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The symbiosis with arbuscular mycorrhizal fungi contributes to plant tolerance to serpentine edaphic stress

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ABSTRACT

Serpentine soils represent stressful environment with low calcium to magnesium ratio, deficiencies of essential macronutrients, increased concentrations of heavy metals and a low water-holding capacity. A considerable potential of arbuscular mycorrhizal fungi (AMF) to promote plant growth and nutrition can be expected under these adverse conditions. We tested a hypothesis that both host plants and AMF have evolved edaphic tolerance, which enable them to cope with the conditions of serpentine habitats. A reciprocal transplant experiment with a full factorial design was conducted, involving serpentine and non-serpentine substrates, host plants (*Knautia arvensis*, Dipsacaceae) and AMF isolates. After three-month cultivation under greenhouse conditions, plant growth and nutrition and AMF development were assessed. Our results evidenced the edaphic differentiation and higher tolerance of both plants and fungi of serpentine origin to serpentine soil. The better performance of serpentine plants was likely based on their tolerance to magnesium accumulation in shoot tissues. The serpentine AMF isolate not only developed higher root colonisation in serpentine conditions, but it was also more efficient in the growth promotion of and phosphorus uptake by the serpentine plants. As it had only a slight impact on magnesium uptake and no influence on calcium and nickel uptake by the serpentine plants, the improved P nutrition seemed to be the crucial mechanism of the mycorrhizal promotion effect. In conclusion, our study proved the tolerance of a plant–mycobiont complex to specific conditions of serpentine soils, which may indicate that not only the individual components of serpentine communities but also their functional interactions are subjected to selective evolutionary forces.

1. Introduction

Serpentine soils represent exceptional habitats characterised by complex physicochemical properties, collectively referred to as the serpentine syndrome (Jenny, 1980). Serpentine soils are found worldwide but have patchy distribution (primarily in California, Cuba, S Africa, S Europe, New Caledonia, SE Asia, W Australia), in total covering less than one percent of the land surface (Coleman and Jove, 1992). The key features of serpentine syndrome include a low calcium to magnesium ratio, deficiencies of essential macronutrients, increased (up to toxic) concentrations of heavy metals (especially nickel, chromium and cobalt) and a low water-holding capacity (for reviews, see Brady et al., 2005; Kazakou et al., 2008; Proctor and Woodell, 1975). Permanent multiple abiotic stresses can promote plant adaptation and accelerate speciation processes, leading to the evolution of distinct serpentine vegetation with low productivity and high levels of endemism (Whittaker, 1954).

Plant performance in serpentine sites is not only shaped by abiotic but also by biotic factors, including communities of soil bacteria and fungi. Arbuscular mycorrhizal fungi (AMF), which colonise the roots of most vascular plants and form an extensive soil mycelial network involved in water and nutrient uptake, play a prominent role in plant–soil and plant–plant interactions (Smith and Read, 2008). Because AMF can alleviate different abiotic stresses (e.g. Entry et al., 2002; Leyval et al., 1997), these symbionts have also been suggested to play a role in plant adaptation to serpentine syndrome (Castelli and Casper, 2003; Schechter and Bruns, 2008). Although the worldwide presence of arbuscular

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mycorrhizal (AM) symbiosis in serpentine habitats is unequivocal (e.g. Goncalves et al., 2001; Hopkins, 1987; Turnau and Mesjasz-Przybylowicz, 2003), little is known about its importance and functional involvement in serpentine plant–soil interactions. There are many comparative ecophysiological studies dealing with serpentine/non-serpentine ecotypes of the same plant species (e.g. Johnston and Proctor, 1981; O'Dell et al., 2006; Wright et al., 2006); however, scarcely any have also considered AMF, despite the fact that most of the investigated plant species form AM symbiosis in their native environment.

Recent mycorrhizal research in serpentine sites has focused on the assessment of AMF species diversity in comparison with non-serpentine soils, using molecular tools (Fitzsimons and Miller, 2010; Schechter and Bruns, 2008). Other studies compared the effects of serpentine and non-serpentine AMF communities on plant growth as well as the nutrient and nickel uptake, via the experimental cultivation of a plant–mycobiont complex under serpentine vs. non-serpentine conditions (Doherty et al., 2008; Ji et al., 2010; Lagrange et al., 2011). The present study followed this research line by conducting a reciprocal transplant experiment, where serpentine and non-serpentine substrates, host plant populations and AMF isolates were combined. This experimental design allowed us to compare the effects of the same inoculation treatments on plants of different origin in serpentine vs. non-serpentine conditions. We addressed the following hypotheses: 1) the serpentine plant populations will grow better under serpentine conditions than their non-serpentine counterparts, 2) similarly, the serpentine AMF isolate will be more tolerant to serpentine conditions than the non-serpentine one, and 3) the tolerance of plant and fungal partners to serpentine syndrome will be combined, resulting in a better performance of native serpentine plant–mycobiont complex under serpentine conditions.

2. Materials and methods

2.1. Plant species

Field scabious, *Knautia arvensis* (L.) J. M. Coult. (Dipsacaceae), is a common perennial herb native to Eurasia, occurring in dry and mesophilous meadows, pastures, shrublands, open woods, forest margins and roadsides (Štěpánek, 1997). It comprises two cytotypes (diploid and tetraploid) that are morphologically only weakly differentiated (the former have slightly smaller outer involucral bracts and achenes) but show distinct distribution pattern in central Europe, with diploids occurring mostly in the east and tetraploids in the west (Kaplan, 1998; Kolář et al., 2009). Several reasons qualify *K. arvensis* as a suitable plant species for the present study: i) it is a mycotrophic plant (Doubková et al., 2011), ii) it encompasses both serpentine and non-serpentine populations in central Europe (Kaplan, 1998), and iii) it harbours both diploid and tetraploid populations on serpentine and non-serpentine soils (Kolář et al., 2009), making it possible to address the interaction of genome copy number and AM symbiosis in plant adaptation to stressful serpentine habitats. The present study is a follow-up of our previous research in which the same *K. arvensis* populations were investigated (Doubková et al., 2011). Briefly, four serpentine and four non-serpentine populations of *K. arvensis* were selected in the Czech and the Slovak Republics (see Fig. S1 in Supplementary data). As a relationship between polyploidy and plant tolerance to environmental stresses has been proposed (Levin, 2002), each substrate type was represented by two diploid and two tetraploid populations in our study (Table 1). Further details on the selected populations, including the chemical characteristics of their original substrates, are provided in Doubková et al. (2011).

Table 1
List of *Knautia arvensis* populations included in the study.

Population origin	Ploidy level	Population code	Geographic coordinates	Altitude (m a.s.l.)
Serpentine	2x	S1	49°40′57.7″ N; 15°07′49.7″ E	400
		S2	49°39′04.9″ N; 15°48′57.3″ E	640
	4x	S3	50°03′01.3″ N; 12°46′24.3″ E	710
		S4	50°03′54.2″ N; 12°45′03.6″ E	790
Non-serpentine	2x	NS1	48°51′43.6″ N; 17°23′23.3″ E	290
		NS2	48°28′29.8″ N; 17°38′59.2″ E	230
	4x	NS3	50°13′12.9″ N; 12°13′19.2″ E	670
		NS4	49°24′39.0″ N; 13°43′55.5″ E	530

2.2. Experimental design

In the summer and autumn of 2008, mature *Knautia* achenes were collected in the field from approx. 50 plants per each population to reduce possible maternal effects. The achenes were surface-sterilised (5% NaClO, 10 min), rinsed with sterilised water and pre-germinated in Petri dishes. The seedlings were grown for four weeks in multipots filled with a γ-sterilised (25 kGy; Bioster, Czech Republic) mixture of garden soil and sand (1:2, v/v). At the stage of three leaf pairs, even-sized seedlings were then planted into 400-ml experimental pots.

Each of the eight *Knautia* populations was subjected to six experimental treatments, resulting from a combination of (i) two substrate types and (ii) three inoculation treatments. We used eight replicates per experimental treatment except for populations S1 and S2, where only three and four replicates, respectively, were possible due to the low germination rates of *Knautia* achenes. (i) Model serpentine (S) and non-serpentine (NS) substrates originated from the localities S1 and NS4 (Table 1), respectively. The substrates were excavated to depths of approx. 30 cm, thoroughly mixed, passed through a 5-mm sieve and sterilised by γ-irradiation (25 kGy). The chemical characteristics of the sterilised substrates together with details on the analytical methods are provided in Table 2. (ii) The three inoculation treatments included (a) non-inoculated plants (referred to as *nm*, non-mycorrhizal), (b) plants inoculated with a reference isolate (*ns*, non-serpentine) and (c) plants inoculated with a serpentine isolate (*s*, serpentine). As the *ns* isolate, *Glomus irregulare* LT (EMBL database, accession number FR828470) was selected due to its relative ability to sustain the adverse edaphic conditions of metal contaminated soils (Sudová et al., 2008). Formerly, this isolate originating from a mesotrophic meadow with soil pH 5.3 was described on the base of spore morphology as *Glomus intraradices* (Sudová and Vosátka, 2008). However, after PCR amplification and cloning of partial SSU, whole ITS and partial LSU rDNA gene (primers and PCR conditions according to Krüger et al., 2009), the subsequent phylogenetic analysis (sequences aligned using MAFFT version 6 together with sequences from public databases, neighbour joining analysis computed with the software MEGA5) revealed that this isolate clusters in the *G. irregulare* clade as defined by Stockinger et al. (2009). The *s* isolate, *Glomus* sp. SR (FR828471), was isolated from the S2 serpentine site and according to the phylogenetic analysis (workflow equal to the *ns* isolate) belongs also to *Glomus* group A (as defined by Schwarzott et al., 2001) and clusters as a sister clade to *Glomus aggregatum* (FJ461812). For the phylogenetic tree showing the position of both isolates, see Fig. S2.

All plants from inoculated treatments were inoculated with 7 ml of a suspension containing colonised root segments, extraradical mycelium and spores of the respective AMF isolate (*ns* or *s*). Both inocula were prepared by wet sieving (Gerdemann and Nicolson, 1962) from mature maize cultures with abundant sporulation and high root colonisation (>80%). The non-inoculated plants were

Table 2
Chemical characteristics of the γ-sterilised cultivation substrates. The elemental data represent plant-available concentrations.

Substrate type	pH_{KCl}	N[a] (%)	C_{org}[a] (%)	P[b] (mg kg^{-1})	Ca[c] (mg kg^{-1})	Mg[c] (mg kg^{-1})	Ca/Mg	K[c] (mg kg^{-1})	Fe[d] (mg kg^{-1})	Mn[d] (mg kg^{-1})	Ni[d] (mg kg^{-1})	Co[d] (mg kg^{-1})	Cr[d] (mg kg^{-1})
Non-serpentine	4.8	0.25	3.8	6.22	1620	207	7.8	67	195	18.5	0.6	0.22	0.20
Serpentine	6.3	0.38	4.2	6.90	1305	3683	0.4	149	71	16.5	86.2	0.61	0.25

[a] Combustion method (CHN Carlo Erba NC2500 analyser, Italy).
[b] 0.5 M sodium bicarbonate-extractable concentration (Unicam UV4-100, UK).
[c] 1 M ammonium acetate-extractable concentration, pH 7.0 (AAS Unicam 9200X, UK).
[d] 0.005 M DTPA–0.1 M triethanolamine–0.01 M $CaCl_2$-extractable concentration (AAS Unicam 9200X, UK).

treated with 7 ml of the autoclaved inoculum (121 °C twice for 25 min). To balance the initial microbial community other than AMF across the different inoculation treatments, all plants received 5 ml of the microbial filtrate from the complementary inoculum/inocula (i.e., *nm* plants were treated with the filtrate from *s* and *ns* inocula, *s* plants with the filtrate from the *ns* inoculum and *vice versa*). Microbial filtrates were prepared by the filtration of soil suspensions (1:10, w/v) through filter paper with a pore size of 15 μm to remove AMF propagules. Plants were grown from the beginning of June to the end of August 2009 in a greenhouse with natural light and supplementary 6-h irradiation provided by metal halide lamps (Philips HPI-T Plus, 400W). All plants were irrigated daily with distilled water until it drained from the pots.

2.3. Plant harvest

All plants were harvested after 12 weeks of cultivation. The shoots were cut off and their total leaf areas were assessed using an area metre (LI-3100, LI-COR, USA). To describe the development of AMF in the inoculated treatments, length of the extraradical mycelium (ERM) and mycorrhizal root colonisation were assessed. The length of ERM was estimated from a small homogenised sample (ca 15 ml) of the substrate cored from each pot, by means of a modified membrane filtration technique (Jakobsen et al., 1992). The total length of ERM was assessed by the grid-line intersect method under a compound microscope at 100× magnification and expressed as metres of hyphae in 1 g of air-dried substrate. The background lengths of dead hyphae remaining in the non-inoculated substrates after γ-sterilisation were subtracted from the ERM lengths recorded for the same substrate in the inoculated pots. The whole root systems were washed to remove soil debris, and a weighted root subsample (~0.5 g) was taken from each plant. These samples were stained with 0.05% trypan blue in lactoglycerol (Koske and Gemma, 1989) and used for the evaluation of mycorrhizal root colonisation by the magnified intersect method under a compound microscope at 100× magnification (McGonigle et al., 1990).

Dry biomass (further referred to as biomass) of the shoots and the remaining roots were recorded after drying for 24 h at 65 °C. Dry biomass of the root subsamples taken for the evaluation of mycorrhizal colonisation was inferred from the dry/fresh biomass ratio of the remaining roots, and the total root biomass was then calculated. Both shoot and root biomass was ground and digested in 65% HNO_3 and 30% H_2O_2. Shoot biomass was analysed for P, N, C, K, Ca, Mg and Ni concentrations. Ca, Mg and Ni concentrations were also determined in the roots to assess whether serpentine and non-serpentine plants differed in the relative allocation of these elements to belowground and aboveground tissues. Nickel concentrations were only analysed in the plants grown in the S substrate (due to the negligible concentrations in plants grown in the NS substrate). P concentration was assessed spectrophotometrically by the ammonium-molybdate ascorbic acid method at 630 nm (Unicam UV4-100, UK). C and N concentrations were determined with a CHN elemental analyser (Carlo Erba NC2500, Italy) and concentrations of K, Ca, Mg and Ni were analysed by an atomic absorption spectrometer (AAS Unicam 9200X, UK).

2.4. Data analysis

Mycorrhizal growth dependence (MGD) was calculated according to Smith et al. (2003) as a percentage increase in shoot biomass of an individual mycorrhizal plant over the mean shoot biomass of non-mycorrhizal plants in the respective treatment [MGD = 100 × (shoot biomass of an individual mycorrhizal

Table 3
The effects of population (nested within origin × cytotype combination), origin (*S*, serpentine vs. *NS*, non-serpentine), cytotype (diploid vs. tetraploid), substrate (*S*, serpentine vs. *NS*, non-serpentine), AM inoculation (*nm*, non-mycorrhizal vs. *ns*, non-serpentine isolate vs. *s*, serpentine isolate) and their interactions on growth parameters and mycorrhizal growth dependence (MGD) of *Knautia arvensis* plants, as well as on AMF development. For significant effects of single factors, the direction of change is indicated by arrows (e.g. ↑*M*,*s* denotes higher values for *M* than for *nm* plants and among *M* plants higher values for *s*- than for *ns*-inoculated plants).

Factor	df	Shoot biomass		Root biomass		Root-shoot ratio[a]		MGD[c]		AM root colonisation[b,c]		ERM length[a,c]	
Population	4	4.4	**	6.3	***	3.6	**	0.3	ns	1.2	ns	3.4	*
Origin	1	<0.1	ns	0.4	ns	1.5	ns	28.8	*** ↑*S*	0.3	ns	0.4	ns
Cytotype	1	0.5	ns	<0.1	ns	<0.1	ns	0.1	ns	1.4	ns	0.9	ns
Substrate	1	71.8	*** ↑*S*	31.9	*** ↑*S*	0.2	ns	39.8	*** ↓*S*	8.7	** ↓*S*	32.6	*** ↓*S*
AM inoculation	2	44.2	*** ↑*M*	21.5	*** ↑*M*,*s*	3.7	* ↓*ns*	3.9	* ↑*s*	6.9	** ↑*s*	0.2	ns
Origin × cytotype	1	16.0	*	3.8	ns	<0.1	ns	3.2	ns	<0.1	ns	1.7	ns
Origin × AM	2	2.9	ns	0.9	ns	<0.1	ns	1.6	ns	1.2	ns	1.3	ns
Substrate × cytotype	1	10.3	**	9.1	**	3.1	ns	3.9	*	<0.1	ns	9.4	**
Substrate × origin	1	17.3	***	54.8	***	47.9	***	7.2	**	<0.1	ns	36.5	***
Substrate × AM	2	21.4	***	9.0	***	0.1	ns	3.9	*	24.4	***	4.0	*
Origin × cytotype × AM	2	0.3	ns	0.4	ns	3.5	*	1.9	ns	<0.1	ns	2.6	ns
Substrate × origin × cytotype × AM	2	2.8	ns	1.2	ns	1.6	ns	1.04	ns	4.7	*	0.3	ns

The data in columns represent *F*-values with significance level marked as follows: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns non-significant. The interactions without any significant effect are not presented.
[a] Data transformed using $\log_{10}$ function.
[b] Data transformed using arcsine function.
[c] Only *M* plants considered (df = 1 for all factors but population where df = 4).

plant − average shoot biomass of non-mycorrhizal plants)/shoot biomass of an individual mycorrhizal plant].

All data were analysed with the Statistica 9.1 software (StatSoft Inc., USA), using the nested ANOVA model (GLM procedures). The model involved population (nested within origin × cytotype combination) as a random factor and plant origin, cytotype, substrate and AM inoculation as fixed factors. Prior to the analyses, all data were checked for normality and homogeneity of variances, and transformed using logarithmic ($\log_{10}$) or square root (sqrt) functions when necessary to meet the ANOVA assumptions (see Tables 3–5 for details). The data on percentage of mycorrhizal root colonisation were arcsine transformed. The post-hoc multiple comparisons were conducted for all significant effects ($P < 0.05$) using the Tukey HSD test. A linear regression was used to test the relationships of mycorrhizal root colonisation and ERM length with plant growth parameters, MGD and nutrient uptake, respectively.

3. Results

3.1. Mycorrhizal colonisation and ERM length

All inoculated plants (further referred to as *M*, mycorrhizal) were highly colonised by AMF, whereas no mycorrhizal structures were recorded in the roots of non-inoculated plants (*nm*, non-mycorrhizal). Both AM colonisation and ERM length were generally significantly lower in the S substrate; however, the substrate effect depended on the AMF isolate (Tables 3 and S1). The *ns* isolate formed significantly higher colonisation in the NS (94 ± 1%; mean ± SE) than in the S substrate (86 ± 1%) whereas the *s* isolate colonised equally in both substrates (*S*: 95 ± 1%; *NS*: 92 ± 1%). In comparison, both isolates formed significantly more ERM in the NS (1.9 ± 0.1 m g^{-1}) than in the S substrate (0.8 ± 0.1 m g^{-1}). Furthermore, the serpentine plants were associated with significantly more mycelium in the S substrate than their non-serpentine counterparts (1.1 ± 0.1 vs. 0.6 ± 0.1 m g^{-1}), while there was no difference between the cytotypes. In the NS substrate, no plant origin effect was observed but the tetraploids associated with significantly more ERM than the diploids (2.0 ± 0.2 vs. 1.6 ± 0.2 m g^{-1}).

3.2. Plant growth parameters

The data on plant growth parameters (leaf area, shoot and root biomass and root-shoot ratio) are summarised in Table S2. Due to a close correlation between leaf area and shoot biomass ($r^2 = 0.76$, $P < 0.0001$), only the latter was considered in further analyses. The inoculation with AMF generally improved plant growth (Table 3), however, the extent of plant benefit from AM inoculation was higher in the NS than in the S substrate (Fig. 1a). In the NS substrate, the inoculation with either AMF isolate significantly positively affected all plant growth parameters, in contrast to the S substrate where aboveground growth was significantly promoted only by the *s* isolate (Fig. 1a). Shoot biomass positively correlated with the percentage of mycorrhizal root colonisation in the NS substrate ($r^2 = 0.23$, $P < 0.0001$) and with the ERM length in both substrates ($r^2 = 0.16$, $P < 0.0001$ for both). As indicated by the significant substrate × origin interaction (Table 3), the serpentine plants had significantly higher root and shoot biomass in their native substrate, while the non-serpentine plants either showed similar (root biomass) or even significantly lower (shoot biomass) values in their native than in the S substrate (Fig. 1b). As a result, in the NS substrate the non-serpentine plants had significantly higher root-shoot ratios (0.67 ± 0.03) than the serpentine ones (0.55 ± 0.02), in contrast to the S substrate (0.49 ± 0.02 vs. 0.70 ± 0.02).

The ploidy level did not influence any growth parameter, but there was a significant substrate × cytotype interaction (Table 3). Both cytotypes grew similarly in the NS substrate, but diploids performed significantly better in the S substrate (shoot biomass of 2.8 ± 0.1 g) than the tetraploids (2.3 ± 0.1 g). Plant growth was affected also by origin × cytotype interaction (Table 3); non-serpentine diploids had significantly higher shoot biomass (2.6 ± 0.1 g) than their serpentine counterparts (1.9 ± 0.2 g) with the opposite being true for the tetraploids (1.8 ± 0.1 vs. 2.4 ± 0.1 g).

3.3. Mycorrhizal growth dependence

The MGD (%) was significantly influenced by plant origin, substrate and their interaction (Tables 3 and S1). Plants grown in the NS substrate showed generally significantly higher MGD (56 ± 3%) than in the S substrate (−16 ± 10%). In the S substrate, serpentine plants showed a considerably higher MGD than their non-serpentine counterparts; in contrast to comparable MGD in the NS substrate (Fig. 2). Moreover, significantly higher MGD was observed for the *s* isolate (6.0 ± 6.2%) under S conditions, compared to the *ns* isolate (−38.6 ± 17.8%). The significant inter-substrate difference in MGD was more pronounced for the tetraploids (65 ± 4 vs. −20 ± 15%) than diploids (44 ± 5 vs. −11 ± 8%).

Table 4
The effects of population (nested within origin × cytotype combination), origin (*S*, serpentine vs. *NS*, non-serpentine), cytotype (2x, diploid vs. 4x, tetraploid), substrate (*S*, serpentine vs. *NS*, non-serpentine), AM inoculation (*nm*, non-mycorrhizal vs. *ns*, non-serpentine isolate vs. *s*, serpentine isolate) and their interactions on Ca, Mg and Ni concentrations in the biomass of *Knautia arvensis* plants. For significant effects of single factors, the direction of change is indicated by arrows (for details, see Table 3).

Factor	df	Shoot Ca[b]	Root Ca[b]	Shoot/root Ca ratio[a]	Shoot Mg	Root Mg	Shoot/root Mg ratio[a]	Root Ni[a,c]
Population	4	11.2 ***	6.3 ***	17.8 ***	1.0 ns	12.1 ***	6.1 ***	0.5 ns
Origin	1	0.1 ns	0.1 ns	0.0 ns	9.2 * ↑*S*	0.1 ns	<0.1 ns	0.1 ns
Cytotype	1	1.0 ns	0.5 ns	0.9 ns	4.6 ns	0.0 ns	0.5 ns	20.5 ** ↑4x
Substrate	1	322.3 *** ↓*S*	159.4 *** ↓*S*	127.5 *** ↓*S*	275.7 *** ↑*S*	642.6 *** ↑*S*	0.3 ns	– –
AM inoculation	2	0.2 ns	13.4 *** ↓*M*	7.5 *** ↑*M*	15.1 *** ↑*M*	6.8 ** ↑*ns*	10.2 *** ↑*M*	1.3 ns
Origin × cytotype	1	0.0 ns	0.6 ns	0.1 ns	0.3 ns	1.0 ns	0.7 ns	20.3 **
Cytotype × AM	2	0.6 ns	3.6 *	2.7 ns	0.4 ns	1.5 ns	0.1 ns	3.0 ns
Substrate × cytotype	1	1.0 ns	1.6 ns	0.0 ns	10.7 **	0.1 ns	5.8 *	– –
Substrate × origin	1	9.2 **	2.3 ns	6.8 *	23.0 ***	14.8 ***	40.4 ***	– –
Substrate × AM	2	0.1 ns	12.1 ***	6.3 **	6.5 **	10.9 ***	23.1 ***	– –
Origin × cytotype × substrate	1	1.1 ns	6.3 *	10.4 **	0.6 ns	7.1 **	0.1 ns	– –

The data in columns represent *F*-values with significance level marked as follows: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns non-significant. The interactions without any significant effect are not presented.

[a] Data transformed using sqrt function.

[b] Data transformed using $\log_{10}$ function.

[c] Only the plants grown in the S substrate considered.

Table 5
The effects of population (nested within origin × cytotype combination), origin (*S*, serpentine vs. *NS*, non-serpentine), cytotype (diploid vs. tetraploid), substrate (*S*, serpentine vs. *NS*, non-serpentine), AM inoculation (*nm*, non-mycorrhizal vs. *ns*, non-serpentine isolate vs. *s*, serpentine isolate) and their interactions on P, N and K concentrations, and N:P and C:N ratios in shoot biomass of *Knautia arvensis* plants. For significant effects of single factors, the direction of change is indicated by arrows (for details, see Table 3).

Factor	df	Shoot P		Shoot N[a]		Shoot N:P[a]		Shoot C:N[a]		Shoot K[a]	
Population	4	4.0	**	11.7	***	4.2	**	10.6	***	5.8	***
Origin	1	6.6	ns	0.2	ns	3.6	ns	0.2	ns	25.2	** ↑*S*
Cytotype	1	0.6	ns	0.0	ns	1.1	ns	<0.1	ns	0.9	ns
Substrate	1	4.9	ns	411.6	*** ↓*S*	290.9	*** ↓*S*	398.8	*** ↑*S*	94.4	*** ↑*S*
AM inoculation	2	128.2	*** ↑*M*	43.7	*** ↓*M*	241.4	*** ↓*M*	44.9	*** ↑*M*	92.7	*** ↓*M*
Origin × cytotype	1	2.5	ns	5.5	ns	1.1	ns	5.4	ns	8.9	*
Origin × AM	2	4.3	*	15.8	***	0.1	ns	16.2	***	1.5	ns
Substrate × origin	1	1.1	ns	3.7	ns	15.5	***	3.5	ns	2.9	ns
Substrate × AM	2	26.3	***	5.6	**	17.1	***	5.4	**	23.4	***
Origin × cytotype × substrate	1	0.5	ns	3.4	ns	5.5	*	4.2	*	6.3	*
Origin × cytotype × AM	2	1.4	ns	3.9	*	0.4	ns	3.7	*	11.2	***
Substrate × origin × AM	2	0.1	ns	0.5	ns	0.2	ns	0.8	ns	3.4	*

The data in columns represent *F*-values with significance level marked as follows: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns non-significant. The interactions without any significant effect are not presented.
[a] Data transformed using $\log_{10}$ function.

3.4. Element concentrations in plant biomass

Both shoot and root calcium concentrations as well as shoot/root Ca ratio were markedly higher in plants grown in the NS substrate (Tables 4 and S3). In contrast to the S substrate, Ca accumulation in plant tissues was however dependent on plant origin and AM inoculation. The non-serpentine plants had significantly higher shoot Ca concentrations and shoot/root Ca ratios than their serpentine counterparts (Fig. 3a). Further, the AM inoculation significantly reduced root Ca concentrations (2828 ± 126 mg kg^{-1} in *nm* plants vs. 2095 ± 72 mg kg^{-1} in *M* plants), resulting in higher shoot/root Ca ratios (Fig. 3b).

In contrast to calcium, plants grown in the NS substrate showed considerably lower shoot and root magnesium concentrations (Tables 4 and S3). The non-serpentine plants had similar shoot but significantly lower root Mg concentrations compared to the serpentine plants in the NS substrate, but significantly lower shoot and higher root Mg concentrations in the S substrate (Fig. 3a). As a result, non-serpentine plants showed significantly higher shoot/root Mg ratios than their serpentine counterparts in the NS substrate, whereas the opposite was true in the S substrate. As for the effect of AM inoculation, the *M* plants showed significantly higher shoot Mg concentrations and shoot/root Mg ratios compared to *nm* plants in the NS substrate (Fig. 3b). In the S substrate, AM inoculation significantly increased root Mg concentrations; this increase was significantly higher for the *ns* than for the *s* isolate. Consequently, the *nm* plants had significantly higher shoot/root Mg ratios in the S than in the NS substrate, while the *M* plants behaved in the opposite way (Fig. 3b). In the NS substrate, the diploids had significantly lower Mg concentrations than the tetraploids (4012 ± 171 vs. 4859 ± 203 mg kg^{-1}); no inter-cytotype difference was recorded in the S substrate.

Shoot Ni concentrations in the S substrate were significantly influenced only by population ($F_{4,146} = 3.15$, $P = 0.016$), as were shoot/root Ni ratios ($F_{4,146} = 2.89$, $P = 0.024$); therefore these parameters are not presented in Table 4. In comparison, root Ni concentrations were significantly affected by cytotype and its interaction with plant origin (Table 4 and S4). Among non-serpentine plants, the diploids had significantly lower root Ni concentrations than the tetraploids (144 ± 7 vs. 209 ± 11 mg kg^{-1}); the serpentine cytotypes did not differ in this respect.

Shoot phosphorus concentrations were significantly increased by AM inoculation in both substrates (Tables 5 and S5). However, both isolates were significantly more effective in P uptake in their native soils (Fig. 4a). Further, the *nm* plants of non-serpentine

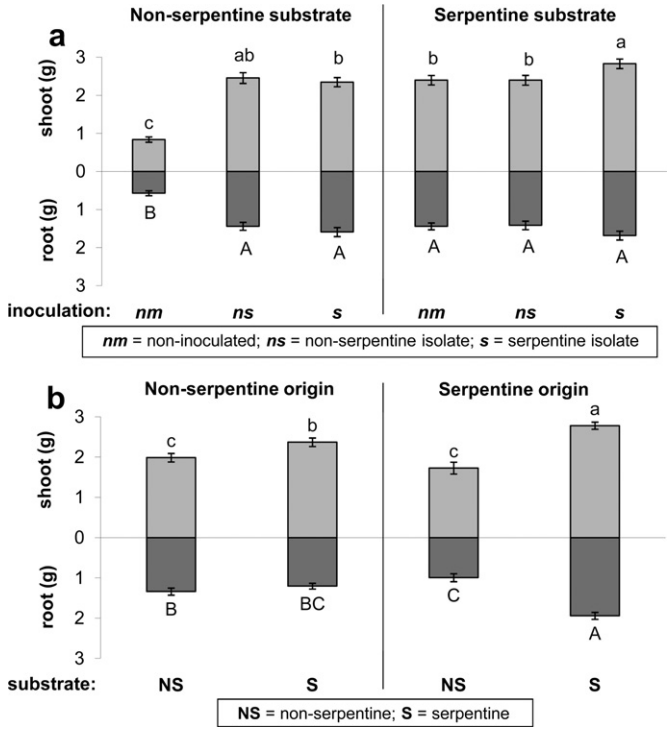

Fig. 1. Shoot and root dry biomass as affected by (a) substrate and AM inoculation and (b) plant origin and substrate. Columns represent means (±SE) of (a) 54–55 replicates; (b) 96 and 66–69 replicates for plants of non-serpentine and serpentine origin, respectively. Columns marked by different letters (a–c for shoot biomass; A–C for root biomass) are significantly different according to Tukey HSD test ($P < 0.05$).

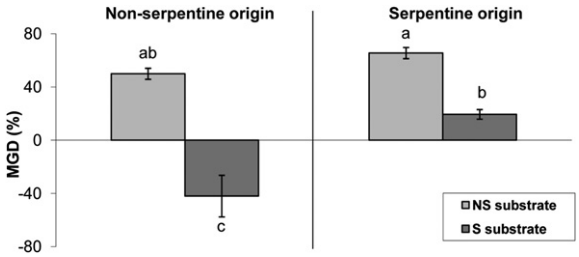

Fig. 2. Mycorrhizal growth dependence as affected by plant origin and substrate. Columns represent means (±SE) of 64 and 44–46 replicates for plants of non-serpentine and serpentine origin, respectively. Columns marked by different letters are significantly different according to Tukey HSD test ($P < 0.05$).

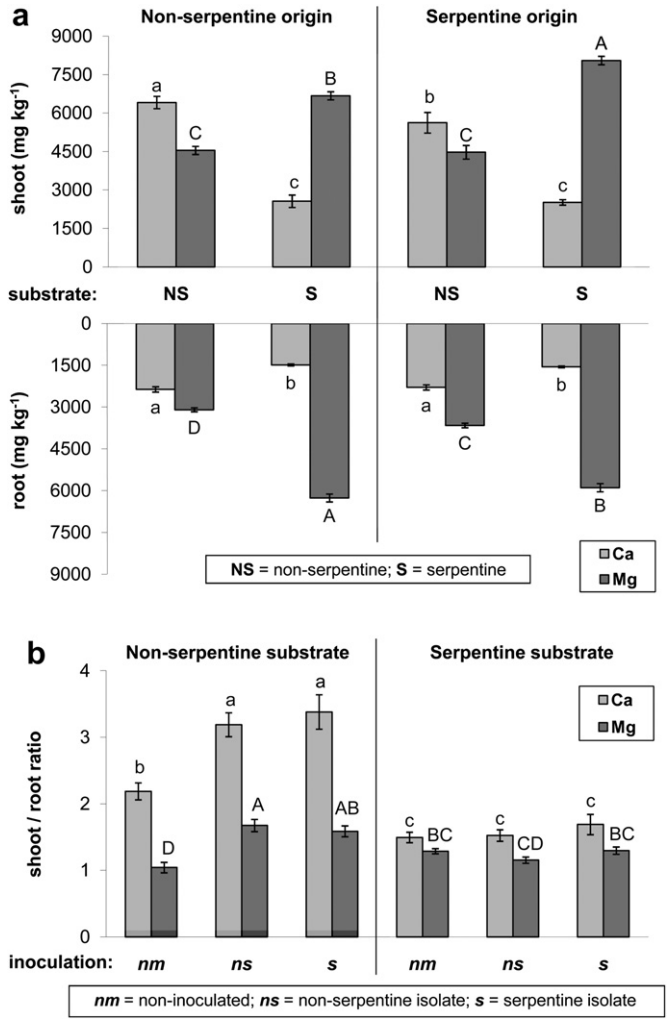

Fig. 3. (a) Calcium and magnesium concentrations in dry shoot biomass as affected by substrate and plant origin. (b) Shoot/root ratios of Ca and Mg concentrations, respectively, as affected by substrate and AM inoculation. Columns represent means (±SE) of (a) 96 and 66–69 replicates for plants of non-serpentine and serpentine origin, respectively; (b) 54–55 replicates. Columns marked by different letters (a–c for Ca concentrations and ratios; A–D for Mg concentrations and ratios) are significantly different according to Tukey HSD test ($P < 0.05$).

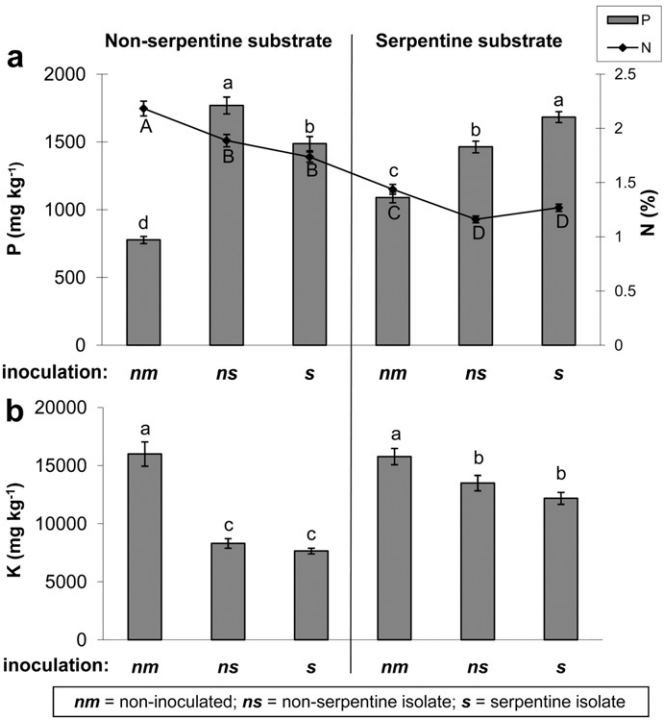

Fig. 4. Phosphorus and nitrogen (a) and potassium (b) concentrations in shoot dry biomass as affected by substrate and AM inoculation. Columns represent means (±SE) of 54–55 replicates. Columns marked by different letters (a–d for P and K concentrations; A–D for N concentrations) are significantly different according to Tukey HSD test ($P < 0.05$).

origin had significantly lower P concentrations (826 ± 24 mg kg^{-1}) than their serpentine counterparts (1097 ± 50 mg kg^{-1}), whereas *M* plants showed comparable P concentrations (averaging 1601 ± 26 mg kg^{-1}), irrespective of the AMF isolate and plant origin. Concerning shoot potassium concentration (Tables 5 and S5), the significant AM-mediated reduction was more pronounced in the NS than S substrate (Fig. 4b). The concentrations of K were significantly higher in serpentine than non-serpentine plants, with a greater difference in the diploids (17,752 ± 1151 vs. 9393 ± 236 mg kg^{-1}) than in the tetraploids (13,405 ± 563 vs. 11,687 ± 599 mg kg^{-1}).

Shoot nitrogen concentrations (Tables 5 and S5) were significantly higher in the *nm* plants compared to their *M* counterparts in both substrates (Fig. 4a). Furthermore, the *nm* plants of serpentine origin had significantly higher N concentrations (1.97 ± 0.10%) than those of non-serpentine origin (1.70 ± 0.06%), whereas *M* plants did not differ in this respect. As for shoot C:N ratio, the *M* plants showed significantly higher values in both substrates (*NS*: 26.5 ± 0.5; *S*: 39.0 ± 0.6) than the *nm* plants (22.3 ± 0.7; 33.5 ± 1.0). Both *ns*- and *s*-inoculated plants of serpentine origin had significantly higher C:N ratios (averaging 34.1 ± 0.9) than their *nm* counterparts (26.3 ± 1.4), whereas only *ns*-inoculated plants of non-serpentine origin (32.5 ± 1.2) differed significantly from *nm* non-serpentine plants (29.0 ± 1.0) in this respect. The significant decrease in shoot N:P ratio caused by AM inoculation was much more pronounced in the NS (*nm*: 29.1 ± 1.2; *M*: 12.1 ± 0.6) than in the S (13.6 ± 0.5; 8.1 ± 0.2) substrate. In the S substrate, the serpentine plants had significantly lower N:P ratios (8.5 ± 0.3) than their non-serpentine counterparts (11.0 ± 0.5); there was no plant origin effect in the NS substrate.

Concerning plant growth in relation to element uptake, the shoot biomass of plants grown in the NS substrate positively correlated with shoot P ($r^2 = 0.15$, $P < 0.0001$) and Mg ($r^2 = 0.26$, $P < 0.0001$) concentrations as well as shoot/root Mg ratio ($r^2 = 0.16$, $P < 0.0001$), but was negatively related with shoot K ($r^2 = 0.40$, $P < 0.0001$) and N ($r^2 = 0.24$, $P < 0.0001$) concentrations. In the S substrate, shoot biomass negatively correlated with root Mg ($r^2 = 0.12$, $P < 0.0001$), root Ni ($r^2 = 0.17$, $P < 0.0001$) and shoot N ($r^2 = 0.36$, $P < 0.0001$) concentrations.

4. Discussion

4.1. Plant edaphic differentiation

The ecological differentiation between serpentine and non-serpentine *K. arvensis* populations was evidenced by the better growth of the serpentine plants in the serpentine substrate compared to those of non-serpentine origin. This finding is in agreement with the results of a previous hydroponic cultivation experiment (F. Kolář et al., unpublished results) in which the same serpentine populations of *K. arvensis* showed higher tolerance to increased Mg concentrations than their non-serpentine counterparts. Despite this differentiation, host plant populations of both

origins surprisingly performed better in the S than in the NS substrate. On one side, the S substrate showed excessive Mg, high Ni and lower Ca concentrations, representing a chemical composition typical of serpentine soils. On the other side, the S substrate contained more organic matter and had higher pH value, slightly higher N and P concentrations and two-fold K concentration than the NS substrate. Although both substrates could be regarded as P-deficient (e.g. Schultz et al., 2001), lower macronutrient availability was the likely reason for the generally lower plant growth in the NS substrate. In fact, serpentine soils represent a highly variable group in terms of their chemical composition (Kazakou et al., 2008); therefore, a higher availability of some macronutrients in serpentine soils compared to non-serpentine ones is not exceptional (e.g. Branco, 2009).

A key aspect of the ecological differentiation between host plant populations under serpentine conditions concerns the Mg and Ca nutrition. Although the plants of different origin did not differ in Ca uptake and/or allocation, they showed clear divergence in within-plant distribution of Mg. The serpentine plants allocated more Mg into shoots, while non-serpentine plants accumulated Mg in roots. These results indicate a tolerance of the serpentine populations to excessive Mg in shoot tissues as shown previously for instance by Johnston and Proctor (1981) and Rajakaruna et al. (2003). In contrast, Mg exclusion was reported for serpentine populations of several plant species and was proposed as one of possible adaptive strategies under serpentine conditions (Main, 1974; O'Dell et al., 2006; Walker et al., 1955).

Concerning elevated Ni availability as another key feature of serpentine syndrome, Ni concentrations in *K. arvensis* biomass were more than four-fold lower in shoots than roots, lying well below the foliar toxicity threshold (50–100 mg kg^{-1}; Marschner, 2002). A strong root-shoot barrier for Ni translocation in both serpentine and non-serpentine plants was previously reported e.g. by O'Dell et al. (2006) and Westerbergh (1994). The lack of plant origin influence might be explained by too low Ni availability in the substrate (86 mg kg^{-1}) to trigger the expression of origin-derived differences in Ni uptake/tolerance, as suggested also by Sambatti and Rice (2007).

4.2. The role of AMF in plant–soil interactions

As for the differences between the two AMF isolates in their response to the S substrate, the serpentine isolate was characterised by higher root colonisation than its non-serpentine counterpart, though both isolates produced comparable length of extraradical mycelium. Reduced sporulation, spore germination and/or root colonisation of AMF were repeatedly shown in response to serpentine chemistry as a whole (Ji et al., 2010) or to its individual components (Amir et al., 2007; Gustafson and Casper, 2004; Jarstfer et al., 1998). Moreover, the AMF isolates also differed in their symbiotic efficiency, as only the combination of the serpentine plant populations and the serpentine isolate led to a positive mycorrhizal plant growth response. This pattern was closely related to the different effect of the AMF isolates on host plant P nutrition. AM symbiosis generally increased shoot P concentrations; however, both AMF isolates showed higher efficiency in their native substrates. These results suggest a functional relationship between particular AMF and edaphic conditions in terms of P uptake efficiency and subsequent plant growth promotion, as previously documented e.g. by Johnson et al. (2010) and van der Heijden et al. (2006).

Although AMF may increase also plant N uptake (Hawkins et al., 2000), the mycorrhizal plants in our study showed lower shoot N concentrations. In fact, the relative contribution of fungal N acquisition to the total plant N uptake largely depends on particular soil N supply, plant N status and AMF-host plant combination (Hodge et al., 2010; Reynolds et al., 2005). The mycorrhizal plants had also higher shoot C:N ratios (similarly to e.g. Chen et al., 2010; Miller et al., 2002), suggesting a relatively higher proportion of structural elements/cell walls and carbon-based secondary metabolites (Lambers et al., 1998). The decreased N concentrations possibly stem from generally higher N demands of AMF as compared to host plants which, in case of N-deficient soils limiting growth of both plants and fungi, likely result in N sequestration in fungal tissues (Johnson, 2010).

A more pronounced mycorrhizal growth promotion in the nutritionally poorer NS substrate is in accordance with a repeatedly recorded shift in mycorrhizal growth dependence and AM-mediated nutrient acquisition in response to changes in soil nutrient availability (e.g. Clark and Zeto, 2000; Koide, 1991), which was suggested as one of the plant-adaptive responses to low- vs. high-nutrient soils (Schultz et al., 2001). It is well established that the functioning of AM symbiosis and derived host plant benefits are affected by both N and P availabilities/limitations (e.g. Johnson, 2010; Sylvia and Neal, 1990; van der Heijden et al., 2006). An important clue for the recognition of limiting nutrient is regarded the N:P ratio in plant tissues (Koerselman and Meuleman, 1996). In our study, N:P ratio of the non-mycorrhizal plants indicates strong P-limitation in the NS substrate (29.4 $\pm$ 1.7) and weak N-limitation in the S substrate (13.1 $\pm$ 0.5). The general decrease of N and increase of P concentrations in response to AM inoculation led to a transition from P- to N-limitation in the NS substrate (N:P ratio of 12.1 $\pm$ 0.6) and a more intense N-limitation in the S substrate (8.1 $\pm$ 0.2). Such a shift of N:P ratio has been repeatedly reported as a consequence of AM-dependent modifications in plant and soil nutritional relations (e.g. Milleret et al., 2009; van der Heijden et al., 2006).

The effects of AM symbiosis on Ca and Mg uptake differed between the S and NS substrates. In general, the impact of AMF on plant Ca and Mg uptake and root-to-shoot translocation varies from positive to negative (e.g. Bermúdez and Azcón, 1996; Kothari et al., 1990; Pairunan et al., 1980). Previously reported concentration-dependent effect of AMF on the plant uptake of different cations (Clark and Zeto, 2000) makes it likely that different soil Ca and Mg concentrations were behind the observed inter-substrate differences in our study. The AM-mediated effect on Ca uptake (namely, decreased root concentrations) was restricted to the NS substrate with higher Ca availability. In comparison, the mycorrhizal plants in the NS substrate, with an almost twenty-fold lower Mg availability, showed increased shoot Mg concentrations and their positive correlation with plant growth. This beneficial effect of AMF on Mg nutrition under low to moderate Mg availability has been previously reported (Doubková et al., 2011; Liu et al., 2002; Taylor and Harrier, 2001). In contrast, no enhancement of shoot Mg (in line with Doubková et al., 2011), but increased root Mg concentrations due to AMF were observed in the Mg-rich S substrate. Moreover, higher Mg concentrations in the roots of mycorrhizal plants suggest that although the AMF were not capable of fully preventing enhanced AM-mediated Mg uptake from Mg-rich soil, they promoted Mg immobilisation in roots, either in intraradical hyphae or in root tissues. Interestingly, higher root Mg accumulation was recorded for the plants inoculated with the non-serpentine isolate, while the serpentine isolate proved a certain capacity to control Mg uptake from the soil. The AM inoculation did not influence plant Ni uptake, similarly to the study of Doherty et al. (2008) who found no effect of native serpentine AMF community on the shoot Ni concentrations of either serpentine or prairie *Sorghastrum nutans* ecotypes.

Unlike the uneven effects of AM inoculation on Mg and Ca uptake in the different substrates, the AM-mediated decrease in

shoot K concentrations was consistently observed in both substrates, although it was more pronounced in the NS substrate with markedly lower K availability. The decrease in shoot K concentrations might be a consequence of K accumulation in fungal structures in response to accumulation of polyphosphate, as proposed by Olsson et al. (2008). This hypothesis is supported by the fact that the AM-mediated increase of P uptake was higher in the NS substrate.

In contrast to classical views on polyploidy-associated changes in plant stature (Levin, 2002), no effect of ploidy level on the growth of *K. arvensis* plants was observed in our study. However, cytotype-specific differences in coping with adverse edaphic conditions of serpentine soils were indicated. When grown in the S substrate, the diploids produced much higher biomass than the tetraploids, which might be related to their lower root Ni concentrations. In the NS substrate, the tetraploids showed higher mycorrhizal growth dependence, presumably as a consequence of higher density of ERM. Similarly, inter-cytotype differences in mycorrhizal growth dependence were previously reported by Sudová et al. (2010). Nevertheless, due to parapatric distribution of different *K. arvensis* cytotypes we cannot exclude the possibility that the observed inter-cytotype differences might, at least partly, be explained by local adaptations of both cytotypes.

4.3. Conclusions

Our reciprocal transplant experiment proved the edaphic differentiation between serpentine and non-serpentine populations of *K. arvensis* as well as AMF isolates. The better performance of serpentine plants in the serpentine substrate was likely based on the tolerance to magnesium accumulation in their shoot tissues. The serpentine AMF isolate developed higher root colonisation in the serpentine substrate and was also more efficient in the growth promotion of and phosphorus uptake by the serpentine plants, suggesting a tolerance of the plant–mycobiont complex to the specific edaphic conditions. Considering only a slight impact of AM symbiosis on magnesium uptake and no influence on calcium and nickel uptake by the serpentine plants, the improved P nutrition seemed to be the crucial mechanism of the mycorrhizal promotion effect. Despite the beneficial effects of AM symbiosis on plant growth under serpentine conditions, the mycorrhizal growth dependence of the *K. arvensis* plants was generally higher in the nutritionally poorer non-serpentine substrate. Due to the antagonism between the AM-mediated effects on plant phosphorus and nitrogen uptake, AM symbiosis led to a switch from P- to N-limitation in the non-serpentine substrate and to increased N-limitation in the serpentine substrate.

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Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.soilbio.2011.09.011.

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