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REGULAR ARTICLE

Arbuscular mycorrhizal symbiosis alleviates drought stress imposed on *Knautia arvensis* plants in serpentine soil

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Abstract
Background and Aims Plants growing on serpentine bedrock have to cope with the unique soil chemistry and often also low water-holding capacity. As plant-soil interactions are substantially modified by arbuscular mycorrhizal (AM) symbiosis, we hypothesise that drought tolerance of serpentine plants is enhanced by AM fungi (AMF).
Methods We conducted a pot experiment combining four levels of drought stress and three AMF inoculation treatments, using serpentine *Knautia arvensis* (Dipsacaceae) plants as a model.
Results AMF inoculation improved plant growth and increased phosphorus uptake. The diminishing water supply caused a gradual decrease in plant growth, accompanied by increasing concentrations of drought stress markers (proline, abscisic acid) in root tissues. Mycorrhizal growth dependence and phosphorus uptake benefit increased with drought intensity, and the alleviating effect of AMF on plant drought stress was also indicated by lower proline accumulation.
Conclusions We documented the role of AM symbiosis in plant drought tolerance under serpentine conditions. However, the potential of AMF to alleviate drought stress was limited beyond a certain threshold, as indicated by a steep decline in mycorrhizal growth dependence and phosphorus uptake benefit and a concomitant rise in proline concentrations in the roots of mycorrhizal plants at the highest drought intensity.

Keywords Abscisic acid · Arbuscular mycorrhizal fungi · Drought stress marker · Phosphorus nutrition · Proline · Water stress

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Introduction

Serpentine soils are scattered worldwide and cover almost 1 % of the total land surface (Coleman and Jove 1992). Plants inhabiting serpentine habitats are exposed to multiple abiotic stresses, including adverse soil chemistry (low calcium to magnesium ratio, elevated concentrations of heavy metals, nutrient deficiencies) and unfavourable physical conditions (coarse texture, shallow soil profile, low organic

matter content). The physical state of serpentine soils often results in their low water-holding capacity and drought stress being exerted on plants (for reviews, see Brady et al. 2005; O'Dell and Rajakaruna 2011).

Common morphological features of serpentine plants that help them to tolerate the adverse soil water conditions include xeromorphic leaves with lower specific leaf area, reduced stature and higher root: shoot ratios (Brady et al. 2005; Anacker et al. 2011). In addition, comparisons of plants from serpentine habitats with different water-holding capacities (Rajakaruna et al. 2003), as well as from serpentine and non-serpentine populations of the same species (e.g., Hughes et al. 2001; Sambatti and Rice 2007; Wu et al. 2010), have revealed other adaptive mechanisms used by serpentine plants to respond to water stress. For example, Sambatti and Rice (2007) report that serpentine populations invest more resources in reproduction than their conspecifics, reflecting a strategy of maximising reproduction within the short period when water availability is not a limiting factor. The reproduction cycle of serpentine plants is also reported to be less impaired by water limitation, as indicated by a low reduction in their seed set relative to well-watered conditions (Wu et al. 2010). Hughes et al. (2001) even conclude that drought is the driving force behind the evolution of serpentine tolerance in the case of the *Mimulus guttatus* complex (Phrymaceae).

One of the mechanisms of plant adaptation to serpentine soils may be the symbiosis with arbuscular mycorrhizal fungi (AMF). As the obligate root symbionts of most vascular plants (Smith and Read 2008), AMF are not only important mediators of host plant interactions with their soil environment, but also contribute to the alleviation of various abiotic stresses, including drought (for review, see Entry et al. 2002). The presence and role of AMF in serpentine soils have recently received increased attention (e.g., Fitzsimons and Miller 2010; Ji et al. 2010; Lagrange et al. 2011; Davoodian et al. 2012; Doubková et al. 2012). The importance of AMF for plant growth and phosphorus nutrition under serpentine conditions has been documented but the potential of AMF to alleviate drought stress imposed on serpentine plants has not been specifically addressed.

In general, the ability of mycorrhizal plants to tolerate water deficit more effectively is based on both direct and indirect mechanisms; water uptake and transport by fungal mycelium, as well as the nutritional and physiological effects of mycorrhization have all been reported (Augé 2001, 2004). Due to their small diameter (2–20 μm), fungal hyphae have access to soil pores inaccessible to plant roots and root hairs, resulting in more efficient water extraction by mycorrhizal than non-mycorrhizal plants (Smith et al. 2010). The nutritional benefits of mycorrhization lie mainly in the enhanced uptake of P and other immobile nutrients, and subsequent promotion of plant growth (Al-Karaki et al. 2004; Subramanian et al. 2006). Physiological effects include modifications of foliar water relationship parameters (Wu et al. 2007b), alterations in root-to-shoot signalling (Duan et al. 1996) and enhanced activity of antioxidative enzymes (Caravaca et al. 2005; Wu et al. 2007b).

Considering the low water-holding capacity of serpentine soils, we hypothesised that AMF contribute to the alleviation of host plant drought stress under serpentine conditions, both in direct and indirect ways. We predicted that: 1) plant benefit from AM symbiosis in terms of growth and water use efficiency (*WUE*) will increase with increasing intensity of drought stress; 2) a limited water supply will increase the role of AMF in plant uptake of immobile nutrients (particularly phosphorus); 3) at the non-nutritional level, the alleviating effect of AM symbiosis on plant drought stress will be reflected in changes in the concentrations of drought stress markers (proline and abscisic acid) in plant tissues; and 4) plants will benefit more from inoculation with a native AMF community than from inoculation with a single native fungal isolate, due to an assumed functional diversity and complementarities of different AMF. To address these hypotheses, we conducted a pot experiment that combined different water regimes and AMF inoculation treatments, using serpentine *Knautia arvensis* (Dipsacaceae) plants as a model.

Material and methods

Plant material

Field scabious, *Knautia arvensis* (L.) J. M. Coult. (Dipsacaceae), is a perennial herb commonly colonised by AMF under natural conditions (Doubková et al. 2011). The species is native to Europe and west Asia, with a secondary distribution in western North America and the Far East (Štěpánek 1997). In Central

Europe, *K. arvensis* encompasses both serpentine and non-serpentine populations (Kolář et al. 2009). For the present study, we selected a serpentine population inhabiting the ecotone community at the margin of an open pine forest (Pluhův Bor, W. Bohemia, Czech Republic; 50°03'01.3" N, 12°46'24.3" E; 710 m a.s.l.; referred to as S3 in Doubková et al. 2011) and experiencing considerable drought stress during summer periods (volumetric soil moisture in the rhizosphere of *K. arvensis* plants ranging from 3.5 to 12.3 % (n=20), according to the field measurements conducted in August 2009; Theta Probe ML2x, Moisture Meter HH2, Delta-T Devices Ltd., UK). Mature *K. arvensis* achenes were collected in August 2009 from ca 50 plants (approx. 10 achenes per plant). The achenes were surface-sterilised (5 % NaClO, 10 min) and germinated in Petri dishes, with the emerged seedlings then grown in multi-pots filled with a γ-sterilised (γ-radiation dose of 25 kGy) mixture of the native serpentine soil and sand (1:2, v/v). After 4 weeks, even-sized seedlings were planted into the experiment.

AMF involved in the study

The experiment involved native AMF originating from the site of plant origin, presuming their adaptation to serpentine conditions, including long-term drought stress. Both the isolate *Glomus* sp. (EMBL database, accession number HE794038) and the complex AMF inoculum were obtained from the rhizosphere soil of *K. arvensis* plants.

Experimental design

The plants were grown in the pots (11 cm in diameter) filled with 300 g of the substrate native to the plant collection site. The substrate was excavated from an area of 30 m^2 having a frequent occurrence of *K. arvensis* plants, to a depth of approx. 30 cm. The entire volume of substrate was then thoroughly mixed, passed through a 5-mm sieve and γ-sterilised (25 kGy). The chemical characteristics of the sterilised substrate, together with details on the analytical methods, are provided in Table 1. The substrate showed typical characteristics of serpentine soils, i.e., low Ca/Mg ratio (0.176±0.002) and elevated concentrations of heavy metals (especially Ni and Co).

The experimental design involved twelve treatments (each containing six replicates), resulting from the combination of four water regimes and three inoculation treatments. Water regimes were defined as a percentage of field capacity (FC) of the cultivation substrate which was initially determined gravimetrically in the laboratory. Plants were assigned to one of four water regimes corresponding to 55, 45, 35 and 25 % FC. These regimes were selected based on preliminary experiments testing a wider moisture range (60–5 % FC) in order to determine a relationship between FC and volumetric soil moisture. The selected range of 25–55 % FC corresponded approximately to the range of field soil moisture values (see above). The various irrigation regimes were initiated six weeks after planting to allow successful establishment of AM symbiosis at 100 % FC. The water regimes were maintained gravimetrically by daily irrigation. The three inoculation treatments involved: (i) non-inoculated plants (referred to as *nm*, non-mycorrhizal) as a control treatment; (ii) plants inoculated with the single serpentine isolate *Glomus* sp., referred to as *SI*; and (iii) plants inoculated with the complex serpentine AMF community, referred to as *COM*.

All inoculated plants were treated with 10 ml of a suspension containing colonised root segments, extraradical mycelium and spores. Both inocula were prepared by wet sieving (Gerdemann and Nicolson 1962), either from a mature maize culture with high mycorrhizal root colonisation (>90 %) and abundant sporulation (*SI*) or from a non-sterile homogenised rhizosphere substrate from the original plant collection site (*COM*). Non-inoculated plants were treated with 10 ml of autoclaved inoculum (121 °C twice for 25 min). Finally, all plants received 5 ml of the microbial filtrate from the complementary inoculum/inocula in an attempt to balance the initial non-AMF microbial communities across all the inoculation treatments. Briefly, non-inoculated plants were treated with both *SI* and *COM* filtrates; *SI*-inoculated plants with *COM* filtrate and vice versa. Microbial filtrates were prepared by filtration of soil suspensions (1:10, w/v) through filter paper of a pore size of 15 μm in order to remove AMF propagules.

Plants were grown in a growth chamber (VB 1014, Vötsch Industrietechnik, Germany) under a 12 h/12 h day/night mode with a 25 °C/13 °C temperature regime, photosynthetically active radiation of 330 $\mu mol m^{-2} s^{-1}$ at plant level, and a stable air humidity of 70 %.

Table 1 Chemical characteristics of the cultivation substrate. The values represent the means and SEM (in parentheses) of three replicates

pH_{KCl}	pH_{H2O}	N [a]	C_{org} [a]	P [b]	Ca [c]	Mg [c]	K [c]	Na [c]	Fe [d]	Mn [d]	Ni [d]	Co [d]	Cr [d]
		(%)		($mg\,kg^{-1}$)									
4.73	5.04	0.62	9.4	11.3	394	2 231	43.0	5.5	125.4	28.1	98.3	3.6	0.32
(0.03)	(0.05)	(0.07)	(0.1)	(0.1)	(23)	(105)	(5.3)	(0.3)	(15.2)	(0.7)	(18.2)	(0.1)	(0.18)

[a] combustion method (CHN Carlo Erba NC 2500 analyser, Italy)

[b] 0.5 M sodium bicarbonate-extractable (Unicam UV 4–100, UK)

[c] 1 M ammonium acetate-extractable, pH7.0 (AAS Unicam 9200X, UK)

[d] 0.005 M DTPA-0.1 M triethanolamine-0.01 M $CaCl_2$ extractable (AAS Unicam 9200X, UK)

Photosynthetic and transpiration parameters, *WUE*

After 12 weeks of cultivation under different water regimes, gasometric measurements were performed prior the harvest on the third youngest leaf (first fully developed) of each plant, using the LI-6400 portable photosynthesis system (LI-COR, USA). Intact leaves were fixed in a standard 6-cm^2 leaf chamber and 15 repeated measurements (30 s each) were conducted at a flow rate of 500 μmol CO_2 mol^{-1}, photosynthetic photon flux density of 800 $\mu mol\,m^{-2}s^{-1}$ and leaf temperature of 20±1 °C. Precise leaf area clipped by the chamber was measured via image analysis using the NIS-Elements AR 3.10 software (Laboratory Imaging Ltd, CR). Net photosynthetic (P_N) and transpiration (E) rate data were then recalculated per square metre of leaf blade and the P_N/E ratio provided information on instantaneous plant *WUE* (mmol CO_2 mol^{-1} H_2O).

Plant harvest

All plants were harvested after 12-week cultivation, at the stage of daughter rosette formation. Shoots were cut off, washed and their leaf areas (LA) were assessed using an area meter (LI-3100, LI-COR, USA). Whole root systems were washed and three root subsamples of known fresh weight (FW) were taken for determination of (i) mycorrhizal root colonisation, (ii) proline and (iii) abscisic acid (ABA) concentrations; ABA was determined for five plants per treatment only. The subsamples (ii) and (iii) were immediately frozen in liquid nitrogen and stored at −80 °C. In addition, a small root subsample (~100 mg FW) from eight *COM*-inoculated plants (two randomly selected individuals per irrigation treatment) was frozen for subsequent determination of native AMF colonising the roots (see below). The dry weight (DW) of remaining shoots and roots was recorded after drying for 24 h at 65 °C. DW of all shoot and root subsamples was inferred from the DW/FW ratio of the remaining shoots and roots, respectively. Both total shoot DW and root DW were then calculated. In addition, mycorrhizal growth dependence (*MGD*) was calculated according to Smith et al. (2003), as the percentage increase in shoot DW of an individual mycorrhizal plant above the mean performance of non-mycorrhizal plants in the same respective water regime [*MGD* = 100 × (DW of an individual mycorrhizal plant—average DW of non-mycorrhizal plants)/DW of an individual mycorrhizal plant].

Nutrient concentrations in shoot biomass, *MPB* and *PPUE*

Shoot biomass was then analysed to assess concentrations of the main nutrients (P, N, K). Dried biomass was ground and a subsample of it was used for determination of N concentration using the flash-combustion method (CHN elemental analyser, Carlo Erba NC2500, Italy). The remaining biomass was digested in 65 % HNO_3 and 30 % H_2O_2 and analysed for P and K concentrations. P concentration was determined spectrophotometrically using the ammonium-molybdate ascorbic acid method at a wavelength of 630 nm (Unicam UV4-100, UK), K concentration was analysed using an atomic absorption spectrometer (AAS Unicam 9200X, UK). By analogy to *MGD*, mycorrhizal phosphorus uptake benefit (*MPB*) was derived from shoot P concentrations in mycorrhizal relative to non-mycorrhizal plants. Furthermore, photosynthetic phosphorus-use efficiency was calculated as *PPUE* (mmol CO_2 $(mol\ P)^{-1}s^{-1}$) = [(net photosynthetic rate × shoot DW × LA × molecular weight of P)/shoot P concentration].

Determination of proline and ABA concentrations

Both leaves and roots were analysed for proline concentrations in the preliminary experiment, with the same effects of drought on proline accumulation found in both tissues. However, as concentration in foliar tissue showed higher variability, only roots were analysed in this study. Proline concentration was determined after a 3 % sulfosalicylic acid extraction based on the acid-ninhydrin method (Bates et al. 1973). The absorbance of the extracts was measured spectrophotometrically at 520 nm (Hach DR4000U, USA) and concentrations were calculated according to the calibration curve for proline standards.

The root samples for (+/−) ABA analysis were homogenised and extracted into distilled water (0.1 g FW/1 ml H_2O), shaken for 16 h under cold (4–5 °C) and dark conditions, and processed by indirect ELISA according to Asch (2000). For each sample, we used three replicates on a microtitre plate. An extinction photometer SUNRISE Remote (Tecan, Germany) was used to measure colour intensity of the final product at 405 nm and the ABA concentration was calculated.

Determination of mycorrhizal parameters and native AMF in the *COM*-treatment

To visualise the intraradical fungal structures, the root samples for determination of mycorrhizal colonisation were stained in 0.05 % trypan blue in lactoglycerol (Koske and Gemma 1989). The frequency of root colonisation (further referred to as mycorrhizal root colonisation), intensity of mycorrhization and relative arbuscule and vesicle abundances in mycorrhizal root fragments were evaluated according to Trouvelot et al. (1986), under a compound microscope at 100× magnification.

The AMF established in the roots of plants inoculated with the native fungal community were identified using molecular tools to enable a more accurate interpretation of potential differences in effects between the two inocula. DNA was extracted from frozen root samples using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was eluted with 50 μl of elution buffer and the DNA extracts were then subjected to a polymerase chain reaction (PCR) after 1:10 dilution with double deionized water. Each PCR reaction was conducted in duplicate. A nested PCR was used to amplify ca 1500 bp fragment covering part of the SSU, the whole ITS and part of the LSU rDNA region using a primer set designed by Krüger et al. (2009). Equimolar mixtures of SSUmAf-LSUmAr primers (each 0.5 μM) were used for the first round of PCR, which was performed using 20 μl reaction mixtures, each containing one unit of *Taq* polymerase (Fermentas, Germany), 1× *Taq* buffer with KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 16 μg of BSA and 1 μl of the diluted DNA extract. The amplification program was as follows: 5 min at 95 °C followed by 38 cycles of 30 s at 95 °C, 90 s at 60 °C and 2 min at 72 °C, and a final 10 min elongation at 72 °C. The PCR products were diluted 1:100 in double deionized water and used as templates in the second amplification step using the primer mixtures SSUmCf and LSUmBr. The PCR reaction and cycling conditions were the same as in the first PCR reactions, except for annealing temperature (63 °C), number of cycles (35) and a 30 min final extension. The amplified products were analysed using agarose gel electrophoresis (1.0 % w/v agarose) and pooled into a single sample which was then gel-purified with Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) and cloned using a pGEM-T cloning kit (Promega, the Netherlands) according to the manufacturer's instructions. The inserts were re-amplified using the M13 forward and M13 reverse vector primers. Twenty-five randomly selected positive clones were purified and sequenced in both directions in Macrogen Inc. (South Korea). The obtained sequences were edited with FinchTV 1.4.0 (Geospiza Inc., USA), manually checked for possible chimeras, and then aligned using MAFFT version 6, together with sequences from public databases. Subsequently, a neighbour-joining analysis based on a Kimura 2-parameter model (1,000 bootstrap replicates) was computed using the software MEGA5; the resulting phylogenetic tree is presented in Fig. S1 in Online Source 1.

Data analysis

The results were analysed using Statistica 9.1 software (StatSoft Inc., USA). Prior to the analyses, all data were checked for normality and homogeneity of variance. If necessary, variables were transformed using logarithmic (log_{10}), square root (sqrt) or arcsine functions to meet the assumptions of analysis of variance (ANOVA). The effects of water regime, AMF inoculation and their interaction were then analysed using a two-way ANOVA; post-hoc comparisons were performed using a Tukey HSD test. A non-parametric Kruskal-Wallis

ANOVA was used to analyse variables which did not meet the ANOVA assumptions even after transformation, followed by a post-hoc Z-value test. The effects of the main factors and their interaction on all plant and mycorrhizal parameters are summarised in Table 2. A linear regression analysis was used to explore the relationships between variables.

Results

Mycorrhizal parameters and determination of native AMF in the *COM*-treatment

All inoculated plants (further referred to as *M*, mycorrhizal) were colonised by AMF, whereas no mycorrhizal structures were found in the roots of non-inoculated plants (*nm*, non-mycorrhizal). Plants inoculated with the native AMF community showed lower mycorrhizal root colonisation, but higher relative arbuscule abundance compared to plants inoculated with the single AMF isolate (Table 2, Fig. 1a, b). The mycorrhizal root colonisation generally decreased with diminishing water supply, whereas relative arbuscule abundance tended to increase, particularly in the case of *COM*-treatment (Fig. 1a, b). The relative vesicle abundance was unaffected by water regime (Table 2), but lower values were recorded for the *COM*-treatment (9.6±1.9 %, mean ± SEM) compared to the *SI* inoculation (19.3±2.4 %). The intensity of mycorrhization was not significantly influenced by any treatment (data not presented).

Three different molecular operational taxonomic units were revealed by molecular identification of

Table 2 The effects of water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation (*nm* – non-mycorrhizal plants, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community) and their interaction on plant and mycorrhizal parameters (DW, dry weight; P_N, net photosynthetic rate; *PPUE*, photosynthetic phosphorus-use efficiency; *MGD*, mycorrhizal growth dependence; *MPB*, mycorrhizal phosphorus uptake benefit; *F*, mycorrhizal root colonisation; *a*, arbuscule abundance; *v*, vesicle abundance). The data represent the results of two-way ANOVA (F-values), except for *MGD* and proline concentrations analysed with non-parametric Kruskal-Wallis ANOVA (H-values). For the significant effects of single factors, the results of post-hoc comparisons are given according to the Tukey HSD test or Z-value test (for the non-parametric analyses), both at $P<0.05$. For *MGD*, *MPB*, *F*, *a* and *v*, only *SI* and *COM* inoculation treatments were compared (AMF inoculation, $df=1$; water × AMF interaction, $df=3$)

parameter	water regime ($df=3$)		AMF inoculation ($df=2$)		water × AMF ($df=6$)
shoot DW	77.78 ***	55>45>35>25	54.54 ***	*nm<SI=COM*	2.74 *
root DW	64.69 ***	55>45>35>25	39.94 ***	*nm<SI=COM*	3.87 **
root:shoot ratio	0.20 ns	–	1.88 ns	–	2.53 *
P_N	0.69 ns	–	4.00 *	*nm≤COM≤SI*	2.19 ns
[a]*E*	1.21 ns	–	4.20 *	*nm≤COM≤SI*	1.46 ns
WUE	1.16 ns	–	0.54 ns	–	2.55 *
[a]*PPUE*	40.37 ***	55=45>35>25	14.04 ***	*nm<COM=SI*	1.75 ns
root proline	21.95 ***	25>35=45=55	30.38 ***	*nm>SI=COM*	–
[b]root ABA	8.81 ***	25≥35≥45≥55	0.43 ns	–	3.40 **
shoot P	4.10 *	55=35≥45≥25	67.28 ***	*nm<SI=COM*	1.52 ns
[b]shoot N	25.53 ***	25>35≥55≥45	60.68 ***	*nm>COM>SI*	2.68 *
[b]shoot K	8.64 ***	25≥35≥45=55	20.63 ***	*nm>SI=COM*	2.00 ns
MGD	17.14 ***	35≥45≥55=25	2.20 ns	–	–
[c]*MPB*	5.92 **	35≥45≥55=25	8.95 **	*SI>COM*	4.13 *
[c]F	6.13 **	55=45≥35≥25	6.37 *	*SI>COM*	1.70 ns
[c]*a*	5.74 **	25≥45≥35≥55	77.51 ***	*SI<COM*	1.45 ns
[a]*v*	1.20 ns	–	12.46 **	*SI<COM*	3.29 ns

The significance level is marked as follows: *** $P<0.001$, ** $P<0.01$, * $P<0.05$, ns non-significant

Data transformed using: sqrt (a), $\log_{10}$ (b) or arcsin (c) function

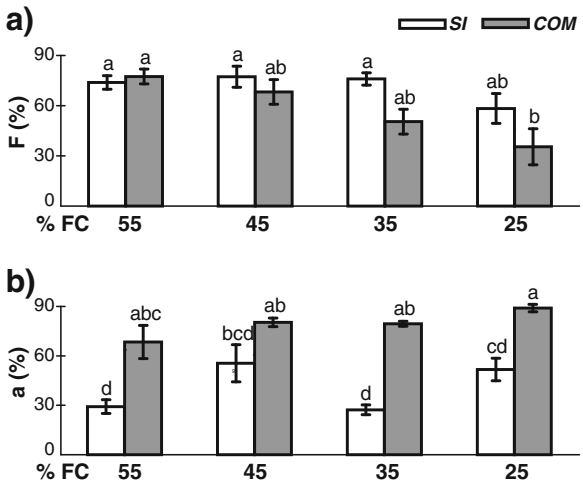

Fig. 1 **a** Mycorrhizal root colonisation (*F*) and **b** relative arbuscule abundance (*a*) in inoculated plants as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns represent the means (± SE) of 6 replicates. Columns marked by different letters are significantly different according to Tukey HSD test ($P<0.05$)

AMF colonising plant roots in the *COM*-treatment inoculated with a non-sterile native substrate. All belonged to the family Glomeraceae, but none could be assigned to any morphologically described AMF species (Fig. S1). Interestingly, the native AMF species used in the *SI* inoculation treatment was also detected in the *COM*-inoculated pots, but nevertheless accounted for only 13 % of the recovered sequences in the clone library.

Plant growth parameters and *MGD*

A diminishing water supply resulted in a gradual reduction in plant growth (Table 2, Fig. 2). In contrast, AMF inoculation had an overall positive effect on shoot and root DW (Fig. 2). Interestingly, biomass production was positively correlated with mycorrhizal root colonisation ($r^2=0.19$, $P=0.002$ for shoot DW). There was a significant water regime × inoculation interaction for both shoot and root DW; the *SI*-inoculated plants tended to perform better than the *COM*-inoculated plants with increasing drought stress (Fig. 2). *MGD* was significantly affected by water regime (Table 2); the benefit of being mycorrhizal gradually increased with decreasing water supply from 55 % FC (*MGD* 39±3 %) to 35 % FC (55±2 %), but fell at 25 % FC (36±7 %). Leaf area was strongly positively correlated with shoot DW ($r^2=0.90$, $P<0.0001$) and these data are therefore not further presented. The root:shoot ratio was affected only by the water regime × inoculation interaction (Table 2). The values were comparable for *nm* and *M* plants irrespective of the intensity of drought stress (averaging 1.41±0.04), except under the most severe drought stress in which *nm* plants showed a higher root:shoot ratio than *M* plants (1.83±0.22 and 1.26±0.08, respectively).

Photosynthetic and transpiration parameters, *WUE*

Both P_N and E were unaffected by water regime (Table 2). With regards to the effect of AMF inoculation, *SI*-inoculated plants showed significantly higher

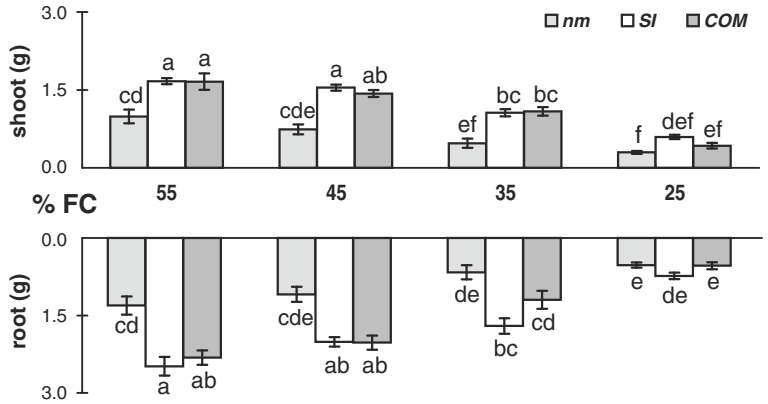

Fig. 2 Shoot and root dry weight as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns represent the means (± SE) of 5–6 replicates. Columns marked by different letters are significantly different according to Tukey HSD test ($P<0.05$)

P_N and E values than *nm* plants; however, this effect was largely caused by a marked difference at 25 % FC (Table 2, Figs. S2a, b). *WUE* was significantly affected only by water regime × inoculation interaction (Table 2). At 55 % and 45 % FC, *M* plants showed lower *WUE* values compared to *nm* plants, while no difference between *M* and *nm* plants was recorded at 35 % and 25 % FC (Fig. S2c).

Nutrient concentrations in shoot biomass, *MPB* and *PPUE*

A diminishing water supply resulted in a significant decline in shoot P concentration and *PPUE*, while shoot K and N concentrations increased (Table 2, Figs. 3a-c, S2d). With regards to the effect of AM symbiosis, inoculation with either AMF consistently increased shoot P concentration and *PPUE* (Table 2, Figs. 3a, S2d). *MPB* increased from 55 % and 45 % FC to 35 % FC (from 73±2 to 84±1 %), while a pronounced fall was recorded at 25 % FC (66±7 %; Table 2). As indicated by the significant water regime × inoculation interaction, *SI*-inoculation was more effective than *COM*-inoculation under the highest drought intensity in terms of promoting P uptake (*MPB* 80±1 vs. 52± 11 %; see also Fig. 3a). The two parameters describing plant benefits of AM symbiosis, i.e., *MPB* and *MGD*, were positively correlated (r^2=0.58, P<0.0001). In addition, both shoot P and *MPB* were positively correlated with mycorrhizal root colonisation (r^2=0.28, P=0.0001 and r^2=0.20, P=0.0015, respectively). In contrast, a negative correlation was found between mycorrhizal root colonisation and shoot N (r^2=0.29, P<0.0001) and K (r^2=0.26, P=0.0002) concentrations, respectively.

Both N and K concentrations were significantly lower in the shoots of *M* plants (Table 2, Fig. 3b-c). While no difference between the two inocula was recorded in terms of K uptake, the *SI*-inoculated plants showed consistently lower shoot N concentration than *COM*-inoculated plants (Table 2, Fig. 3b). N uptake was also significantly affected by water regime × inoculation interaction; the difference between *nm* and *M* plants decreased with the diminishing water supply (Fig. 3b).

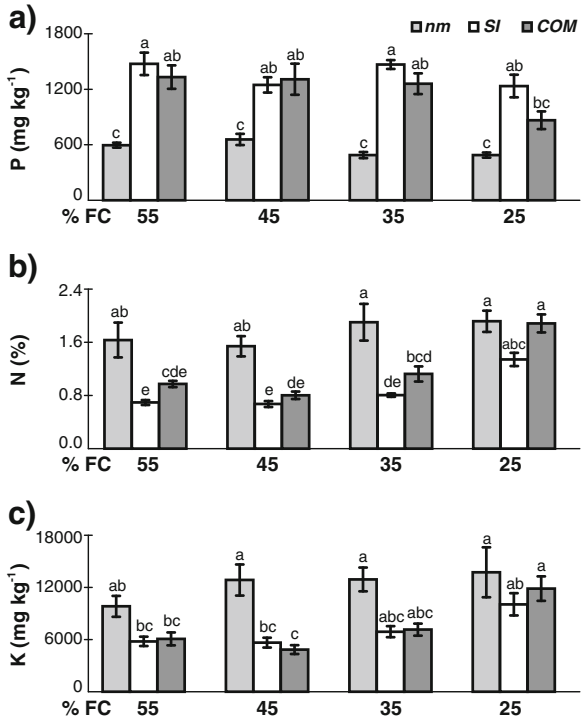

Fig. 3 **a** Phosphorus, **b** nitrogen and **c** potassium concentrations in shoot biomass as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns represent the means (± SE) of 5–6 replicates. Columns marked by different letters are significantly different according to Tukey HSD test (P<0.05)

Proline and ABA concentrations

Root proline concentration was significantly affected both by water regime and AMF inoculation (Table 2). In *nm* plants, proline gradually increased with diminishing water supply, while *M* plants displayed constantly low proline concentration at 55–35 % FC, with no difference observed between the two inoculation treatments (Fig. 4a). However, at 25 % FC, proline also increased markedly in the roots of *M* plants, although levels remained lower than in *nm* plants. Interestingly, this increase was less pronounced in the case of *SI*-inoculated plants.

ABA concentration in root biomass also gradually increased with diminishing water supply (Table 2). AMF inoculation had no effect on ABA accumulation per se, but there was a significant water regime × inoculation interaction. With intensification of drought stress, increase in ABA concentration was more pronounced in *nm* than *M* (particularly in the *SI*-inoculated) plants (Fig. 4b).

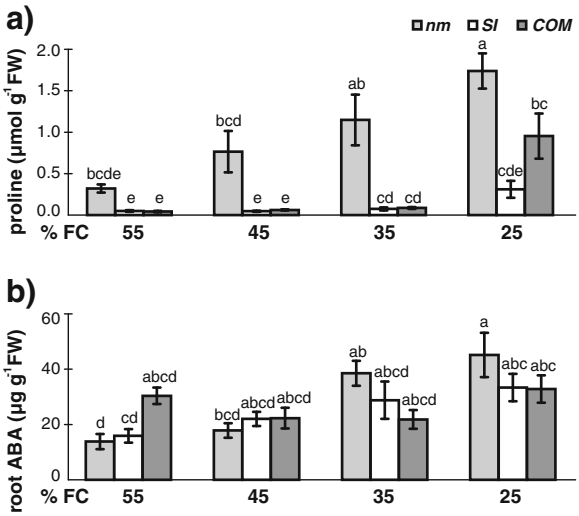

Fig. 4 Root **a** proline and **b** ABA concentrations as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns represent the means (± SE) of 5–6 replicates. Columns marked by different letters are significantly different according to Z value test (proline) and Tukey HSD test (ABA; both at $P<0.05$)

Discussion

Effects of AM symbiosis on plant growth and *WUE* under increasing drought stress

In line with our prediction, the overall positive effect of AM symbiosis on *K. arvensis* growth in serpentine soil increased with intensification of drought stress. For different soil types, similar results were reported also by Subramanian et al. (2006) and Bolandnazar et al. (2007). However, a drop in *MGD* recorded in our study under the most severe drought conditions, probably in relation to a reduced mycorrhizal root colonisation, indicates that the potential of AMF to alleviate drought stress in host plants is limited to a certain water supply threshold. In contrast to the total mycorrhizal colonisation, arbuscule abundance slightly increased in response to diminishing water supply which might be related to increased ABA concentration in plant tissues (Ludwig-Müller 2010). Interestingly, first AMF aquaporin genes have recently been identified (Aroca et al. 2009) and their high expression in arbuscule-containing root cells (along with extraradical mycelium) has recently been recorded under drought stress, pointing to the active role of AMF in the alleviation of water deficit (Li et al. 2012).

In contrast to our hypothesis, we did not document a positive AMF-effect on water use efficiency under limited water supply. A lower *WUE* observed in mycorrhizal *K. arvensis* plants under well-watered conditions might be attributable to a more efficient exploitation of soil water by larger root systems connected to the extraradical mycelial network, as was suggested by Duan et al. (1996) and Davies et al. (2002). The involvement of extraradical mycelium in plant water uptake might be inferred from the relatively lower investment of mycorrhizal plants into the root biomass compared to non-mycorrhizal plants under the highest intensity of drought stress.

Effects of AM symbiosis on plant nutrition under increasing drought stress

In line with our previous study (Doubková et al. 2012), the results of elemental analysis of plant biomass clearly confirm that a key role of AM symbiosis in the performance of *K. arvensis* in serpentine soils consists in improved phosphorus acquisition. Importantly, we proved that the relative impact of AMF on plant P nutrition was larger in dry compared to well-watered soil, likely due to an overall lower nutrient mobility under drought conditions. Similarly to the relative mycorrhizal growth benefit, also the relative P nutritional benefit dropped under the conditions of an extremely low water supply, thus indicating the limits of AMF-beneficial influence under drought stress.

Contrary to phosphorus, AMF inoculation considerably impaired the N nutrition and this distinct negative effect markedly weakened at the most intensive drought stress where mycorrhizal colonisation declined. In contrast, other studies reported relatively greater role of AM symbiosis in N acquisition under limited water supply conditions (e.g., Subramanian and Charest 1999; Lee at al. 2012). However, in agreement with our recent data, negative AMF-effects on plant N concentration were consistently observed for *K. arvensis* plants under well-watered conditions, both in N-limited and P-limited substrates (Doubková et al. 2012 and unpublished data). Based on this cumulative evidence, we assume that N-sink in AMF-tissues is higher compared to plant-tissues, as previously suggested by

Johnson (2010). Nevertheless, considering the negative correlation of N concentration with shoot dry weight and with mycorrhizal root colonisation, a biomass-dilution effect of AMF inoculation cannot be excluded.

The opposite direction of drought- and mycorrhiza-induced changes in shoot potassium concentration (gradual increase and decrease, respectively) points to the importance of K-nutritional status for plant drought tolerance. Plants exposed to environmental stresses, including drought, have repeatedly been reported to have an increased requirement for K due to the importance of this element for plant osmotic adjustment and maintenance of photosynthetic CO_2 fixation (Römheld and Kirkby 2010).

AMF-mediated alleviation of plant drought stress as indicated by stress markers

Consistent with our hypothesis, both the intensity of plant stress responses to a limited water supply and the positive role of AM symbiosis in plant drought tolerance were clearly indicated by the plant tissue concentration of proline, one of the so-called compatible solutes which is considered a common biochemical marker of various types of abiotic stress. Specifically, proline concentration increased with diminishing water supply and decreased in response to AMF inoculation, analogously to shoot K concentration. The reports of AMF-mediated changes in proline concentration under drought stress range from increase (Ruiz-Lozano et al. 1995; Fan and Liu 2011) to decrease (Wu et al. 2007a; Manoharan et al. 2010), leading to ambiguous interpretation (for review, see Augé 2001). Considering the crucial role of proline in scavenging of reactive oxygen species, in the protection of proteins against denaturation and in the stabilisation of membranes and subcellular structures (for review, see Kishor et al. 2005), our observation of lower proline accumulation in well-watered as well as inoculated plants supports the view that AM symbiosis effectively alleviated drought and, consequently, oxidative stress in host plants.

Increasing intensity of drought stress was also marked by a gradually rising concentration of ABA in root tissues. Accumulation of abscisic acid is a signal for initialisation of adaptive mechanisms against drought, indicating root sensitivity to soil water status and stomata closure (Sauter et al. 2001). Moreover, ABA involvement in signalling and control of stomata closure has been proposed as one of the possible non-nutritional explanations of the mycorrhizal promotion effect on drought-stressed plants (Ludwig-Müller 2010). The fact that non-mycorrhizal plants responded to limited water supply by increasing their production of ABA more than mycorrhizal plants may indicate that they experienced more intense drought stress than mycorrhizal plants. Higher ABA accumulation in the root tissues of non-mycorrhizal plants has previously been recorded under limited water supply conditions (Duan et al. 1996; Goicoechea et al. 1997). Also the trend towards higher ABA concentration observed in our experiment in mycorrhizal plants under less intense drought stress corresponds with the findings of previous studies reporting higher or unchanged ABA concentration due to AMF under well-watered conditions (Danneberg et al. 1993; Goicoechea et al. 1997). Nevertheless, the considerable variability in our data and differences between both inocula preclude any broader generalisation.

Comparison of the effects of the single AMF isolate and complex AMF community

The lack of any clear differences between the two inoculation treatments in terms of their plant growth promotion effects contrasts with our hypothesis that the AMF community would provide a "higher buffer capacity" against environmental stress. It might be a consequence of the generally low diversity of the AMF community established in the *COM*-inoculation treatment. Also the fact that the AMF used in the single-isolate treatment was a component of the fungal community might have contributed to blurring the differences between both inocula. Although functional complementarities of AMF species/isolates have been documented (Schreiner and Bethlenfalvay 1997; Caravaca et al. 2005), the results of Jansa et al. (2008) show that synergistic effects of individual AMF in mixed inocula may, but do not have to, occur and that the outcome of the interactions depends on the particular plant-fungus/fungi combination.

In spite of the general similarity in the effects of the two inocula, they tended to diverge with the increasing intensity of drought stress, particularly in terms of *MPB*, proline accumulation and photosynthetic rate. The higher efficiency of the single AMF isolate in this regard might be related to its ability to maintain relatively high mycorrhizal root colonisation under the

stressful conditions. Besides differences in extra- and intraradical fungal development, vitality and activity (Marulanda et al. 2003, 2007), the variation among AMF isolates in their ability to alleviate plant drought stress has generally been attributed also to the different impact of AMF isolates on plant physiological processes, including water use efficiency and transpiration and photosynthetic rates (Ruiz-Lozano et al. 1995; Wu et al. 2007b; Huang et al. 2011).

Conclusion

The role of AM symbiosis in plant drought tolerance in serpentine habitats where drought is only one of numerous abiotic stresses imposed on the vegetation has not yet been studied. Our results provide strong evidence that the role of AM symbiosis in the performance of *Knautia arvensis* plants in stressful environment of serpentine soils consists not only in the improved phosphorus nutrition, but also in the alleviation of drought stress. Notably, the benefit to plants of being mycorrhizal increased with diminishing water supply, both in terms of growth and phosphorus uptake. In spite of the evident relation between plant growth, P uptake and drought tolerance, the influence of AMF on plant drought tolerance was also based on mechanisms independent of plant nutritional status. The mycorrhizal alleviating effect was also evident from the lower accumulation of drought stress markers in plant tissues and from different root-to-shoot biomass allocation under extreme drought indicating the contribution of extraradical mycelium to water uptake. Nevertheless, the potential of AMF to alleviate drought stress was limited beyond a certain water supply threshold, as indicated by a steep decline in mycorrhizal growth dependence and phosphorus uptake benefit and a concomitant rise in proline concentration at the most severe drought stress level.

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