase (NR) (Oliver et al. 1983; Azcón et al. 1996) and glutamine synthetase (GS) activities (Lea et al. 1990; Azcón et al. 1996; Subramanian and Charest 1999) in AM plants have been suggested to be associated with the enhanced P nutritional status. In addition, it is widely suggested that an improved drought tolerance of mycorrhizal plants (higher soil water uptake and more rapid recovery from water stress) is attributed to higher root hydraulic conductivity because of improved P nutrition (Graham et al. 1987; Kothari et al. 1990; Bryla and Duniway 1997; Maiquetía et al. 2009). However, comparisons between mycorrhizal and P-fertilised non-mycorrhizal plants of similar size and equal P status have shown no difference in root hydraulic conductivity (Andersen et al. 1988; Kothari et al. 1990), and Davies et al. (1993) reported that improvement of water status by AM fungi during drought was independent of host plant P status. Other studies have shown increased activities of NR (Ruiz-Lozano and Azcón 1996) and GS (Azcón and Tobar 1998) in AM plants regardless of the P content. Recently, Smith and Smith (2011) reviewed that P and N are delivered to roots via the mycorrhizal pathway regardless of plant growth responses (positive or negative), but the contribution to total N requirement and the costs to the plant are not clear. Taken together, P-mediated effects on physiological and metabolic responses in relation to water stress tolerance have not yet been conclusive.

In this study, we hypothesised that the effect of AM symbiosis on N, P uptake and N assimilation is higher than that of P supplement, especially under drought-stressed conditions. To test this hypothesis, direct quantification of NO_3^- uptake, amino acid and protein synthesis from newly absorbed NO_3^- via ^{15}N tracing was performed in mycorrhizal, non-mycorrhizal P-supplemented and non-mycorrhizal perennial ryegrass plants under well-watered and drought-stressed conditions. The responses of leaf water status, photosynthetic ability, and growth and nutrition parameters were also addressed.

Materials and methods

Experimental design and plant culture

The experiment consisted of three main treatments [*Glomus intraradices* infection (AM), P supplement in non-mycorrhizal plants (P), neither mycorrhizal infection nor P supplement (control)] and two sub-treatments [well-watering (WW) and drought stress (DS)]. The experiment was set up in a randomised bock design with three replicate pots per block.

Surface-sterilised seeds of perennial ryegrass (*Lolium perenne* L.) were sown in sterilised sand. Three uniform seedlings were transplanted after 15 days to each experimental pot filled with 1,000 g sterilised mix of quartz sand and soil (1:1, v/v). The experimental soil had a pH of 7.6, 1.9% organic matter, 0.09% total N, 4.0 μg NO_3^-–N g^{-1}, 1.2 μg NH_4^+–N g^{-1}, 37 μg P g^{-1} ($NaHCO_3$-extractable P) and 351 μg K g^{-1}. For mycorrhizal treatments, 5 g of mycorrhizal inoculum was placed directly below the seedling. The inoculum consisted of a mixture of soil, hyphae, spores and infected root fragments from a 6-month open pot culture of *Sorghum sudanense* S_{TAPF} inoculated with *Glomus intraradices* Schenck and Smith isolated by the Soil Microbiology Laboratory, Sunchon National University, Korea. Non-mycorrhizal treatments received the same amount of autoclaved inoculum. For the P-supplemented non-mycorrhizal plants, 10 ml KH_2PO_4 solution (48.3 g l^{-1}) per pot were supplied weekly to give a total P supplement of 110 μg P g^{-1} soil. This P application was selected from a previous experiment to match growth and P concentration of AM plants. Non-mycorrhizal plants (control and P) were watered daily to full capacity with 100 ml of a ready-made nutrient solution (Kim et al. 1991), whereas control and AM plants were supplied with the same volume of P-free nutrient solution (i.e. P sources were removed from the basal nutrient solution supplied to control plants) to ensure adequate nutrient supply in all treatments. The seedlings were grown in a greenhouse with a day/night mean temperature of 27/18°C and a relative humidity of 65/80%. Natural light was supplemented by metal halide lamps which generated approximately 400 μmol photons m^{-2} s^{-1} at the canopy height for 16 h per day.

Water-deficit imposition and $^{15}NO_3^-$ labelling

The pots of all treatments were watered regularly to field capacity until full vegetative stage (approximately 10 weeks after sowing). For each treatment, one half of the plants were subjected to drought stress by reducing the volume of irrigation water supplied per day. In preliminary experiments, changes in leaf water potential (Ψ_w) occurring in response to daily irrigation with different volumes of water were monitored. The resultant data indicated that predawn Ψ_w was abruptly reduced from the first 2 days when soil was irrigated with <10 ml per pot per day, while Ψ_w was maintained between −0.31 and −0.55 MPa for 15 days with 100 ml. For these reasons, 100 ml of daily irrigation per pot was supplied to the well-watered [non-mycorrhizal control (WW), mycorrhizal (AMWW) and non-mycorrhizal P-supplemented (PWW) plants] and 10 ml to drought-stressed [non-mycorrhizal control (DS), mycorrhizal (AMDS) and non-mycorrhizal P-supplemented (PDS) plants], respectively, with half-volume of the daily irrigation for each treatment being supplied at 10:00 h and the other half at 16:00 h.

For the ^{15}N feeding of the well-watered treatment, 50 ml of ^{15}N solution (1 mM K^{15}NO$_3$ with 10.0 at.% excess) were evenly administered via three porous plastic tubes placed on each pot at 10:00 h and 16:00 h, respectively (Kim et al. 2004). For the drought treatment, 5 ml of ^{15}N solution, containing the same percentage of excess ^{15}N atom and the same amount of N, was applied to the control pot. The $^{15}NO_3^-$ feeding was conducted every day throughout the entire 12-day experiment period.

Determination of mycorrhizal colonisation

The percentage of mycorrhizal root colonisation was estimated microscopically after clearing washed roots in 10% KOH and staining with 0.05% trypan blue following the method of Phillips and Hayman (1970). Quantification was performed using the grid-line intersect method (Giovannetti and Mosse 1980) and expressed as intensity of infection (percentage of root cortex with infection) or arbuscule frequency (percentage of root cortex with arbuscules).

Measurements and sampling

Leaf water potential (Ψ_w) was immediately evaluated as the xylem-pressure potential determined using a pressure chamber (PMS Instrument Co., Corvallis, OR, USA). The net photosynthetic rate was measured using a portable photosynthesis measurement system LI-6400 (LICOR, Lincoln, NE, USA) at 22°C on leaves under their ambient CO$_2$ atmospheric concentrations (approximately 400 ppm) at 1000 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by a LED light. It was measured in the youngest fully expanded leaf 4 h after the beginning of the photoperiod under greenhouse conditions. The first sampling for day 0 was conducted immediately prior to water stress treatment (the first ^{15}NO$_3$ feeding) at 10:00 h. An additional sampling was done after 12 days of water treatment. The harvested plants were separated into shoots and roots. All plant samples were frozen immediately in liquid nitrogen, and then freeze-dried, weighed, ground and stored in vacuum desiccators for further analysis.

Sample fractionation, isotope and chemical analysis

Freeze-dried powdered samples (1–5 mg) were weighed (±10 μg) into tin capsules for the determination of total N. For the fractionation of nitrogenous compounds, about 200 mg of finely ground freeze-dried samples was extracted twice with 100 mM sodium-phosphate buffer (pH 7.5) at 4°C. The proteins in the combined supernatant were precipitated with 80% (v/v) acetone and centrifuged for 10 min at 10,000×*g* at 4°C. The resultant pellets, which corresponded to the soluble protein fractions, were re-suspended in 0.5 ml of extraction buffer (Kim et al. 2004). NO_3^- and amino acids in the soluble fraction were separated further with a Dowex 50 H$^+$ column, as described by Kim et al. (2004). The aliquots obtained after protein precipitation were evaporated under vacuum at 4°C, precipitated with 95% ethanol at −20°C and centrifuged. The resultant supernatant was passed through a H$^+$ column (Dowex 50 W×8). The pH of solution collected from the H$^+$ column was adjusted to neutral pH, and this solution was concentrated to a final volume of 0.5 ml (NO_3^- fraction). The amino acids were eluted from the Dowex 50 W×8 column with 25 ml of 0.5 N HCl and concentrated to 1 ml. For the fractionated liquid samples, an appropriate sample volume (30–100 μl) was dropped into a tin capsule that was cooled with liquid nitrogen. The contents of the tin capsules were then freeze dried. The N content and ^{15}N at.% of all fractions was determined using N single mode analysis on a continuous flow isotope mass spectrometer (IsoPrime; GV Instrument, Manchester, UK) linked to a C/N analyser (EA 3000; EuroVector, Milan, Italy). The obtained ^{15}N abundance in the nitrate, amino acid and protein fractions was converted to relative specific activities (RSA, i.e. percentage of recently incorporated atoms relative to the total numbers of atoms) by Eq. 1 (Kim et al. 2004):

$$\mathrm{RSA} = \left({}^{15}\mathrm{N\ at.\%\ measured\ in\ a\ given\ compound} - \mathrm{natural}\ {}^{15}\mathrm{N\ at.\%}\right) / \left({}^{15}\mathrm{N\ at.\%\ \ of\ administered}\ {}^{15}\mathrm{NO}_3^- - \mathrm{natural}\ {}^{15}\mathrm{Nat.\%}\right) \times 100 \tag{1}$$

in which the natural ^{15}N at.% was adopted from the ^{15}N at.% of non-^{15}N-fed plants.

The amounts of newly absorbed N (NAN) incorporated into nitrate, amino acids and proteins from the onset of water treatment (day 0, when ^{15}N labelling began) were calculated by Eq. 2 (Kim et al. 2004; Lee et al. 2005):

$$\mathrm{NAN} = (\mathrm{RSA} \times \text{N content measured in a given N compound})/100 \qquad (2)$$

Total amount of N incorporated into reduced N compounds was determined by subtracting NAN in nitrate fraction from NAN in total N (Yoneyama and Takeba 1984). To evaluate AM or P-supplement effects in response to drought stress, the resulting data were normalised to the difference (Δ) between the values measured in the non-mycorrhizal control, mycorrhizal (AM) and P-supplemented non-mycorrhizal (P) plants under well-watered conditions and those of each treatment corresponding under drought-stressed conditions.

For total P determination, the samples were digested in $HClO_4$–H_2O_2 (v/v 7:3) mixture for 30 min in a sealed chamber under fume hood. The aliquots were filtered after solubilisation with 5 ml of 1 N HCl and adjusted to a final volume of 50 ml. The prepared sample solution reacted with P-colour reagent and incubated at 30°C for 30 min. The absorbance was measured at 470 nm. The specific absorption rate of P (SPAR) was defined as the amount of P absorbed per unit of root mass (Azcón et al. 2003) and calculated as follows: SARP=plant P content (mg)/root mass (g).

Statistical analysis and presentation of data

A randomised block design was used with three replicates for six treatments and two sampling dates. An individual pot containing three plants represented a replicate. Duncan's multiple range test was employed to compare the means of separate replicates. Unless otherwise stated, conclusions are based on differences between means significant at $P \leq 0.05$. The correlative analysis was also performed. All statistical measures were performed using SAS 9.1 (SAS Institute Inc., 2002–2003).

Results

Mycorrhizal colonisation, leaf water potential, growth and nutritional status

Root colonisation by *G. intraradices* was 55.8% at the beginning of water treatment (Table 1). Compared to the initial level, mycorrhizal colonisation and arbuscular frequency decreased by 23.7% and 28.4%, respectively, under drought-stressed conditions, while they were unchanged under well-watered conditions. At the beginning of water treatment (day 0), leaf water potential (Ψ_w) and photosynthesis rate were not significantly different for the three treatments. However, biomass, N and P content in AM and P plants were higher than in non-AM (control) plants, without any difference between AM and P treatment (Table 1). At the end of water treatment (day 12), under well-watered (WW) conditions, photosynthesis rate, biomass and P content in AM and P plants were significantly higher than in control plants whereas Ψ_w and N content were not affected by the treatments (Table 1). Under drought-stressed (DS) conditions, the Ψ_w in control and P plants decreased sharply below −2.10 MPa, whereas the value in AM plants declined less to −1.24 MPa (Table 1). The photosynthetic activity was decreased by 93.8% and 85.2% in control and PS plants, respectively, but only by 47% in AM plants under DS conditions compared to corresponding well-watered plants at day 12. Drought stress also significantly decreased biomass, N and P content in all three treatments compared to the well-watered plants (Table 1). The decrease in growth, N and P content by drought was consistently less pronounced in AM than in control or P plants. After 12 days of drought treatment, AM plants had significantly higher biomass, N and P content in shoots and roots than non-AM plants (control and P). However, P supply alone was beneficial to the plants because P plants had higher biomass, N and P content than control plants (except for biomass and N content in shoots).

N and P absorption

To better understand the role of mycorrhiza in N and P nutrition under drought stress, we determined the total amount of newly absorbed N from the onset of water treatment (day 0), as calculated by the ^{15}N content of whole plant, and the specific P absorption rate (SPAR), defined as the amount of P absorbed per unit of root mass (Fig. 1). Under well-watered conditions, the amount of N absorbed in AM plants increased by 25.5% compared to the control, but by only 6.0% in P plants (Fig. 1a). Drought decreased N absorption by 34.0% and 34.3% in control and P plants but only by 22.8% in AM plants. The SPAR in AM and P plants, under well-watered conditions, increased by 43.8% and 55.1%, respectively, compared to control plants (Fig. 1b). Drought decreased the SPAR by 11.4% and 33.8%, respectively, in the control and P plants, while no change occurred in AM plants.

Partitioning of the newly absorbed nitrogen

To investigate the effects of AM symbiosis and P supplement on the fate of newly absorbed N, the amount of $^{15}NO_3^-$-derived N in the nitrate, amino acid and protein fractions in shoot and root tissues was quantified after 12 days of $^{15}NO_3^-$ feeding (Fig. 2). Under well-watered

Table 1 Mycorrhizal colonisation, leaf water potential and photosynthesis rate in non-mycorrhizal control, mycorrhizal (AM) and P-supplemented non-mycorrhizal (P) perennial ryegrass under well-watered (WW) or drought-stressed (DS) conditions at the beginning (day 0) and the end (day 12) of water treatment

Measurements/treatment		Day 0			Day 12		
		Control	AM	P	Control	AM	P
Mycorrhizal colonisation (%)							
WW		–	55.8 a (8.1)[a]	–	–	52.4 a (8.5)	–
DS		–	–	–	–	42.6 b (5.8)	–
Leaf water potential (MPa)							
WW		−0.48 a	−0.41a	−0.40 a	−0.45 a	−0.39 a	−0.41 a
DS		–	–	–	−2.31 c	−1.26 b	−2.10 c
Photosynthesis rate (μmol CO_2 m^{-2} s^{-1})							
WW		8.26 c	8.78 bc	8.67 c	9.90 b	11.57 a	11.84 a
DS		–	–	–	0.61 f	6.08 d	1.80 e
Biomass (g DW $plant^{-1}$)							
Shoot	WW	2.38 d	2.77 c	2.64 c	3.06 b	3.47 a	3.31 a
	DS	–	–	–	1.93 e	2.72 c	2.04 e
Root	WW	0.98 c	1.15 ab	1.11 b	1.11 b	1.28 a	1.26 a
	DS	–	–	–	0.89 d	1.03 bc	0.98 c
N content (mg N $plant^{-1}$)							
Shoot	WW	60.2 c	65.5 b	64.8 b	74.4 a	72.0 a	73.9 a
	DS	–	–	–	50.0 def	54.9 d	52.3 de
Root	WW	9.9 c	11.2 b	11.6 b	13.1 ab	14.0 a	13.8 a
	DS	–	–	–	6.7 a	10.9 bc	8.8 bc
P content (mg N $plant^{-1}$)							
Shoot	WW	2.41 e	3.86 c	4.05 bc	2.62 e	4.52 b	5.16 a
	DS	–	–	–	1.75 f	3.25 d	2.77 e
Root	WW	0.85 e	1.18 c	1.21 c	1.04 cd	1.52 ab	1.69 a
	DS	–	–	–	0.84 e	1.43 b	1.08 cd

Values are the means of three replicates with three plants each. Values in a vertical column or a horizontal row followed by different letters at each sampling date are significantly different at $P \leq 0.05$ according to Duncan's multiple range test

[a] The value in parentheses is arbuscular frequency

conditions, the amount of newly absorbed N (NAN) in the nitrate pool (^{15}N-nitrate) of shoots was increased by AM symbiosis (+31%) or the P supplement (+38.1%) compared to control plants (Fig. 2a), but no significant changes occurred in roots (Fig. 2b). Drought did not affect the size of the ^{15}N-nitrate pool in control plants, but ^{15}N-nitrate increased by 13% and 10.3% in shoots and roots of AM plants, respectively, and decreased by 14.5% and 14.9% in shoots and roots of P plants.

The amount of newly absorbed N incorporated into amino acids (^{15}N-amino acids), under the well-watered condition, was increased by the AM symbiosis by 8.6% and 19.3%, respectively, in shoots and roots, while it was not significantly affected by the P supplement when compared to control plants (Fig. 2c, d). Drought treatment significantly decreased the ^{15}N-amino acid in both shoots and roots for all three treatments. The ^{15}N-amino acid in P plants decreased by 32.6% and 33.2% in shoots and roots, respectively, while only by 22.4% and 20.3% in AM drought-stressed plants compared to well-watered plants. Comparing the two non-AM (control and P) treatments under drought stress, a slight increase (+3.1%) in ^{15}N-amino acids of P plants was observed only in shoots.

The amounts of newly absorbed N incorporated into proteins (^{15}N-protein), under the well-watered condition, was significantly increased in both shoots (+34.3%) and roots (+14.3%) by AM colonisation, but only in shoots (+9.6%) by the P supplement when compared to control plants (Fig. 2e, f). Drought decreased the ^{15}N-protein fraction in all three treatments. The decrease in ^{15}N-protein was less pronounced in AM plants (−29.4% and −28.7% in shoots and roots) than in the control (−40.8% and −43.0%) or P plants (−40.7% and −41.1%).

Mycorrhizal symbiosis and P-supplement-dependent changes in N and P uptake and N assimilation

The effects of AM infection or P supplement on N and P absorption and the amount of N incorporated into amino acids, proteins and reduced N compounds were compared by calculating the percentage of difference between the values measured in AM or P plants and those of control

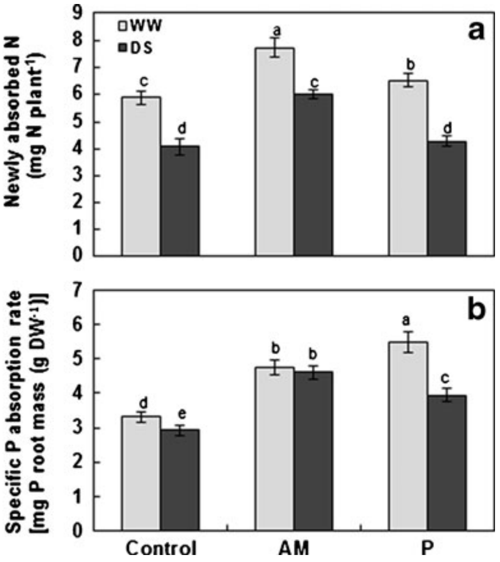

Fig. 1 Total amount of newly absorbed N (NAN, **a**) and specific P absorption rate (SPAR, **b**) in non-mycorrhizal control, mycorrhizal (AM) and P-supplemented non-mycorrhizal (P) perennial ryegrass under well-watered (*WW*, *grey*) or drought-stressed (*DS*, *black*) conditions at the end of water treatment on day 12. Values are means ± SE. Values not sharing same letter for each treatment in common differ significantly ($P \leq 0.05$) according to Duncan's multiple range test

plants under well-watered or drought-stressed conditions (Table 2). In general, the effects of AM symbiosis and the P supplement were positive for all measurements at both water regimes. The effect of AM symbiosis on N absorption, defined as total amount of newly absorbed N, was greater than that of P supplement under both water regimes. The beneficial effect of AM colonisation on N absorption was highly enhanced under the drought-stressed condition, and the small positive effect of the P supplement under well-watered conditions was lost under drought stress. The effect of a P supplement on SPAR was significantly higher than that of AM colonisation under the well-watered condition, whereas it was lower in the drought treatment. AM symbiosis also had a stronger positive effect on the amount of N incorporated into amino acids, proteins and reduced N than the P supplement under both water regimes (Table 2). The effectiveness of the AM symbiosis for increasing N assimilation was greatly enhanced under the drought-stressed condition. At the end of the drought stress treatment, on day 12, the effects of AM colonisation on the amount of N incorporated into amino acids, proteins and reduced N compounds were 5.6-, 4.6- and 5.4-fold higher than those of the P supplement.

Fig. 2 The amount of newly absorbed N (NAN) in the nitrate, amino acid and protein fraction in the shoots (**a**, **c**, **e**) and roots (**b**, **d**, **f**) of non-mycorrhizal control, mycorrhizal (AM) and P-supplemented non-mycorrhizal (P) perennial ryegrass under well-watered (*WW*, *grey*) or drought-stressed (*DS*, *black*) conditions at the end of water treatment on day 12. Values are means ± SE. Values not sharing same letter for each treatment in common differ significantly ($P \leq 0.05$) according to Duncan's multiple range test

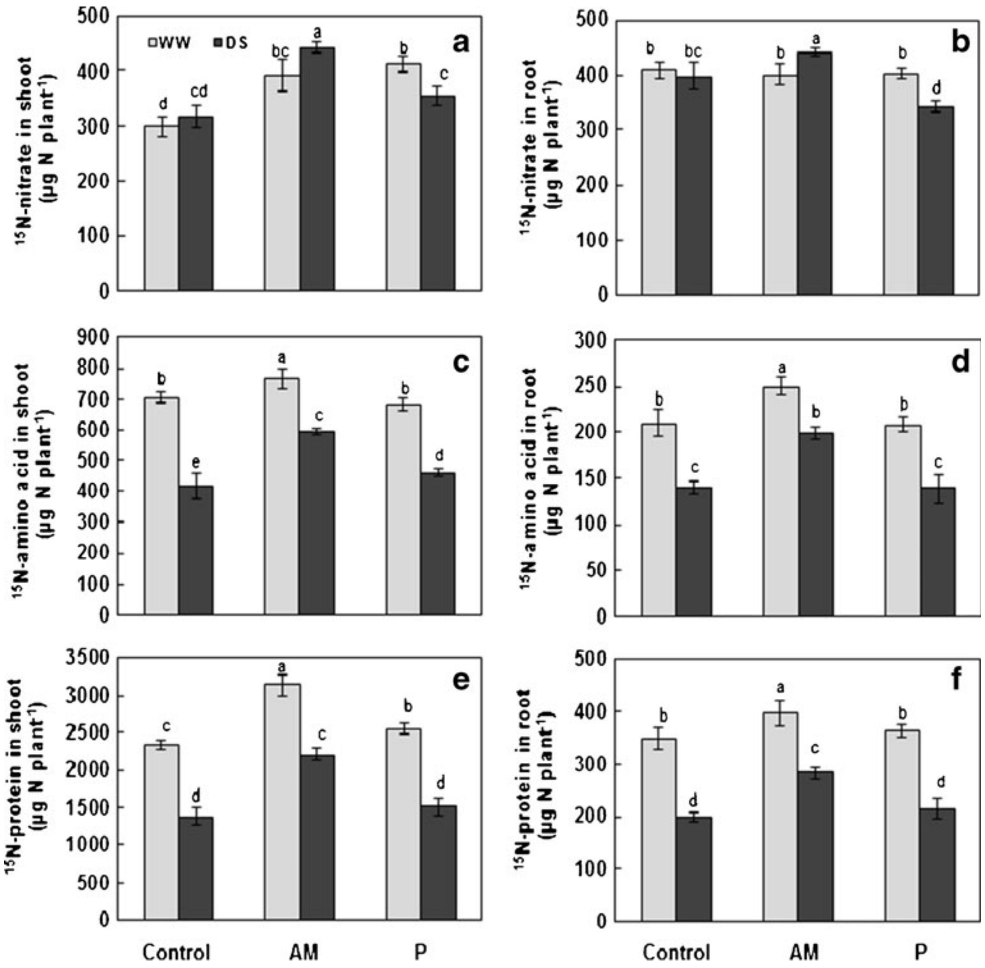

Table 2 Mycorrhizal colonisation or P-supplement effects on N, P absorption and N assimilation under well-watered or drought-stressed conditions at the end of water treatment

Measurements	Well-watered	Drought-stressed
Total amount of newly absorbed N		
Mycorrhizal effect	+25.5% b	+43.4% a
P-supplement effect	+6.0% c	ns[a]
Specific P absorption rate		
Mycorrhizal effect	+43.8% c	+57.6% ab
P-supplement effect	+55.5% a	+36.3% d
Amount of N incorporated into amino acids		
Mycorrhizal effect	+11.0% b	+42.8% a
P-supplement effect	ns	+7.7% c
Amount of N incorporated into proteins		
Mycorrhizal effect	+32.2% b	+47.9% a
P-supplement effect	+9.4% c	ns
Total amount of N incorporated into reduced N compounds		
Mycorrhizal effect	+26.7% b	+44.9% a
P-supplement effect	+6.3% d	+8.3% c

Data are expressed as percentage of values observed in control plants. Values are the means of three replicates with three plants each. Values in a vertical column or a horizontal row followed by different letters are significantly different at $P \leq 0.05$ according to Duncan's multiple range test

[a] *ns* no significant difference compared to the control

To examine the response of AM, P and control plants to drought treatment, the Ψ_w, N absorption, SPAR and total amount of N incorporated into reduced N compounds from newly absorbed N in whole plant level were compared in a scatter plot (Fig. 3). The data in Fig. 3 were normalised by taking the difference (Δ) between the values measured in the control, AM and P plants under well-watered condition and those of each corresponding treatment under the drought-stressed condition. The decrease in the Ψ_w, N absorption, SPAR and total amount of N incorporated into reduced N compounds from newly absorbed N (designated as total reduced N) by drought stress was less negative in AM plants than in non-AM (control and P) plants. Comparing the two non-AM (control and P) treatments, $\Delta\Psi_w$, ΔN absorption and Δ total reduced N in P plants were similar or slightly higher than those of control plants, whereas the ΔSPAR was notably lower. It was found that $\Delta\Psi_w$ was significantly associated with ΔN absorption (r^2=0.904, $P \leq 0.05$, Fig. 3a) and Δ total reduced N (r^2=0.929, $P \leq 0.05$, Fig. 3b), respectively. A significant relationship between Δ total reduced N and ΔN absorption (r^2=0.958, $P \leq 0.05$, Fig. 3c) was also observed, but not with ΔSPAR (r^2= 0.447, $P > 0.05$, Fig. 3d). These data indicate that the AM symbiosis provides significant benefit to plant N nutrition

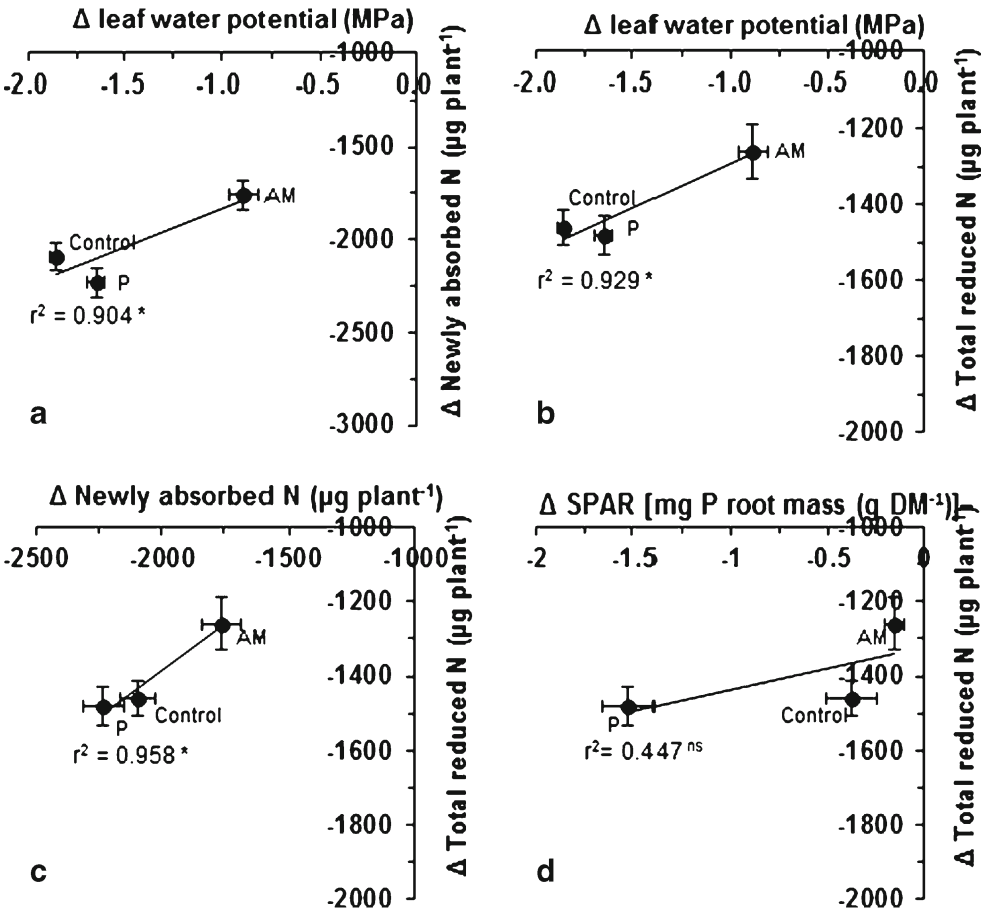

Fig. 3 Scatter plot of total amount of newly absorbed N (NAN, **a**) and total amount of reduced N (**b**) in relation to leaf water potential, and scatter plot of NAN (**c**) and specific P absorption rate (SPAR, **d**) in relation to total amount of reduced N. Values are normalised to the difference (Δ) between the values measured in the non-mycorrhizal control, mycorrhizal (AM) and P-supplemented non-mycorrhizal (P) plants under well-watered condition and those of each treatment corresponding under drought-stressed condition. Values are means ± SE. Significance levels of the liner correlation coefficients were denoted by $^*P<0.05$. *ns* no significant difference

particularly during drought stress and that this effect is much higher than that caused by improved P nutrition alone.

Discussion

As revealed by Ψ_w values, drought stress occurred after 12 days of water treatment. The Ψ_w in drought-stressed plants significantly decreased, while it was maintained at the initial level (day 0) under well-watered conditions. The minimum value of −2.31 MPa in drought-stressed control plants is similar to that recorded (−2.23 MPa) in white clover exposed to 9 days of drying (Singh et al. 2000). Mycorrhizal colonisation and arbuscule frequency *G. intraradices* under well-watered conditions were 23% and 46.5% higher than those under drought-stressed conditions. These results are in agreement with previous findings that water stress significantly decreased mycorrhizal colonisation by *G. clarum* in water melon (Kaya et al. 2003) and by *G. versiforme* in citrus (Wu and Xia 2006), suggesting a negative effect of water stress on mycorrhizal development in host plants (Morte et al. 2000). However, root colonisation has sometimes been observed to increase rather than decrease under water-stressed conditions (Augé 2001).

In the present study, a negative effect of drought stress on Ψ_w and photosynthetic ability was alleviated to a much greater extent in AM plants than in non-AM (P and control) ones. The less negative Ψ_w values in AM plants may be due to an increased water uptake by hyphae or an improved root conductance to water flow (Morte et al. 2000; Khalvati et al. 2005). Increased photosynthetic activity has been widely found in mycorrhizal plants exposed to drought stress (Subramanian and Charest 1998; Sanchez-Blanco et al. 2004; Khalvati et al. 2005; Wu and Xia 2006). Higher photosynthetic rates result in higher concentration of soluble sugars and other photosynthetic metabolites in the leaf symplasm, which can lead to an increased cytoplast osmolality in AM plants compared to non-AM plants (Augé 2001; Porcel and Ruiz-Lozano 2004; Khalvati et al. 2005). Such mycorrhizal effects appear to be the results of additive mechanisms involving nutrient acquisition and physiological processes (Subramanian and Charest 1999; Mathur and Vyas 2000).

Consistent with our results, it has been widely suggested that AM improve water relationship and growth of plants exposed to water-limited or osmotic stress (Subramanian and Charest 1998; Tobar et al. 1994; Morte et al. 2000; Sharifi et al. 2007). The improved water-stress tolerance, which is evidenced by a higher root hydraulic conductivity and more rapid recovery from water stress, has often been attributed to improved P nutrition (Graham et al. 1987; Kothari et al. 1990; Bryla and Duniway 1997; Maiquetía et al. 2009). Increased N uptake (Tobar et al. 1994; Subramanian and Charest 1999) and N assimilatory enzymatic activities such as NR (Oliver et al. 1983; Azcón et al. 1996) and GS (Lea et al. 1990; Azcón et al. 1996; Subramanian and Charest 1999) in AM plants has been also suggested to be associated with the enhanced P nutritional status. These trends led us to question whether increased P supply could match AM effects on N acquisition and its further assimilation under drought-stressed conditions. The significant higher biomass, N and P contents of P-supplemented plants as compared to controls at the beginning of water treatment (day 0), together with the lack of differences between AM and P plants under well-watered conditions, show that the level of P supplementation used in this study was appropriate to match the effects of AM. However, most of the parameters in AM plants increased as compared to P-supplemented plants at the end of the drought treatment (day 12).

Many interacting mechanisms may be involved in these phenomena and the effect of AM symbiosis on N metabolism may be indirect. The present data suggest an additional AM effect which is especially evident in the absorption of $^{15}NO_3^-$-derived N and its assimilation into amino acids and proteins. Under drought conditions, the effects of the AM symbiosis (defined as percent increases compared to control plants) on N absorption, amino acid and protein synthesis were considerably higher (42.8–47.9% of increase) than those in P plants (7.6–8.3%). In this way, SPAR and total P content of AM plants was increased by only 16.7% and 21.6%, respectively, compared to P-supplemented plants, whereas N absorption and its assimilation to amino acid and protein increased more than 40%. From these data, we deduce that the AM contribution to N uptake and its assimilation is additional to the effects of improved P nutrition, and that other as yet unknown effects of the AM symbiosis are important for improving N nutrition under drought-stressed conditions. In this context, additional AM effects on the activity of the N assimilatory enzymes NR and GS, compared to direct P effects derived by P fertilisation, have been previously reported (Azcón et al. 1996; Azcón and Tobar 1998).

The data of N absorption and SPAR confirm the well-established effect of AM symbiosis increasing acquisition of poorly diffusing nutrients under water-stressed conditions (Tobar et al. 1994; Subramanian and Charest 1998; Morte et al. 2000; Sharifi et al. 2007). However, the effect of the P supplement on improving N absorption was negligible (e.g. non-significant difference between P and control plants). Given that the additional contribution of AM compared to the P supplement (i.e. percentage of difference between AM and P plants) to N absorption (+39.3%) was much higher than that of SPAR (+16.7%), the improved N uptake can be mainly attributed to additional AM effects. This presumably results from a direct role of the AM fungal external mycelium in transport of NO_3^- to roots (Tobar et al. 1994;

Subramanian and Charest 1999) and in enhancement of hydraulic conductivity to water flow (Kothari et al. 1990; Khavati et al. 2005).

In previous studies with white clover, we demonstrated that the decreased N uptake under drought-stressed conditions reflects water status in soil and plant tissues and it in turn restricts de novo synthesis of amino acids and proteins (Kim et al. 2004; Lee et al. 2005). In the present work on ryegrass, quantification of the amount of N incorporated into amino acids and proteins clearly showed that the AM symbiosis alleviates the negative effect of drought on de novo synthesis of amino acids and proteins, whereas the effect of P supplement is negligible or low. Our data, in conjunction with others, suggest that AM infection assisted the host plant to maintain higher protein concentration regardless of moisture regime (Tobar et al. 1994; Subramanian and Charest 1999; Morte et al. 2000). Although improved N assimilation in AM plants is considered a consequence of increased N uptake via the external mycelium (Tobar et al. 1994; Subramanian and Charest 1999) and/or the enhanced activity of N assimilative enzymes in the host plants (Ruiz-Lozano and Azcón 1996; Subramanian and Charest 1998), it is still not clear whether the enhanced enzyme activity results from an AM-related improved host plant P status (Oliver et al. 1983; Lea et al. 1990; Subramanian and Charest 1999) or is independent of P nutrition (Ruiz-Lozano and Azcón 1996; Azcón and Tobar 1998). In the present study, a significant relationship ($r^2=0.904$, $P\leq 0.05$) between $\Delta\Psi_\mathrm{w}$ and ΔN absorption, caused by the drought treatment, was observed. It was also found that Δ total reduced N is closely related with $\Delta\Psi_\mathrm{w}$ ($r^2=0.929$, $P\leq 0.05$) and ΔN absorption ($r^2=0.958$, $P\leq 0.05$), but has a much lower correlation with ΔSPAR ($r^2=0.447$, $P>0.05$). Taken together, these results suggest that the AM symbiosis contributes directly to the metabolic and/or nutritional status of N in host plants exposed to drought stress by improving water relationship and N uptake which consequently elevate de novo synthesis of amino acids and proteins. This effect, which is largely independent of the symbiosis-related improved P supply, may be a key factor that enables the plants to withstand drought conditions.

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