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The role of arbuscular mycorrhizal symbiosis in plant performance under the specific edaphic conditions of serpentine soils

Ph.D. Thesis

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Declarations:

I hereby declare that the thesis documents my own work and is not a subject of any other defending procedure. Throughout the thesis, I have properly acknowledged and cited all sources used. The thesis involves set of original results published in the international peer reviewed scientific journals. Those who contributed substantially to this work are noted as co-authors of the respective publications.

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I hereby declare that Pavla Doubková has substantially contributed to all publications, which represent an integral part of this Ph.D. thesis. She participated in designing, set-up and performance of the experiments, in data gathering, analyses and interpretation and manuscript preparation and writing (if not stated otherwise).

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Summary

Serpentine soils represent a unique environment characterized by unfavourable physicochemical properties involving low calcium to magnesium ratio, increased concentrations of heavy metals, often also deficiencies of essential macronutrients, and low water-holding capacity. Under these adverse conditions, a considerable potential of arbuscular mycorrhizal fungi (AMF) to promote plant growth was hypothesized due to the importance of arbuscular mycorrhizal (AM) symbiosis for plant nutrition and alleviation of various types of abiotic stress. On a model host plant species, *Knautia arvensis* (Dipsacaceae), we examined: i) occurrence of AM symbiosis and species richness and composition of the native AMF communities; ii) role of AM symbiosis in plant growth, element uptake and drought stress tolerance; iii) edaphic differentiation in plant populations or in AMF symbionts under serpentine vs non-serpentine conditions.

Generally, *K. arvensis* plants showed lower frequency of mycorrhizal root colonization at serpentine compared to the non-serpentine sites. Adjacent serpentine and non-serpentine populations also differed in AMF species assemblages colonizing their roots. Both, species composition and richness of these AMF communities depended primarily on edaphic parameters of the native soils (pH value and nickel concentration for composition; pH value, and soil chromium and potassium concentrations for richness).

AM symbiosis showed an overall beneficial effect on *K. arvensis* growth, with the mycorrhizal growth dependence of host plants determined mainly by nutritional status of the soil, regardless of its serpentine vs non-serpentine character. In contrast, the identity and complexity of AMF inocula generally played a minor role in determining the extent of mycorrhizal growth and nutrient uptake promotion. In serpentine substrates, improved phosphorus acquisition and drought stress alleviation were likely the crucial mechanisms of the beneficial influence of AM symbiosis, instead of any substantial modifications in calcium and magnesium nutrition or nickel uptake. The phytotoxicity effect of nickel seemed to be even increased by AM symbiosis at considerably elevated nickel availability. In the reciprocal transplant experiment, edaphic differentiation was proved for the selected serpentine and non-serpentine AMF isolates. The serpentine isolate developed higher root colonization and it was more efficient in growth promotion of and phosphorus uptake by the serpentine plants. Edaphic differentiation of *K. arvensis* populations was found under serpentine conditions in terms of plant growth and element uptake. No differences in calcium nutrition were recorded, while a tolerance to accumulation of magnesium in shoot tissues seemed to be one of the essential adaptive traits of serpentine *K. arvensis* plants.

Souhrn

Hadcové půdy představují specifické prostředí s nepříznivými fyzikálně-chemickými vlastnostmi, které zahrnují především vysoký obsah hořčíku, spolu s nízkým poměrem vápníku vůči hořčíku, zvýšené koncentrace některých těžkých kovů a často také nedostatek základních živin či nízkou vododržnou kapacitu půdy. Předložená práce vycházela z předpokladu, že za těchto nepříznivých podmínek může hrát významnou pozitivní roli v růstu a výživě rostlin jejich symbióza s arbuskulárně mykorhizními (AM) houbami. Ty zlepšují příjem živin hostitelskými rostlinami a pomáhají jim překonávat různé typy abiotického stresu. U zvolené modelové hostitelské rostliny – chrastavce rolního (*Knautia arvensis*, Dipsacaceae) jsme zjišťovali: i) přirozený výskyt AM symbiózy a druhovou bohatost a složení společenstev AM hub kolonizujících kořeny; ii) význam AM symbiózy v hadcových podmínkách pro rostliny z hlediska růstu, příjmu prvků a odpovědi při vystavení vodnímu stresu; iii) diferenciaci hadcových a nehadcových populací *K. arvensis*, resp. AM hub v reakci na hadcové podmínky.

Rostliny *K. arvensis* na hadcových lokalitách vykazovaly obecně nižší kolonizaci kořenů AM houbami než rostliny v nehadcových půdách. Přilehlé hadcové a nehadcové populace se přitom lišily druhovým složením společenstev AM hub kolonizujících jejich kořeny. AM společenstva byla v první řadě ovlivněna mikrostanovištními půdními parametry – hodnoty pH a koncentrace niklu působily na druhové složení AM společenstev; hodnoty pH, koncentrace chromu a draslíku na jejich bohatost.

AM symbióza měla celkově pozitivní vliv na růst rostlin *K. arvensis*, přičemž míru mykorhizní růstové odpovědi určoval především obsah živin v půdě, bez ohledu na její hadcový vs. nehadcový charakter. Identita AM izolátů a složení použitých AM inokul (jednotlivé izoláty vs. společenstva AM hub) měly, na rozdíl od obsahu živin v půdě, pouze minimální vliv na to, do jaké míry symbióza podpořila růst a příjem živin u hostitelské rostliny. Pozitivní působení AM symbiózy v hadcové půdě se pravděpodobně zakládalo především na zvýšení příjmu fosforu a zmírnění vodního stresu, nikoli na modifikaci příjmu či transportu hořčíku, vápníku nebo niklu. Při výrazně zvýšené dostupnosti niklu AM symbióza dokonce zřejmě ještě zesílila jeho toxický účinek na rostliny. V přesazovacím experimentu prokázaly vybraný hadcový a nehadcový AM houbový izolát edafickou diferenciaci, kdy hadcový izolát dosahoval v hadcové půdě vyššího procenta mykorhizní kolonizace kořenů a ve větší míře podporoval růst a příjem fosforu u hadcových rostlin. Dále byla doložena také edafická diferenciace populací *K. arvensis* – na základě jejich růstu a příjmu prvků. Zatímco v příjmu/transportu vápníku se mezi populacemi neprojevily rozdíly, pro hadcové rostliny byla jako jedna ze zásadních adaptací na růst v hadcové půdě rozpoznána tolerance k akumulaci hořčíku v pletivech nadzemní části rostlin.

Table of contents

1. Introduction **11**

1.1. Serpentine soils – specification of edaphic conditions 11

1.2. Impact of specific serpentine characteristics on plants 11

1.3. Adaptations and edaphic differentiation of serpentine plants 12

1.4. Involvement of AM symbiosis in plant performance under serpentine conditions 14

2. Aims, hypotheses and subjects of the study **17**

2.1. Main aims of the thesis 17

2.2. Working hypotheses 18

2.3. Model plant species and arbuscular mycorrhizal fungi 19

3. Overview of the conducted surveys and experiments **24**

4. Synthesis and discussion of the results **30**

4.1. Edaphic conditions at selected serpentine and non-serpentine sites **30**

4.2. Arbuscular mycorrhizal fungi native to the *K. arvensis* sites **30**

4.2.1. Mycorrhizal colonization in the roots of *K. arvensis* plants 30

4.2.2. Diversity of AMF colonizing *K. arvensis* roots 31

4.3. Effects of AM symbiosis on *K. arvensis* tolerance to serpentine syndrome **34**

4.3.1. Plant growth, nutrition and drought tolerance 34

4.3.2. Role of AMF identity 38

4.4. Edaphic differentiation of *K. arvensis* populations **39**

4.4.1. Plant growth and element uptake 39

4.4.2. Role of ploidy level in serpentine plant tolerance and interaction with AMF 41

4.5. Conclusions **43**

5. Manuscripts **44**

6. References **163**

7. Supplementary **172**

1. Introduction

1.1. Serpentine soils – specification of edaphic conditions

Serpentine soils are derived from the weathering of ultramafic rocks rich in ferromagnesian silicate minerals – the latter constitute more than 90% of the rock (Alexander et al., 2006). These soils form unique habitats found worldwide, but with patchy distribution (they occur primarily in California, Cuba, S Africa, S Europe, New Caledonia, SE Asia and W Australia), in total covering less than 1% of the Earth's land surface (Coleman and Jove, 1992).

Serpentine soils are characterized by specific physicochemical properties, collectively referred to as the serpentine syndrome (Jenny, 1980). Based on the chemistry of the source rock/minerals, the key feature of serpentine syndrome constitutes a low calcium to magnesium ratio – stemming typically from both low Ca and high Mg concentrations. The edaphic properties further include elevated, potentially phytotoxic concentrations of heavy metals (HM), especially nickel, chromium, cobalt or manganese. Frequently, serpentine soils are also deficient in essential macronutrients: phosphorus, nitrogen and potassium. The inhospitality of serpentine soils can be further increased by a low content of organic matter, coarse texture, shallow soil profile and vulnerability to erosion, which may all result also in a low soil water holding capacity. In general, serpentine soils represent a highly variable group concerning exact macro- and micronutrient plant-available concentrations, particular HM phytotoxicity, and intensity or frequency of drought. For reviews on edaphic characteristics of serpentine soils, see Proctor and Woodell (1971), Brady et al. (2005), Alexander et al. (2006), Kazakou et al. (2008), O´Dell and Rajakaruna (2011).

1.2. Impact of specific serpentine characteristics on plants

The low supply of soil Ca, together with the minimal mobility of this nutrient in plant tissues often results in plant Ca deficiency. Because of the very limited within-plant translocation, Ca deficiency symptoms (tissue necroses) develop at first in actively growing regions (new shoots and leaves, leaf margins, root tips; Taiz and Zaiger, 2010). The effect of high Mg availability on plant physiology, on the other hand, is based mainly on indirect mechanisms. This concerns primarily

antagonistic interactions among elements at the level of root uptake. Excessive Mg^{2+} ions compete with other cations, including Ca^{2+}, for sorption sites on the plasma membrane of root cells and can consequently induce or further enhance Ca deficiency (Yermiyahu et al., 1994; Shabala and Hariadi, 2005). In addition, the concentration of cytosolic Mg influences cytosolic pH and therefore can alter enzymatic activity, among others of key photosynthetic enzymes (Marschner, 2002; Shaul, 2002). The resultant effect of a low Ca:Mg ratio on plant performance thus depends on the precise soil Ca and Mg concentrations, and their combination (McGahan et al., 2009). Moreover, excessive Mg is stored in vacuoles, which can have detrimental consequences under drought stress conditions when accumulation of Mg ions increases plant osmotic stress (Taiz and Zeiger, 2010).

General mechanisms of HM toxicity primarily include the induction of oxidative stress (production of reactive oxygen species) and displacement of essential metal ions from biomolecules, for example from metalloenzymes containing metal atoms/molecules in positions important for their activity (e.g. displacement of Mg^{2+} in the Rubisco by Co^{2+}, Ni^{2+} or Zn^{2+} cations) leading to their functional inactivation (for reviews, see Palit et al., 1994; Schützendübel and Polle, 2002; Mithöfer et al., 2004). In serpentine soils, special attention has been paid to nickel as a metal with high plant-available concentrations, relatively high rate of root-to-shoot translocation and considerable plant toxicity (Seregin and Kozhevnikova, 2006; Kazakou et al., 2008). Although nickel ranks among essential micronutrients, plants need only a very low tissue Ni concentration for normal growth (on average in the order of 10^{-1} μg g^{-1}; Marschner, 2002). Based on the above-mentioned mechanisms, excessive Ni availability can lead to restricted cell division and expansion, disruption of photosynthesis, inhibition of root growth and branching, interveinal chlorosis of young leaves, necrosis, and wilting (Schützendübel and Polle, 2002; Chen et al., 2009; Nagajyoti et al., 2010).

1.3. Adaptations and edaphic differentiation of serpentine plants

The combination of the mentioned aspects of serpentine syndrome limits plant growth, resulting in low plant productivity and in a range of plant morphological and/or physiological adaptations (Brady et al., 2005). Moreover, multiple permanent abiotic stress conditions can accelerate speciation processes, which have lead to the evolution of serpentine vegetation distinct from

adjacent areas and with high levels of endemism (Tilstone and Macnair, 1997; O'Dell and Rajakaruna, 2011).

Serpentine plants evolve various strategies to cope with the unfavourable chemical and physical conditions of the soil, which has repeatedly been evidenced by the intraspecific ecotypic differentiation between serpentine and non-serpentine populations, varieties or subspecies (O'Dell and Rajakaruna, 2011) – in terms of their germination, growth, survival ability or changed pattern of element uptake (Nagy and Proctor, 1997; Taylor and Levy, 2002; Nyberg Berglund et al., 2003; Rajakaruna et al., 2003b). As suggested for example by Main (1974) and Rajakaruna et al. (2003b), edaphic differentiation among each pair of non-serpentine and serpentine populations can be based on diverse parallel mechanisms (responding to more aspects of edaphic growth conditions), not a single one. Considering the variability in serpentine chemistry, it seems plausible that particular edaphic parameters constituting the decisive selective pressure on the local vegetation differ depending both on individual plant species (Lazarus et al., 2011) and the serpentine site (Chiarucci, 2003; Nyberg Berglund et al., 2003; Kazakou et al., 2008). The latter is supported also by the fact that edaphic adaptation may be induced by serpentine soil heterogeneity within each field site in terms of, for example, water content or N, Ca, Mg or K availability (Reynolds et al., 1997; Yost et al., 2012).

Modifications of element uptake or within-plant element translocation are among the ways how plants respond/adapt to either low or excessive soil availabilities of different elements. Serpentine plants vary in their response to low values of the Ca:Mg ratio, ranging from a plain tolerance to Ca deficiency and Mg toxicity, to selective uptake of low-available Ca and/or exclusion of excessive Mg or even to permanently increased plant Mg requirement (Brady et al., 2005; Kazakou et al., 2008). They have also adopted various mechanisms of HM avoidance or tolerance – exclusion, tolerance to toxic effects or compartmentalization of accumulated HM in certain tissues or cellular structures (Kazakou et al., 2008; O´Dell and Rajakaruna, 2011). As for Ni, its deposition into leaf epidermal cells (particularly into vacuoles) or retention in the root cortex (especially in the apoplast) have so far been documented for hyperaccumulating plants, using different microscopic, spectrometric and microanalytical approaches (Psaras et al., 2000; Mesjasz-Przybylowicz et al., 2007; Moradi et al., 2010).

Xeromorphic leaves, reduced stature and higher root:shoot ratios are considered to be common morphological features of serpentine plants that help them cope with the adverse soil water conditions in serpentine habitats (Brady et al., 2005; Anacker et al., 2011). Changes in

reproduction timing or the size of the seed set can also constitute parameters subjected to the adaptive response of serpentine populations/races to varying water holding capacity of their native soil (e.g. Rajakaruna et al., 2003a; Sambatti and Rice, 2007; Wu et al., 2010).

1.4. Involvement of AM symbiosis in plant performance under serpentine conditions

In addition to various morphological and physiological adaptations, serpentine plants may also exhibit variation in their affinity to or dependence on symbiotic partnership with arbuscular mycorrhizal fungi (AMF) as compared to their counterparts growing in non-serpentine soils. AMF belonging to the phylum Glomeromycota (Schüssler et al., 2001) form the most widespread type of symbiosis between plant roots and fungi, being obligate symbionts colonizing roots of most vascular plants (Smith and Read, 2008). These AMF entirely depend on host plants to supply them with photosynthetically derived carbon (accounting for up to 20% of total fixed C of host plants; Smith and Read, 2008). Among the soil biota, AMF have a special position because they constitute a large fraction of soil microbial communities (Hodge and Fitter, 2010) and provide a direct link between plant roots and soil, and thus play a prominent role in plant-soil and plant-plant interactions (Smith and Read, 2008). The extensive soil network of extraradical hyphae of AMF increases the volume of soil available for the acquisition of immobile nutrients, especially phosphorus, outside their depletion zones along roots. Therefore, taking into account also AMF-induced modifications in gene expression of certain nutrient transporters on both plant and fungal membranes, mycorrhizal roots take up these nutrients more efficiently than non-colonized root systems (Smith and Read, 2008). Several fungal phosphate transporters, both in intra- and extraradical hyphae of AMF, and also plant phosphate transporters expressed strictly in response to AM symbiosis have been identified (for a review, see Javot et al., 2007). Guether et al. (2009) also reported on plant ammonium transporter preferentially expressed in cells containing arbuscules. Furthermore, AMF also play a role in the maintenance of water balance (Augé, 2001), soil structure improvement through better soil aggregation (Rillig and Mummey, 2006), plant-pathogen and plant-herbivore interactions etc. (Smith and Read, 2008). As a result, AMF have frequently been found to alleviate different abiotic (e.g. nutrient deficiency, heavy metal or organic pollutant toxicity, drought or flooding) and biotic (pathogens, plant competition)

stresses under various conditions (for reviews, see e.g. Leyval et al., 1997; Augé, 2001; Pozo et al., 2010; Smith et al., 2010). All these facts contribute to a substantial influence of AMF on the structure and productivity of plant communities (van der Heijden et al., 1998, 2008).

The presence of AMF in serpentine habitats worldwide is unequivocal (e.g. Hopkins, 1987; Goncalves et al., 2001; Turnau and Mesjasz-Przybylowicz, 2003) and has even been reported for serpentine populations of plant families with very low or zero affinity to mycorrhiza such as Brassicaceae, Proteaceae or Cyperaceae (Goncalves et al., 2001; Boulet and Lambers, 2005; Lagrange et al., 2011). AMF diversity in serpentine habitats has recently been addressed in several studies. AMF species distribution and host plant specificity was at first determined based on the abundance of their spores in the rhizosphere of the serpentine and non-serpentine host plants (Castelli and Casper, 2003; Ji et al., 2010). However, AMF spores extracted from the rhizosphere soil represent only a part of AMF communities colonizing roots (Clapp et al., 1995). Moreover, determination of AMF based on morphology of their spores and hyphae has certain constraints, since only about 150 species have been distinguished by this approach (Smith and Read, 2008). Due to the huge progress of molecular tools in the past decade, current molecular techniques allow more detailed AMF identification and the taxonomy of Glomeromycota is currently undergoing a revision based on phylogenetic analyses of fungal DNA (Schüssler and Walker, 2010; Krüger et al., 2012). Fitzsimons and Miller (2010) and Schechter and Bruns (2008, 2013) were thus able to compare the AMF species diversity associated with serpentine and non-serpentine ecotypes of the same plant species using extracted fungal DNA. Such comparison was also addressed in the present study.

Serpentine AMF were suggested to have evolved various adaptive strategies parallel to serpentine vegetation, and thus to contribute to host plants in their coping with the conditions of serpentine soils (Castelli and Casper, 2003; Amir et al., 2008; Schechter and Bruns, 2008, 2013). Nevertheless, both beneficial and negative effects of AMF on serpentine plant growth have been reported (e.g. Ji et al., 2010; Lagrange et al., 2011 vs Castelli and Casper, 2003; Doherty et al., 2008). Although there are many comparative ecophysiological studies dealing with differences between serpentine and non-serpentine ecotypes/populations of plant species naturally forming AM symbiosis in their native environment (e.g. O'Dell et al., 2006; Wright et al., 2006; Wu et al., 2010), none of the studies have also considered the importance and functional involvement of AMF. Nevertheless, increased interest in the research of AM symbiosis in

serpentine habitats in the past few years promises substantial progress in this respect (Orłowska et al., 2011; Amir et al., 2013; Lagrange et al., 2013).

Generally, positive effects of AMF on host plant performance in serpentine soils may consist in modified macroelements uptake, amelioration of HM (mainly Ni) toxicity or alleviation of drought stress. Recently, enhanced P uptake (Amir et al., 2013) together with increased Ca but also Mg uptake (Orłowska et al., 2011) by mycorrhizal plants has been reported under serpentine conditions. AMF-induced reduction of Ni phytotoxicity may be reached by decreased Ni uptake and root-to-shoot translocation (Orłowska et al., 2011; Amir et al., 2013). Besides reduced leaf concentration of Ni, Orłowska et al. (2011) also observed AMF-induced modifications in the uptake of other HM, Co and Zn. Generally, AMF can immobilize heavy metals in the rhizosphere by secreted glycoprotein glomalin or other chelating compounds, by adsorption of metals to fungal cell walls or their chelation inside the hyphae (for reviews, see Göhre and Paszowski, 2006; Miransari, 2011). The role of AMF in serpentine plant tolerance to drought stress has not yet been addressed. In general, the alleviative effect of AM symbiosis under drought conditions is being achieved both directly and indirectly. Direct mechanisms include modifications of soil water retention properties, more efficient water uptake by fungal mycelium, either down-regulated (water conservation strategy) or up-regulated (to increase water flow) expression of AMF aquaporin genes in arbuscule-containing root cells, or modified foliar water relationship (Augé, 2001, 2004; Wu et al., 2007b; Smith et al., 2010). The indirect effect consists in better nutritional status, and thus stimulated plant growth/larger root systems of mycorrhizal plants (Al-Karaki et al., 2004; Subramanian et al., 2006), or in AMF involvement in the host plant drought response, including enhanced activity of antioxidative enzymes (Caravaca et al., 2005; Wu et al., 2007a) and lower accumulation of drought stress markers such as proline and abscisic acid (see e.g. review by Augé, 2001).

2. Aims, hypotheses and subjects of the study

2.1. Main aims of the thesis

I. Comparison of serpentine and non-serpentine sites with regard to the occurrence, species richness and composition of AMF communities, using a model host plant species.

II. Assessment of the role of AMF in growth, element uptake and drought tolerance of the model host plant species. Comparison of the relative beneficial effects of AM symbiosis under serpentine and non-serpentine conditions.

III. Assessment of the influence of edaphic origin and ploidy level on serpentine tolerance of the model plant species and its interactions with AMF.

2.2. Working hypotheses

I. Roots of both serpentine and non-serpentine individuals of the model plant species are colonized by AMF in the field.

- The frequency of mycorrhizal root colonization, species richness and composition of AMF communities colonizing roots differ between serpentine and non-serpentine sites.

II. AM symbiosis contributes to the adaptation of model plant species to serpentine soils.

- AM symbiosis has a generally positive effect on growth, nutrition and drought tolerance of the model host plant under serpentine conditions.
- The mycorrhizal benefits are generally higher under the adverse serpentine edaphic conditions than in non-serpentine soils.
- Serpentine AMF isolates show higher tolerance to the serpentine syndrome than their non-serpentine counterparts.
- Single AMF isolates obtained from native serpentine sites provide generally lower mycorrhizal benefit to their host plants than whole native AMF communities.

III. Both edaphic origin and ploidy level shape the serpentine tolerance of the model plant species and its interactions with AMF.

- Serpentine populations of the model plant species show higher tolerance to the serpentine syndrome compared to their non-serpentine counterparts.
- The highest serpentine tolerance is achieved when both plants and AMF of serpentine origin are combined.
- Diploid and tetraploid serpentine populations of the model plant species differ in their tolerance to serpentine conditions as well as in the profit derived from being mycorrhizal.

2.3. Model plant species and arbuscular mycorrhizal fungi

To fulfil the presented aims, we searched for a model mycorrhizal plant species inhabiting both serpentine and non-serpentine habitats in the Czech Republic. Based on previous taxonomical and karyological research reported by Kaplan (1998) and Kolář et al. (2009), we selected field scabious, *Knautia arvensis* (L.) J. M. Coult. (Dipsacaceae), for which initial evidence on ecotypic differentiation among serpentine and non-serpentine populations was available from a hydroponic experiment (F. Kolář, pers. comm.).

Knautia arvensis is a perennial herb common throughout most of Europe, inhabiting dry and mesophilous meadows, pastures, shrublands, open woods, forest margins and roadsides (Štěpánek, 1997). The species encompasses both serpentine and non-serpentine populations in Central Europe; serpentine and non-serpentine ecotypes are sometimes treated as separate subspecies or varieties (Kaplan, 1998; Kolář et al., 2009, 2012).

On both soil types, *K. arvensis* comprises two cytotypes – diploid and tetraploid (Fig.1). Diploid plants have been suggested to have evolved serpentine tolerance in response to the expansion of forest vegetation in interglacial periods of the Holocene when heliophilous plants like *K. arvensis* were restricted to small, isolated refugia devoid of forest cover such as serpentine outcrops. As indicated by molecular markers, serpentine tetraploids likely represent direct local autopolyploid derivatives of relict diploids (Kolář et al., 2012). Generally, polyploids differ from their diploid ancestors in phenotype as well as physiology, resulting in their different ecological niches (Levin 2002) and tolerance to abiotic stresses such as nutrient deficiency, drought, cold or salinity (e.g., Saleh et al., 2008; Liu et al., 2011; van Laere et al., 2011; Deng et al., 2012). We therefore hypothesized that the ploidy level might influence the plant's ability to sustain serpentine stress and interact under these stressful conditions with AMF, which substantially influence coping with abiotic stress and plant nutrient acquisition.

Initially, eight model populations of *K. arvensis* were selected in the Czech Republic and Slovakia to meet the full-factorial design, that is to involve both diploid and tetraploid populations originating from both serpentine and non-serpentine sites (S1–S4 and NS1–NS4 in Fig. 2). Because the distribution of non-serpentine populations is largely parapatric (tetraploids in the west give way to diploids in the east; Kolář et al., 2009), tetraploid populations originated from W Bohemia whereas their diploid counterparts were collected in Moravia and W Slovakia.

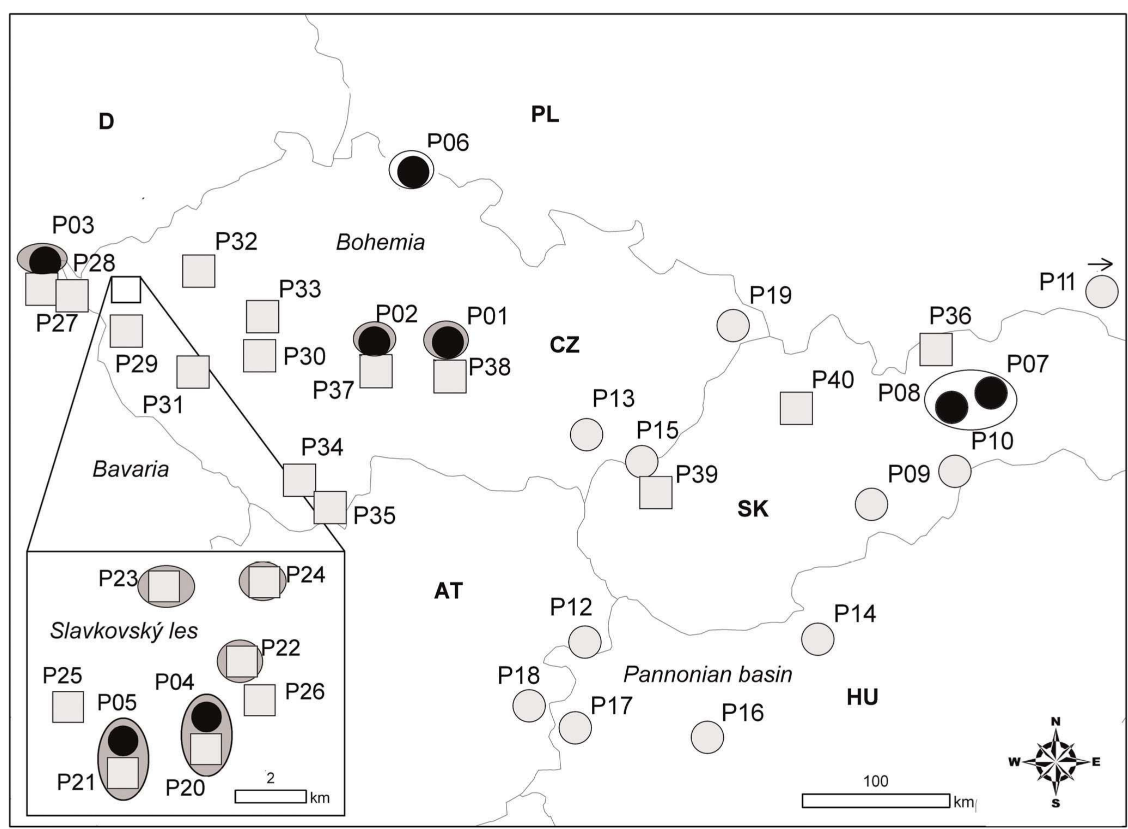
Due to extensive interspecific hybridization of tetraploid *K. arvensis* with closely related *K. kitaibelii* in the zone of their sympatry in Moravia and SE Bohemia (Štěpánek, 1997), we deliberately selected spatially distant non-serpentine tetraploids from W Bohemia to guarantee their non-hybrid status. Serpentine populations show a disjunct, island-like distribution, which mirrors the occurrence of major serpentine outcrops. Tetraploids are restricted to serpentine sites of the Slavkovský les Mts while the centre of distribution of serpentine diploids is located in few isolated outcrops in E Bohemia (Kaplan, 1998; Kolář, 2009). Recently, a few diploid patches were also found in the Slavkovský les Mts (Kolář et al., 2009, 2012); however, these were unknown at the beginning of the Ph.D. thesis.

In addition to the aforementioned eight model populations selected on a large geographic scale across edaphic origins and ploidy levels, fine-scale sampling was adopted when addressing species richness and composition of AMF communities in roots of tetraploid *K. arvensis* plants at adjacent serpentine and non-serpentine sites in the Slavkovský les Mts (S4–S6 and NS5–NS7).

As concerns the fungi, native AMF communities from the *K. arvensis* sites and/or single AMF isolates originating directly from *K. arvensis* rhizosphere soil were primarily used for plant inoculation in cultivation experiments. The AMF were isolated via a sequence of trap and multispore cultures, with *K. arvensis* being used as a host plant species. The obtained pure AMF isolates of serpentine origin were then permanently maintained in a mixture of serpentine soil and inert substrate (1 : 1, v/v) to maintain a selection pressure of the original serpentine conditions. In contrast to fungi originating from serpentine *K. arvensis* sites, the long-term process to obtain pure AMF isolates from the selected non-serpentine sites failed, so a reference non-serpentine AMF isolate (obtained from a collection of AMF isolates at the Institute of Botany) had to be used in the cultivation experiments. The reference AMF isolate originated from a mown mesotrophic meadow in the vicinity of a broad-leaved forest (Litožnice, Central Bohemia, Czech Republic, 50°04′10″ N, 14°36′30″E; altitude 230 m; Sudová and Vosátka, 2008). For the list of serpentine AMF isolates used for inoculation of *K. arvensis* plants, see Table 1; more details are presented in the respective publications in the Section 5. Both the AMF isolates and fungi colonizing the roots of plants inoculated with native AMF communities were identified using molecular techniques and subsequent phylogenetic analyses based on the partial SSU, whole ITS and partial LSU regions of nuclear ribosomal DNA.

Fig. 1 Survey of *Knautia arvensis* agg. distribution in Central Europe (Kolář et al., 2012)

Ploidy levels and habitat differentiation of populations of *K. arvensis* agg. were examined in a study by Kolář et al. (2012) based on a selection of 40 populations.



tetraploids (semiruderal grasslands)

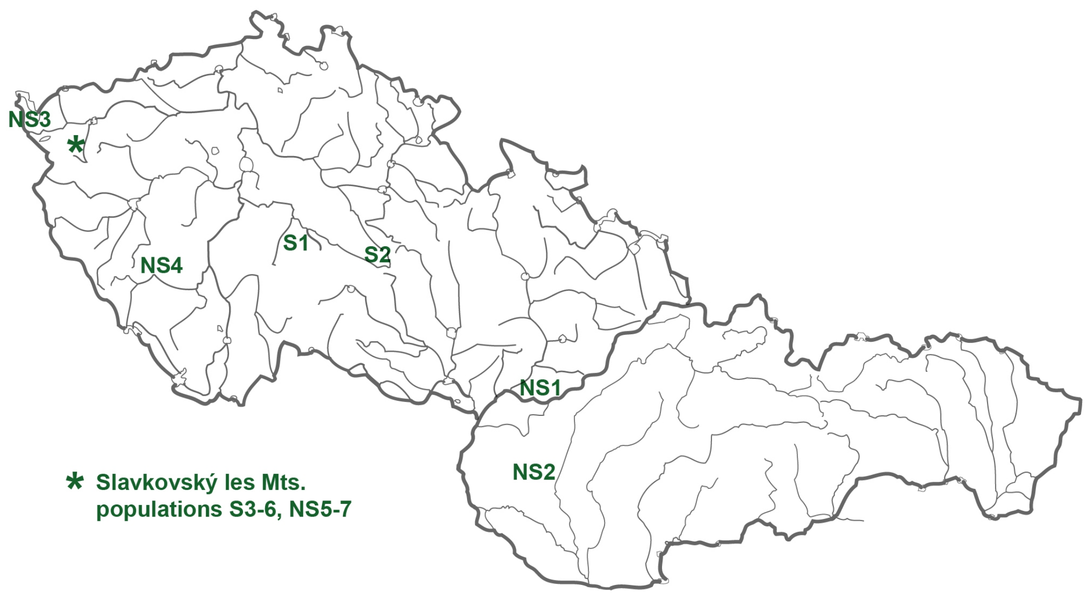
diploids from 'non-relict' genome size group (semiruderal grasslands)

diploids from 'relict' genome size group

relict limestone habitats (open pine forests or subalpine grasslands)

relict serpentine pine forests

Fig. 2 Serpentine and non-serpentine populations of *Knautia arvensis* involved in this thesis. The legend and other details are summarized in the table below.



Substrate type	Ploidy level	Population code	Site	Geographic coordinates	Altitude (m asl)	Manuscript No. (see Section 3.)
Serpentine	2x	**S1**	Borovsko (E Bohemia)	49°40'57.7" N, 15°07'49.7" E	400	1, 2
		S2	Staré Ransko (E Bohemia)	49°39′04.9" N, 15°48′57.3" E	640	1, 2
	4x	**S3**	Pluhův Bor (W Bohemia)	50°03′01.3" N, 12°46′24.3" E	710	1, 2, 3, 4
		S4	Křížky (W Bohemia)	50°03'54.2" N, 12°45'03.6" E	790	1, 2, 4, 5, 6
		S5	Dominova skalka (W Bohemia)	50°04′17.70″N, 12°47′11.10″E	750	6
		S6	Grünská kyselka (W Bohemia)	50°03'31.6"N, 12°47'15.8"E	680	6
Non-serpentine	2x	**NS1**	Tvarožná Lhota (S Moravia)	48°51'43.6" N, 17°23'23.3" E	290	1, 2
		NS2	Lajdovci (W Slovakia)	48°28'29.8" N, 17°38'59.2" E	230	1, 2
	4x	**NS3**	Aš (W Bohemia)	50°13'12.9" N, 12°13'19.2" E	670	1, 2, 4
		NS4	Chanovice (SW Bohemia)	49°24'39.0" N, 13°43'55.5" E	530	1, 2, 4, 5
		NS5	Křížky (W Bohemia)	50°03'45.4"N, 12°45'15.9"E	770	6
		NS6	Dominova skalka (W Bohemia)	50°04'26.4"N, 12°46'52.8"E	730	6
		NS7	Grünská kyselka (W Bohemia)	50°03'51.4"N, 12°47'39.6"E	750	6

Table 1 Serpentine AMF isolates used in the presented experiments and originating from the rhizosphere soil of *K. arvensis* plants.

AMF isolate	Code of source population	EMBL accession No.*	Manuscript No. (see Section 3.)
***Glomus* sp. SR**	S2	FR828471	2, 4,5
***Glomus* sp. PB**	S3	HE794038	3, 4
***Glomus* sp. DS**	S5	HE794039	4
***Acaulospora* sp. K**	S4	HE794040	4

* EMBL Nucleotide Sequence Database accession numbers

3. Overview of the conducted surveys and experiments

My contribution: As regards the manuscripts MS1–5, I substantially participated in designing, setting up and performing the experiments, analysing the data, interpreting the results and writing of the manuscripts.

MS 1. Initial field survey of AMF occurrence and mycorrhizal root colonization at both serpentine and non-serpentine *K. arvensis* sites, and pilot pot experiment involving native combinations of soils, plants and AMF communities.

Doubková P, Suda J, Sudová R (2011) Arbuscular mycorrhizal symbiosis on serpentine soils: the effect of native fungal communities on different Knautia arvensis *ecotypes. Plant and Soil 345: 325–338*

Field survey:

- eight *K. arvensis* populations: four serpentine (diploid S1 and S2, tetraploid S3 and S4), four non-serpentine (diploid NS1 and NS2, tetraploid NS3 and NS4)
- chemical analyses of rhizosphere soil
- evaluation of mycorrhizal root colonization

Pot experiment:

- eight *K. arvensis* populations (the same as above)
- each plant population grown in its native substrate
- two inoculation treatments per plant population: non-sterilized vs. γ-sterilized native substrate
- three-month greenhouse cultivation
- assessment of plant growth and leaf concentrations of photosynthetic pigments, shoot P, Ca, Mg and Ni concentrations, mycorrhizal root colonization

MS 2. Reciprocal transplant pot experiment combining serpentine and non-serpentine substrates, host plant populations and AMF isolates to evaluate edaphic differentiation of *K. arvensis* populations and AMF isolates.

Doubková P, Suda J, Sudová R (2012) The symbiosis with arbuscular mycorrhizal fungi contributes to plant tolerance to serpentine edaphic stress. Soil Biology and Biochemistry 44: 56–64

Pot experiment:

- eight *K. arvensis* populations: four serpentine (diploid S1 and S2, tetraploid S3 and S4), four non-serpentine (diploid NS1 and NS2, tetraploid NS3 and NS4)
- reciprocal transplant experiment with model serpentine and model non-serpentine substrate
- three inoculation treatments: control (inactivated inoculum), model serpentine AMF isolate, model non-serpentine AMF isolate
- three-month greenhouse cultivation
- assessment of plant growth, shoot P, N and K concentrations and C/N ratio, both shoot and root Ca, Mg and Ni concentrations, mycorrhizal root colonization and length of extraradical mycelium

MS 3. Pot experiment testing AMF role in alleviation of plant drought stress under serpentine conditions.

Doubková P, Vlasáková E, Sudová R (2013) Arbuscular mycorrhizal symbiosis alleviates drought stress imposed on Knautia arvensis *plants in serpentine soil. Plant and Soil 370: 149–161*

Pot experiment:

- serpentine *K. arvensis* population (S3) experiencing drought stress under natural conditions
- native serpentine substrate
- four water (irrigation) regimes: 55, 45, 35 and 25% of soil field capacity
- three inoculation treatments: control (inactivated inoculum), serpentine AMF isolate and complex AMF community, both native to the plant population site
- three-month cultivation in a growth chamber
- assessment of plant growth, net photosynthetic rate and transpiration, root concentrations of proline and abscisic acid, shoot P, N and K concentrations, mycorrhizal root colonization

MS 4. Pot experiment comparing the effects of different serpentine AMF isolates and native AMF communities on serpentine and non-serpentine *K. arvensis* plants in their native substrates.

Doubková P, Kohout P, Sudová R (2013) Soil nutritional status, not inoculum identity, primarily determines the effect of arbuscular mycorrhizal fungi on the growth of Knautia arvensis *plants. Mycorrhiza 23: 561–572*

Pot experiment:

- four *K. arvensis* populations: two serpentine (S3, S4) and two non-serpentine (NS3, NS4)
- each of the population grown in its native substrate
- six inoculation treatments: control (inactivated inoculum), four different serpentine AMF isolates, complex AMF community native to the respective population
- six-month greenhouse cultivation
- assessment of plant growth and flowering, shoot P, N, Ca, Mg and Ni concentrations, mycorrhizal root colonization

MS 5. Semi-hydroponic study testing the influence of elevated Ni availability on *K. arvensis* plants and AMF isolates performance with respect to their edaphic origin.

Doubková P, Sudová R (2014) Nickel tolerance of serpentine and non-serpentine Knautia arvensis *plants as affected by arbuscular mycorrhizal symbiosis, Mycorrhiza 24: 209–217*

Semi-hydroponic experiment:

- selected serpentine (S4) and non-serpentine (NS4) *K. arvensis* population
- both populations grown in an inert substrate, being supplied with nutrient solutions
- three Ni treatments: control (0 µM Ni), 50 µM Ni- and 100 µM Ni-supplemented nutrient solutions
- three inoculation treatments: control (inactivated inoculum), model serpentine AMF isolate, model non-serpentine AMF isolate
- three-month greenhouse cultivation
- assessment of plant growth and leaf concentrations of photosynthetic pigments, both shoot and root P, Ca, Mg and Ni concentrations, mycorrhizal root colonization

MS 6. Comparison of species richness and composition of AMF communities colonizing *K. arvensis* roots at adjacent serpentine and non-serpentine sites.

Kohout P, Doubková P, Bahram M, Suda J, Tedersoo L, Voříšková J, Sudová R.: Niche partitioning in arbuscular mycorrhizal communities in temperate grasslands: a lesson from adjacent serpentine and non-serpentine habitats

Field survey:

- six *K. arvensis* populations: three pairs of adjacent serpentine (S4, S5, S6) and non-serpentine (NS5, NS6, NS7) populations
- survey of surrounding vegetation
- chemical analyses of rhizosphere soil
- evaluation of mycorrhizal root colonization
- assessment of species richness and composition of AMF communities colonizing the roots of *K. arvensis* plants using 454 sequencing

My contribution: I was involved in field sampling and processing of soil and root samples (DNA isolation and PCR amplification, preparation of samples for 454 sequencing) and participated in the preparation of manuscript.

4. Synthesis and discussion of the results

4.1. Edaphic conditions at selected serpentine and non-serpentine sites

Generally, chemical analyses of soil samples collected from the selected serpentine *Knautia arvensis* sites proved lower Ca and higher Mg available concentrations resulting in a lower Ca:Mg ratio accompanied by higher concentrations of heavy metals (Ni, Cr, Co, Fe, Mn). In contrast to often reported macronutrient limitation and low content of organic matter in serpentine soils (Kazakou et al., 2008; O´Dell and Rajakaruna, 2011), N and organic C contents were generally higher in our serpentine compared to the selected non-serpentine habitats, while the concentrations of P highly varied among sites, regardless of soil type.

For the serpentine populations, both among- and within-site variations in edaphic parameters were found in our data sets in accordance with the reported variability of serpentine soils (Kazakou et al., 2008; McGahan et al., 2009; Lazarus et al., 2011). The differences in concentrations of some elements recorded for native substrates excavated independently for the subsequent experiments from the same sites were likely behind the observed variation in plant and fungal performance. This variation in serpentine soil properties has to be taken into account when interpreting results of the presented experiments.

4.2. Arbuscular mycorrhizal fungi native to the K. arvensis sites

4.2.1. Mycorrhizal colonization in the roots of K. arvensis plants

All *K. arvensis* plants sampled in the field (MS1, MS6) were colonized by AMF, and the mycorrhizal root colonization averaged 76 ± 2 % (mean ± SE) of root length. On a larger geographical scale (MS1), serpentine plants were generally colonized to a lower extent (72 ± 3 %) compared to non-serpentine plants (88 ± 1 %). We suppose that, lower development of AMF in the roots of *K. arvensis* in serpentine soils might be ascribed specifically to elevated Ni availability (Vivas et al., 2006; Amir et al., 2007; Doherty et al., 2008; see also the results of semi-hydroponic experiment in MS5), to high Mg (inducing low tissue Ca:Mg ratio; Jarstfer et al.,

1998) or low Ca availability (Gustafson and Casper, 2004). In accordance with the latter two studies, mycorrhizal root colonization of *K. arvensis* plants in our field survey positively correlated with soil Ca concentration and Ca/Mg ratio.

On a smaller geographical scale (MS6), the role of serpentine vs non-serpentine habitat in determining the level of *K. arvensis* mycorrhizal colonization was confirmed after eliminating the effects of other studied soil parameters. From these particular soil parameters, only the available concentration of Fe was identified to have a significant independent (reducing) effect on the level of root colonization. Due to the origin in ferromagnesian silicate minerals, serpentine soils are often characterized by elevated Fe concentrations (Alexander et al., 2006); however, evidence of a significant negative impact of higher Fe availability on AMF performance under serpentine conditions has not yet been submitted. Generally, the toxicity of high Fe concentrations has been suggested because of the involvement of iron in redox reactions, possibly leading to oxidative stress (Meharg, 2003; Smith and Read 2008). In contrast to the larger-scale survey (MS1), the relationship between the level of mycorrhizal colonization of *K. arvensis* roots and soil Ca availability or Ca/Mg ratio was not confirmed in MS6. Therefore, rather interplay between more soil parameters (or complex serpentine syndrome) seems to be responsible for the difference in mycorrhizal root colonization recorded between the serpentine and non-serpentine sites.

4.2.2. Diversity of AMF colonizing K. arvensis roots

In the adjacent serpentine and non-serpentine *K. arvensis* populations (MS6), both richness and composition of AMF communities colonizing their roots were primarily affected by soil characteristics. After eliminating the influence of individual studied soil parameters (discussed below), serpentine vs non-serpentine habitats did not differ in the AMF richness, but they were characterized by AMF communities with distinct species composition. The other studies comparing AMF assemblages associated with selected plant species in serpentine vs non-serpentine soils provided quite conflicting findings. Schechter and Bruns (2008, 2013; for AMF colonizing the roots of a model plant species), and Ji et al. (2012; for AMF spore communities in the rhizospere soil of a model plant species) reported a clear distinction between both soil types in different regions of the United States, whereas Fitzsimons and Miller (2010; for AMF in the roots) found no such difference in Portuguese serpentine soils. The latter authors compared their study with the results of Schechter and Bruns (2008), and provided two possible explanations of the

contradiction. Their first hypothesis stems from theories of island biogeography and metapopulation dynamics, considering the substantially larger area of serpentine soils in California than in Portugal as a feature generally supporting higher endemic richness. However, our results do not support this hypothesis because serpentine sites in the Czech Republic (or generally in Central Europe) show the same patchy distribution and limited areas as in Portugal. In the second explanation, they considered a higher pH value, reduced Ca:Mg ratio and cation-exchange capacity (CEC) in the Californian compared to the Portuguese serpentine soils as potentially significant factors shaping composition of AMF communities, possibly more important than relative differences in overall edaphic chemistry between serpentine and neighbouring non-serpentine sites. The same trends for pH and especially Ca:Mg ratio values can be seen also in the study of Ji et al. (2012), CEC were not evalulated in their survey.

In accordance with Fitzsimons and Miller (2010), our study provides evidence of the substantial role of soil pH value in shaping both the composition and richness of AMF communities in roots of *K. arvensis*; the same has also been documented, for example, by Fitzsimons et al. (2008), Toljander et al. (2008) and Dumbrell et al. (2010) for different soil types. Generally, the importance of the pH value stems from its impact on the availability of certain elements, on their solubility or bonds in the soil environment (Marschner, 2002; Helgason and Fitter, 2009). The differences in serpentine vs non-serpentine AMF communities of *K. arvensis* plants likely originated from different pH optima of individual AMF species. AMF variability in the tolerance to or preference of particular soil pH was previously evidenced based on differences between AMF isolates in spore production or germination, growth of and enzymatic activity in extraradical mycelium, in mycorrhizal root colonization, and arbuscule and vesicle formation (Vosátka et al., 1999; van Aarle et al., 2002; Klugh and Cumming, 2007).

In addition to serpentine conditions in general (i.e. when freed from effects of particular soil characteristics) and soil pH value, the concentration of soil Ni was identified as another significant factor structuring AMF assemblages in *K. arvensis* roots (as reported also by Ji et al., 2012, for four grass species). This likely originates from the aforementioned effects of Ni on AMF development (4.2.1.) and also from differences in Ni tolerance among AMF species/isolates, as documented for spore germination and mycorrhizal root colonization (Amir et al., 2008, 2013; see also MS5 study).

Although we also detected a significant effect of the site (i.e. geographical distance of sampling points) on AMF community composition, the influence of edaphic factors prevailed.

On a broader geographic scale, also Ji et al. (2012) identified environmental conditions (particularly soil chemical properties) as the driving force in shaping AMF spore communities at serpentine vs prairie sites, in contrast to the lack of impact of host plant identity. Similarly, Schechter and Bruns (2013) excluded host genotype-AMF specificity between serpentine and non-serpentine ecotypes of a model plant species based on the results of a pot experiment, suggesting that soil characteristics have a substantial influence on the composition of AMF communities.

As stated above, soil parameters were also identified to have the main influence on richness of AMF communities colonizing *K. arvensis* roots in our study. Their richness was positively affected by soil pH, which might stem from a higher proportion of AMF species with higher pH optima within the range of pH 3.6–5.8 recorded at *K. arvensis* sites. The same trend (i.e. higher number of AMF species with increasing pH) has also been reported by Toljander et al. (2008) for a similar pH range of 3.85–6.28. The species richness of AMF communities associated with *K. arvensis* plants negatively correlated with the available concentration of chromium, in accordance with the results of Khan (2001) and Nakatani et al. (2011). Considering the lack of any significant effect of Cr on AMF species composition in our study, we suppose that the decrease in AMF diversity might represent an indirect result of potential negative impact of Cr on plant fitness (as a heavy metal, chromium has a direct detrimental impact on plant physiology; Babula et al., 2008; Hayat et al., 2012), and their lower "investment" into symbiotic fungi. In addition, more diverse AMF communities were also associated with lower soil K concentration, which is, as far as we know, the first report of such a relationship. In case of excessive K availability, plants might be exposed to imbalance in cation acquisition/nutrition caused by an interference of K^+ with other cations (Ca^{2+}, Mg^{2+}) at the level of root uptake, transport or metabolism (Egilla et al. 2001; Marschner, 2002; Shabala and Hariadi 2005).

Besides being influenced by edaphic properties, the richness of AMF communities associated with *K. arvensis* plants was also affected by the composition of their neighbouring plant community. There was no general connection between plant and AMF species richness, as could have been anticipated from the reported existence of plants-AMF relationship in this respect (see e.g. van der Heijden et al., 1998; Johnson et al. 2003, 2010; Meadow and Zabinski, 2012). Instead, four plant species had a significant impact on the richness of AMF associated with *K. arvensis* in their presence (two of them showing positive influence – *Veronica chamaedrys* and *Hypericum maculatum*; the other two negative – *Avenella flexuosa* and *Galium saxatile*). However, our data provided no clue to reveal potential mechanisms standing behind

these relationships. Similarly, König et al. (2010) proved a positive effect of plants of particular functional types, namely grass and tall herb species, on AMF community richness in a large-scale experiment manipulating plant biodiversity at natural site. And a stimulative effect of the occurrence of particular herb species on richness of AMF colonizing roots of model grass species was recorded also by Mummey et al. (2005). Therefore, based on our results and taking into consideration the local environmental conditions at *K. arvensis* sites, the particular plant species might serve as indicators of AMF diversity in the studied type of grasslands.

4.3. Effects of AM symbiosis on *K. arvensis* tolerance to serpentine syndrome

4.3.1. Plant growth, nutrition and drought tolerance

A beneficial effect of AM symbiosis on *K. arvensis* growth prevailed in most of the experiments. In contrast to our hypothesis, the relative importance of AM symbiosis was either comparable for both substrate types (MS1) or even higher in the non-serpentine compared to serpentine substrates (MS2, MS4), reflecting generally lower macronutrient availability in the non-serpentine substrates. These results are in accordance with a repeatedly recorded shift in mycorrhizal growth dependence and AMF-mediated nutrient acquisition in response to changes in soil nutrient availability (e.g. Clark and Zeto, 2000; Schultz et al., 2001; Smith and Read, 2008). The most obvious example of the relationship between nutrient availability and the extent of AMF-induced plant promotion was provided in the extremely nutritionally deficient non-serpentine substrate in the MS4 study. The non-mycorrhizal plants were not even able to survive in this substrate, while the mycorrhizal plants counterbalanced the nutrient limitation by higher investment into roots and generally high nutrient uptake efficiency, as indicated by considerably high P, N, Ca and Mg accumulation ratios (shoot/soil concentration ratios). Soil nutritional status also might explain the wide range of mycorrhizal growth effects reported in the MS1 study, where mycorrhizal growth promotion was recorded for two serpentine populations grown in their native soils with relatively low P availability and the lowest Ca:Mg ratios. Based on the results of pot experiments, soil P availability, together with the Ca:Mg ratio or absolute Ca and Mg concentrations were assumed to be the likely decisive factors determining the extent of the mycorrhizal benefit for particular *K. arvensis* populations.

In general, the functioning of AM symbiosis and derived host plant benefits are affected both by P and N availabilities or rather limitations (e.g. Sylvia and Neal, 1990; van der Heijden et al., 2006; Johnson, 2010). According to the N:P ratio in shoot tissues, which is used as an important clue for the recognition of limiting nutrient in particular soil (Koerselman and Meuleman, 1996; Güsewell, 2004; Johnson, 2010), *K. arvensis* plants grown in the non-serpentine substrate (MS2) were generally exposed to strong P limitation which was converted to weak N limitation by AMF inoculation. Analogously, the weak N-limitation in the serpentine substrate (MS2) was even intensified by AM symbiosis. Such a shift in the N:P ratio has been repeatedly reported to be a consequence of AMF-dependent modifications in plant and soil nutritional relations (e.g. van der Heijden et al., 2006; Milleret et al., 2009). For *K. arvensis* plants in our experiments, the changes in the N:P ratio actually stemmed primarily from inverse effects of AMF on P and N uptake.

In fact, improved phosphorus nutrition seemed to be predominantly one of the main causes of the beneficial effect of AM symbiosis on *K. arvensis* growth. Improved P nutrition was generally one of the most significant AMF-induced effects on plant element acquisition under both serpentine and non-serpentine conditions (MS1, MS2, MS3). Across all pot experiments, the overall pattern of rising mycorrhizal P uptake benefit with decreasing soil P availability can be noted. However, our data did not enable differentiation between relative contributions of the direct plant and AM pathways to plant P uptake. Actually, both pathways may act simultaneously, and their relative participation depends on soil nutrient availability (for review, see Smith and Smith, 2011) as well as on the identity of both plant and fungal symbionts (Pearson and Jakobsen, 1993; Smith et al., 2004; Facelli et al., 2010).

Similarly, the relative contribution of fungal nitrogen acquisition to total plant N uptake largely depends on the particular soil N supply, plant N status and AMF-host plant combination (Reynolds et al., 2005; Hodge et al., 2010). Although AMF may increase plant N uptake (e.g. Hawkins et al., 2000; Govindarajulu et al., 2005; Lee et al., 2012), mycorrhizal *K. arvensis* plants had mostly consistently lower shoot N concentrations compared to non-mycorrhizal plants, regardless of either N or P limitation of the cultivation substrates (MS2, MS3, MS4). Nevertheless, considering the consistently recorded negative correlation of shoot N concentrations with shoot dry weight, we cannot exclude the role of the biomass-dilution effect. However, an alternative explanation might lay in the generally higher N sink in AMF tissues compared to host plant tissues, leading to N sequestration in fungal tissues (Hodge and Fitter, 2010; Johnson, 2010).

The beneficial mycorrhizal effect on growth of *K. arvensis* plants from the S3 population recorded in all experiments under well-watered conditions even increased under limited water supply (MS3). This finding corroborates the results of Bolandnazar et al. (2007) and Subramanian et al. (2006) and evidences the rising importance of AM symbiosis with the intensification of drought stress. Nevertheless, when the soil water availability fell under a certain level, neither plants nor fungi were able to ensure sufficient water uptake. In our experimental system, 25% of the soil field capacity was identified as such a threshold leading to a major drop in mycorrhizal growth dependence, which was possibly related to a decrease in mycorrhizal root colonization. Based on our results, two simultaneously acting fundamental mechanisms were likely involved in the recorded beneficial mycorrhizal influence on *K. arvensis* performance under limited water supply, namely improved nutrition and involvement of AMF in drought response of host plants (Augé, 2001). Under drought conditions, when the mobility of inorganic ions is generally reduced (e.g. Augé, 2001; Lee et al., 2012), the role of extraradical mycelium of AMF in partial substituting roots in nutrient uptake (mainly P; Smith and Read, 2008) increases, as evidenced by higher values of mycorrhizal P uptake benefit in water-limited than in well-watered *K. arvensis* plants. Moreover, the lower investment of mycorrhizal *K. arvensis* plants into root biomass under a high intensity of drought stress indicates that extraradical mycelium might also be directly involved in plant water uptake. Considering the role of proline and abscisic acid as drought stress markers (Sauter et al., 2001; Kishor et al., 2005), the lower increase in their accumulation (together with lower concentration of osmoprotective potassium) clearly evidences less intense drought stress of mycorrhizal plants compared to their non-mycorrhizal counterparts under the same irrigation regime in serpentine substrate.

Regardless of the drought stress conditions, mycorrhizal *K. arvensis* plants were characterized by consistently lower foliar concentrations of potassium compared to non-mycorrhizal plants in both substrate types (MS2, MS3), which is in accordance with the generally prevailing decrease in plant K concentrations induced by AMF (Smith and Read, 2008). One of the potential mechanisms involved concerns K accumulation in fungal structures in response to accumulation of polyphosphate (Olsson et al., 2008; Orłowska et al., 2013). However, as data on K concentrations in roots of the experimental *K. arvensis* plants are not available, this relationship could not have been tested.

The dependence of the mycorrhizal effect on soil nutrient availability was also recorded for magnesium nutrition, but only partly for calcium nutrition, of *K. arvensis* plants (MS1, MS2, MS4).

Specifically, a more pronounced AMF-induced increase in shoot Mg concentrations was generally recorded for plants growing in non-serpentine substrates compared to either decrease or no effect in Mg-rich serpentine substrates. A concentration-dependent mycorrhizal effect on plant Mg uptake has previously been reported, for example, by Taylor and Harrier (2001) and Liu et al. (2002), with a beneficial AMF effect evidenced under low to moderate Mg availability. Regarding Ca, AMF-mediated decrease in root Ca concentration prevailed in plants grown in the non-serpentine substrate with higher Ca availability, while no significant AMF effect was observed in this respect in the serpentine substrate. On the other hand, Ca concentrations in shoots of *K. arvensis* grown in the native substrates were either not influenced or decreased by AMF inoculation, regardless of the particular soil Ca availability. To our knowledge, these results represent the first report on the effects of AMF on Ca and/or Mg nutrition under serpentine conditions.

A broad range of mycorrhizal effects on shoot nickel concentrations was recorded for *K. arvensis* plants growing in the serpentine substrates with no obvious relation to soil Ni availability. Based on the lack of obvious phytotoxicity effects, the elevated available concentrations of Ni did not likely exceed a threshold of Ni phytotoxicity, as suggested also by Sambatti and Rice (2007). Any general AMF effect on Ni uptake or translocation was not recorded even in the semi-hydroponic study with considerably higher Ni concentrations in nutrient solutions; only serpentine plants inoculated with the serpentine AMF isolate translocated Ni relatively more into their shoots than those inoculated with the non-serpentine fungus (see also 4.3.2.). Doherty et al. (2008) recorded no impact of serpentine AMF on Ni content in aboveground biomass; whereas Vivas et al. (2006) evidenced a decrease in plant Ni concentration induced by AMF isolate from Ni-contaminated soil (both studies involved plants grown in Ni-supplemented soils). Collectively, these results support the view that the effect of AMF on Ni uptake depends both on soil element availability and the particular host plant-AMF combination. Besides a lack of AMF-induced modifications in Ni uptake, AM symbiosis did not ameliorate the toxic effects of Ni on *K. arvensis* plants in any other obvious way; moreover, mycorrhizal plants showed even lower relative tolerance to Ni (MS5); similarly to Doherty et al. (2008). Under the stressful conditions of the Ni-supplemented solution, a shift in the balance of symbiotic relationship might have lead to the lack of mycorrhizal promotion of plant growth and nutrition, if the host plant C-investment exceeded the benefits provided by the fungal partner (Johnson et al., 1997, Johnson and Graham, 2013).

4.3.2. Role of AMF identity

A clear edaphic differentiation between model serpentine and non-serpentine AMF isolates in their response to serpentine conditions was recorded in the reciprocal transplant experiment (MS2). The serpentine isolate was characterized by higher root colonization and also showed higher symbiotic efficiency in the serpentine substrate, as only the combination of serpentine plant and fungal partners led to a positive AMF effect on plant growth. In addition, each of the AMF isolates showed higher P uptake benefit in its native substrate. Therefore, a functional relationship between particular AMF and edaphic conditions in terms of P uptake efficiency and subsequent *K. arvensis* plant growth promotion were suggested, in accordance with van der Heijden et al. (2006) and Johnson et al. (2010). Also when the same two AMF isolates used in the reciprocal transplant experiment were included in the semi-hydroponic study (MS5), inoculation with the isolate of serpentine origin resulted in a less pronounced growth inhibition caused by Ni enrichment. Nevertheless, this difference between the isolates seemed to be based on the relatively stronger Ni-induced decrease in root colonization of the serpentine fungus, likely resulting in a lower sink for host plant carbon. Additionally, in serpentine plants inoculated with the serpentine isolate, the interaction between increased shoot Ni and Ca concentrations might have played a role in the alleviation of Ni phytotoxicity to leaf tissues (Gabbrielli and Pandolfini, 1984; Chaney et al., 2008) and thus in the maintenance of plant aboveground growth. Regarding edaphic differentiation between the model isolates, the influence of different taxonomical position of the particular serpentine vs non-serpentine isolate could not be eliminated, though both are representatives of fast-sporulating r-strategists from the family Glomeraceae (pairs of AMF isolates of the same species and different edaphic origin were not available at the onset of the study). Therefore, no general conclusions concerning the possible co-adaptation of serpentine AMF and *K. arvensis* host plants to specific edaphic stress can be derived from these two studies, unless an experiment involving a higher number of AMF isolates of both edaphic origins is conducted. Unfortunately, establishment of such an experiment with more complex design was precluded by a limited number of host plant seedlings because of the generally low germination rate of *K. arvensis* achenes.

When we compared four AMF isolates of serpentine origin in terms of their effects on plant growth in native substrates (MS4), we found no significant differences in any *K. arvensis* population, regardless of the differences in fungal intraradical development. The results further

evidenced the substantial influence of substrate nutritional status on the extent of mycorrhizal growth promotion. Neither AMF identity nor the level of mycorrhizal root colonization were the overriding factors in determining plant mycorrhizal benefit. Also the effects of AMF on nutrient uptake were mostly independent of the root colonization level (e.g. with regard to phosphorus, as previously reported also by Newsham et al., 1995, and Clark et al., 1999, for this element). We found a divergence between the effects of particular AMF in terms of both P uptake and growth promotion, which was previously reported, for example, by Smith et al. (2004) and Jansa et al. (2008).

The benefits provided by native AMF communities and single AMF isolates were comparable, showing no convincing evidence for the hypothesized prominent role of native AMF communities in conferring growth and nutrient-uptake benefits or drought stress alleviation to *K. arvensis* plants (MS3, MS4). The lack of expected differences between the single isolates and native communities might be caused by potential imbalance in the development of individual members of AMF communities in the experimental pots, leading to the establishment of only a part of originally coexisting AMF species. While the average number of AMF species (OTUs) per plant individual reached 15 ± 1 in the field survey (MS6), only two to four species were identified in the roots of plants inoculated with native soils or native AMF communities in pot experiments. In addition to low species richness, the AMF communities established in the pots were also characterized by dominance of a single taxon, mainly representing r-strategy; also shown by Sýkorová et al. (2007) and Verbruggen et al. (2012). Therefore, the fungal complementarity and selection relationships (Wagg et al., 2011) evolved in the long term at native *K. arvensis* sites might have been disrupted.

4.4. Edaphic differentiation of *K. arvensis* populations

4.4.1. Plant growth and element uptake

The reciprocal transplant experiment evidenced edaphic differentiation between *K. arvensis* populations of serpentine and non-serpentine origin but did not support the assumption of generally lower plant growth under serpentine conditions (MS2). Although serpentine plants grew better in the serpentine substrate than their non-serpentine counterparts, still all populations performed better in the serpentine substrate with generally higher macronutrient availability (see section 4.3.1.). Based on the N:P ratio in shoot tissues, *K. arvensis* plants grown in the model serpentine

substrate were limited by N, not by P availability. It can be concluded that both nutrient limitation and some of the features specific for serpentine chemistry played the role in plant growth in the reciprocal transplant experiment.

For *K. arvensis* plants grown in their respective native substrates (MS1, MS4), the ability to adapt to low macronutrient (P, Ca, Mg, N) availabilities by raising their accumulation ratios was evidenced. The most striking example concerns the substantially higher accumulation ratios of all four elements recorded for the non-serpentine population subjected to extreme soil nutrient limitation (MS4). The Ni accumulation ratio, by contrast, tended to increase with rising soil Ni availability across the experimental substrates (MS1, MS2, MS4); however, as suggested above (see section 4.3.1.), concentrations of Ni in the substrates likely did not reach levels with toxic effects on *K. arvensis* plants.

We identified a decrease in magnesium uptake as a general response of *K. arvensis* plants to serpentine conditions, when expressed by the relationship between soil availability and shoot concentrations (i.e. plant accumulation ratios; MS1, MS2, MS4). However, higher relative allocation of Mg into shoots and consequently considerably higher Mg accumulation ratio was proven for serpentine compared to non-serpentine plants under serpentine conditions (MS2). Serpentine *K. arvensis* plants thus seem to have evolved a tolerance to excessive Mg in shoot tissues under conditions of elevated Mg availability, which has previously been reported as one of the possible strategies of serpentine adaptation (e.g. Kazakou et al., 2008; O'Dell and Rajakaruna, 2011). On the other hand, Mg retention in roots of serpentine plants was recorded when grown in a non-serpentine substrate (MS2) or in a nutrient solution with a high Ca:Mg ratio in the semi-hydroponic study (MS5). This finding indicates that the accumulation of Mg is not a constitutive trait of serpentine *K. arvensis* plants; in other words, they tolerate, but do not require, high Mg concentration in their shoot tissues (Brady et al., 2005; Kazakou et al., 2008). The involvement of an adaptive rather than a constitutive strategy in *K. arvensis* tolerance to high Mg concentration is also supported by results of a hydroponic study performed by Kolář et al. (2014) with regard to growth under elevated Mg concentrations.

In contrast, plants were rather unresponsive to soil calcium availability in the above-mentioned experiments (MS2, MS5), except for the most Ca-limited population. Generally, we recorded neither selective Ca uptake nor intensified root-to-shoot Ca translocation for *K. arvensis* plants growing in a serpentine substrate (MS1, MS2, MS4). *Knautia arvensis* populations thus showed no edaphic differentiation in this respect; instead, tolerance towards low internal

Ca concentrations seems to be their actual adaptive mechanism to Ca limitation under serpentine conditions (see also Kruckeberg, 1984; O´Dell et al., 2006; Veatch-Blohm et al., 2013). Actually, it is in accordance with the fact that the Ca:Mg ratio in biomass of field-sampled plants did not exceed a unit in any of the serpentine populations, which means *K. arvensis* plants did not evolve an adaptation to increase their leaf Ca:Mg ratio (O'Dell et al., 2006; Kazakou et al., 2008).

As indicated above, Ni availability in the serpentine substrate was presumably too low to trigger any plant edaphic differentiation in Ni uptake/tolerance according to shoot Ni concentrations (MS2). When the impact of Ni was tested separately in a semi-hydroponic study with Ni-enriched nutrient solutions (MS5), Ni-induced plant growth depression showed to be independent of plant edaphic origin (see also Doherty et al., 2008; Ghasemi and Ghaderian, 2009), but the serpentine plants were able to maintain relatively higher concentrations of chlorophylls, indicating their better physiological state and thus higher Ni tolerance. Moreover, their higher concentrations of carotenoids with antioxidative effects and lower carotenoids to chlorophyll ratio suggests a more intense defensive response to Ni toxicity and lower Ni-induced oxidative stress (see e.g. Krupa et al., 1996; Drazkiewicz and Baszynski, 2010; Martínez-Peñalver et al., 2011). As suggested from the results of hydroponic study with Ni- and/or Mg-enriched nutrient solutions by Kolář et al. (2014), the toxic impact of Ni on growth of *K. arvensis* roots seems to be partly alleviated by elevated Mg concentration. This finding also provides a possible explanation of the pronounced negative impact of Ni addition in our semi-hydroponic study, considering the relatively low Mg concentration in the Ni-supplemented solutions. When cultivated in the serpentine substrate, both serpentine and non-serpentine plants showed a strong barrier to root-to-shoot Ni translocation. However, in the semi-hydroponic experiment with higher Ni concentrations in the nutrient solution, serpentine plants restricted the translocation of Ni to aboveground biomass more efficiently. The results thus demonstrated only a limited ability of the non-serpentine *K. arvensis* plants to regulate Ni transport.

4.4.2. Role of ploidy level in serpentine plant tolerance and interaction with AMF

Although our field survey revealed certain differences between *K. arvensis* diploids and tetraploids in their rhizosphere soil chemistry (MS1), large geographical distances of the eight selected populations precluded general statements about specific edaphic preferences of each cytotype.

The differences in mycorrhizal colonization (i.e. lower values in the roots of tetraploids, which were recorded both in the field and subsequent experiment conducted in native substrates) might therefore be related rather to specific edaphic conditions of the sites than to the effects of ploidy level *per se*. This hypothesis was supported by the absence of intercytotype difference in mycorrhizal colonization in the experiment where the plants were grown in model serpentine and non-serpentine substrates (MS2). The lack of cytotype-specific colonization pattern is in accordance with the results recorded for another ploidy-heterogeneous host plant species, *Aster amellus* (Sudová et al., 2010, 2014).

Regarding the host plant response to inoculation with AMF, the mycorrhizal growth response was independent of ploidy level in the experiment conducted in substrates native to *K. arvensis* populations (MS1), suggesting the role of edaphic conditions was superior to plant ploidy level. When cultivated in model serpentine and non-serpentine substrates, the interaction between cytotype and substrate significantly influenced mycorrhizal growth dependence, but the effect of cytotype alone remained non-significant (MS2). The fact that polyploidy can affect the mycorrhizal growth response of host plants but still plays a minor role in plant-soil-AMF interactions compared to edaphic conditions has recently been documented also for *A. amellus* (Sudová et al., 2014).

As concerns cytotype-specific differences in coping with adverse conditions of serpentine soils, diploids of *K. arvensis* showed generally higher serpentine tolerance than their tetraploid counterparts, regardless of their edaphic origin, while no intercytotype growth difference was recorded in the non-serpentine substrate (MS2). In a hydroponic study involving the same populations, Kolář et al. (2014) demonstrated a significant interaction between plant ploidy level and edaphic origin in response of *K. arvensis* seedlings to Mg stress, but did not record any direct effect of ploidy level on plant tolerance to elevated concentrations of Mg or Ni. Based on these results, they concluded that polyploidy alone played a rather minor role in serpentine differentiation of *K. arvensis* plants. Because of the parapatric distribution of different *K. arvensis* cytotypes included in the studies outlined above, it cannot be excluded that the recorded intercytotype differences might, at least partly, be explained by local adaptations of individual populations. Importantly, the interpretation of the intercytotype differences might be confounded by different genetic distances between diploids and tetraploids of either serpentine or non-serpentine *K. arvensis* populations, as was recently revealed by Kolář et al. (2012). Based on genome size and several molecular markers, non-serpentine diploids and tetraploids represent genetically distinct lineages, while serpentine tetraploids were found to be genetically close to serpentine diploids, corresponding with their common adaptive responses and supporting the autopolyploid origin of serpentine tetraploids (Kolář et al., 2012, 2014).

In spite of the mentioned limitations, our findings concerning intercytotype differences in serpentine tolerance and interactions with AMF provided hypotheses to be tested on a smaller geographical scale, using newly discovered mixed-ploidy serpentine *K. arvensis* populations in the Slavkovský les Mts (Kolář et al., 2009, 2012), which were unknown at the onset of our experiments. Recently, a detailed follow-up study of within-site edaphic heterogeneity and AMF species richness and composition in two mixed-ploidy *K. arvensis* populations is being conducted; however, this research topic is beyond the scope of the present Ph.D. thesis.

4.5. Conclusions

I. AMF communities colonizing *Knautia arvensis* roots at native sites

- Serpentine *K. arvensis* plants showed a generally lower frequency of mycorrhizal root colonization compared to their non-serpentine counterparts.
- Adjacent serpentine and non-serpentine *K. arvensis* populations differed in the communities of AMF species colonizing their roots. Both species composition and richness of these AMF communities were determined primarily by edaphic parameters.
- Regarding species composition of the AMF communities, soil pH value and nickel concentration were the most significant explanatory factors, together with presence of certain co-existing plant species. AMF species richness increased with soil pH (in the range of 3.6–5.8) and decreased with rising soil chromium and potassium concentrations.

II. Involvement of AM symbiosis in serpentine adaptation of *K. arvensis* plants

- AM symbiosis showed overall beneficial effects on *K. arvensis* growth. The mycorrhizal growth dependence of host plants was governed mainly by nutritional status of the soil, independently of its serpentine character.
- Improved phosphorus acquisition and drought stress alleviation were likely the crucial mechanisms of the beneficial effect of AM symbiosis in serpentine soils.
- AMF inoculation either decreased or had no effect on both plant magnesium and calcium acquisition and generally did not influence Ni uptake of serpentine *K. arvensis* plants under serpentine conditions. On the contrary, AMF seemed to increase the Ni-phytotoxicity effect in the case of considerably elevated Ni availability in a hydroponic solution.

- Selected serpentine and non-serpentine AMF isolates proved their differentiation under serpentine conditions in the reciprocal transplant experiment. The serpentine AMF isolate developed higher root colonization and was more efficient in growth promotion of and phosphorus uptake by the serpentine plants.
- The identity and complexity (isolates vs communities) of the AMF inoculum, however, generally had a minor role in determining the extent of mycorrhizal plant growth and nutrient uptake promotion compared to the nutritional status of the substrate.

III. The effects of edaphic origin and ploidy level on serpentine tolerance of *K. arvensis* plants and their interactions with AMF

- Populations of *K. arvensis* showed edaphic differentiation in terms of plant growth and element uptake under serpentine conditions. No differences in calcium uptake and/or allocation were recorded between serpentine and non-serpentine populations, while the tolerance to magnesium accumulation in shoot tissues seemed to be one of the essential adaptive traits of serpentine *K. arvensis* plants.
- Regardless of their serpentine or non-serpentine origin, *K. arvensis* populations proved their adaptation to macronutrient deficiency or excess of their native substrates, as indicated by plant accumulation ratios of elements.
- In the reciprocal transplant experiment, only the combination of serpentine plant and fungal partners led to a promotional mycorrhizal effect on plant growth in the serpentine substrate.
- The ploidy level of *K. arvensis* plants seemed to play no fundamental role in their adaptation to serpentine edaphic conditions or in their responsiveness to AM symbiosis.

5. Manuscripts

MS1. Doubková et al., Plant and Soil 2011

MS2. Doubková et al., Soil Biology and Biochemistry 2012

MS3. Doubková et al., Plant and Soil 2013

MS4. Doubková et al., Mycorrhiza 2013

MS5. Doubková and Sudová, Mycorrhiza 2014

MS6. Kohout et al.

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REGULAR ARTICLE

Arbuscular mycorrhizal symbiosis on serpentine soils: the effect of native fungal communities on different *Knautia arvensis* ecotypes

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Abstract Serpentine soils represent a unique environment that imposes multiple stresses on vegetation (low Ca/Mg ratios, macronutrient deficiencies, elevated heavy metal concentrations and drought). Under these conditions, a substantial role of arbuscular mycorrhizal (AM) symbiosis can be anticipated due to its importance for plant nutrition and stress alleviation. We tested whether serpentine and non-serpentine populations of *Knautia arvensis* (Dipsacaceae) differ in the benefits derived from native AM fungal communities. Four serpentine and four non-serpentine populations were characterised in terms of mycorrhizal colonisation and soil characteristics. The serpentine populations showed significantly lower mycorrhizal colonisation than their non-serpentine counterparts. The mycorrhizal colonisation positively correlated with soil pH, Ca and K concentrations and Ca/Mg ratio. Seedlings from each population were then grown for 3 months in their sterilised native substrates, either uninoculated or reinoculated with native AM fungi. Two serpentine and two non-serpentine populations responded positively to mycorrhizal inoculation, while no significant change in plant growth was observed in the remaining populations. Contrary to our hypothesis, serpentine populations of *K. arvensis* did not show higher mycorrhizal growth dependence than non-serpentine populations when grown in their native soils and inoculated with native AM fungi.

Keywords Arbuscular mycorrhizal fungi · Ca/Mg ratio · Edaphic stress · Nickel · Mycorrhizal colonisation · Ploidy level · Serpentine

Abbreviations

AM	Arbuscular mycorrhiza, arbuscular mycorrhizal
CEC	Cation exchange capacity
DMF	Dimethylformamide
DW	Dry weight
MGD	Mycorrhizal growth dependence
NS	Non-serpentine
PCA	Principal component analysis
S	Serpentine
SEM	Standard error of the mean

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Introduction

Serpentine soils represent a unique microcosm for studying plant edaphic speciation and adaptation to stressful conditions. Serpentine substrates derived from the weathering of ultramafic rocks are characterised by specific physico-chemical properties referred to as the 'serpentine syndrome' (Jenny 1980). These properties limit plant growth and result in low plant productivity, a high level of endemism and vegetation that is distinct from adjacent areas. Serpentine vegetation must cope with deficiencies of essential macronutrients (i.e., P, N and K), low Ca and excessive Mg availability, resulting in low Ca/Mg ratios, and elevated (often phytotoxic) concentrations of heavy metals such as Ni, Co and Cr. The inhospitality of serpentine soils is often further increased by a low content of organic matter, low water-holding capacity, shallow soil profile and vulnerability to erosion (for reviews, see Proctor and Woodell 1975; Brady et al. 2005; Kazakou et al. 2008). The combination of these factors imposes severe requirements on the plants that inhabit serpentine sites and elicits a range of morphological and/or physiological adaptations that include lower stature, high root–shoot ratio, xeromorphic foliage and modified ion uptake and translocation (Brady et al. 2005).

In contrast to the extensive information available concerning vegetation composition and plant edaphic speciation in serpentine localities, studies dealing with arbuscular mycorrhizal (AM) associations in these sites are scarce and the role of AM fungi in plant adaptation to serpentine soils remains to be understood (Schechter and Bruns 2008). This is surprising in view of the fact that AM symbiosis is the most widespread type of symbiosis between plant roots and fungi. It is formed in the roots of the vast majority of vascular plants by obligate symbiotic fungi from the phylum Glomeromycota (Schüssler et al. 2001). Among soil biota, AM fungi have a special position because they provide a direct link between plant roots and the soil. It is well established that the extraradical hyphae of AM fungi play an essential role in increasing the volume of soil available for the acquisition of immobile nutrients such as P, Zn and NH_4, and that, as a result, mycorrhizal roots take up these nutrients more efficiently than uncolonised root systems (Smith and Read 2008). In addition, numerous studies have demonstrated the importance of AM fungi in alleviating environmental stresses in plants, including heavy metal stress and drought (for reviews, see Leyval et al. 1997; Smith et al. 2010). Moreover, a significant role of the extensive network of extraradical mycelium of AM fungi in soil aggregation has been demonstrated, with important consequences for the prevention of erosion (Rillig and Mummey 2006). These facts lead us to propose the hypothesis that AM fungi act as buffering agents, alleviating the effects of multiple serpentine stresses on host plants.

All field surveys and experimental studies conducted in serpentine soils to date show that symbiosis with AM fungi is common in serpentine vegetation worldwide. The majority of these studies have addressed either the mycorrhizal status of the serpentine vegetation (e.g., Hopkins 1987; Goncalves et al. 2001; Boulet and Lambers 2005) or general questions of AM ecology such as the spatial distribution of AM propagules, plant/fungal feedback and response to manipulation of growth conditions (Koide and Mooney 1987; Castelli and Casper 2003; Gustafson and Casper 2004). Two recent studies have focused on the species diversity of AM fungi that colonise the roots of serpentine and non-serpentine individuals of the same plant species (Schechter and Bruns 2008; Fitzsimons and Miller 2010).

The increasing number of publications describing patterns and processes in natural serpentine AM communities stands in contrast to the lack of comparative functional studies aimed at elucidating the role of AM fungi in plant adaptation to serpentine soils. The fact that intensive AM colonisation has also been reported for serpentine populations of "normally" non-mycorrhizal plant families such as Brassicaceae, Proteaceae or Cyperaceae (Hopkins 1987; Boulet and Lambers 2005; Lagrange et al. 2011) might indicate that AM symbiosis is important in these stressful habitats. Amir et al. (2008) reported a higher Ni tolerance of serpentine AM fungi in spore germination tests, but no amelioration of Ni toxicity to the host plants was observed in response to inoculation with serpentine AM fungi (Boulet and Lambers 2005; Doherty et al. 2008). Recently, Ji et al. (2010) reported the results of a reciprocal transplant experiment in which AM fungal communities originating from serpentine soil were more efficient in the promotion of plant growth in serpentine soil compared to non-native AM fungi, thus providing evidence for the ecological matching of whole AM fungal communities to the local plant-soil environment.

The hypothesis on which the present study is based is that AM symbiosis is one of mechanisms by which plants adapt to serpentine soils. More specifically, we wanted to compare serpentine and non-serpentine populations of the same mycorrhizal plant species, *Knautia arvensis*, with respect to (1) rhizosphere soil conditions, (2) mycorrhizal root colonisation, and (3) relative importance of native AM fungal communities for plant growth in their native serpentine or non-serpentine substrates. To match the native soil–plant–AM fungi combinations, complex AM fungal communities were used. We hypothesised that AM symbiosis is of higher significance under the adverse edaphic conditions associated with serpentine soils and that serpentine populations would therefore exhibit a greater benefit from mycorrhizal association than their non-serpentine counterparts.

Materials and methods

The plant species under study

Knautia arvensis (L.) J. M. Coult. (Dipsacaceae) is a common perennial herb, inhabiting dry and mesophilous meadows, pastures, shrublands, open woods, forest margins and roadsides throughout most of Europe (Štěpánek 1997). It harbours two more-or-less spatially separated (parapatric) but morphologically only weakly differentiated ploidy levels, diploid and tetraploid, which are sometimes treated as separate subspecies or varieties (Kaplan 1998; Kolář et al. 2009). *Knautia arvensis* was selected as a good model system for studying the role of AM symbiosis in plant adaptation to serpentine conditions because: (1) it is a mycorrhizal species (Wang and Qiu 2006); (2) it inhabits both serpentine and non-serpentine substrates in Central Europe (Kaplan 1998); and (3) the serpentine populations (ecotypes) showed higher tolerance to elevated Mg concentrations (lower Ca/Mg ratio) in a hydroponic experiment than the non-serpentine populations (F. Kolář et al., unpublished data). Furthermore, both diploid and tetraploid cytotypes occur on serpentine as well as on non-serpentine sites (Kolář et al. 2009), thus providing an opportunity for assessment of the interactions between mycorrhizal inoculation and genome copy number by comparison of plants of different ploidy level within the same species.

The field survey

Four serpentine and four non-serpentine populations of *K. arvensis* in the Czech and the Slovak Republics were sampled in order to compare mycorrhizal colonisation in the roots of individuals of different origin (Table 1). For each substrate type tested, two diploid and two tetraploid populations were selected to avoid a potential bias due to differences in genome copy number of the host plant. Ploidy levels were determined by flow cytometry, as described by Kolář et al. (2009). The plants were sampled in the post-flowering stage within a 1-week period at the beginning of August 2008. Ten plants per population were randomly collected in an area of approximately 100 m^2. The intact plant root systems were carefully excavated and transported to the laboratory. The attached rhizosphere soil was shaken off gently and saved for subsequent use. The roots were separated from the rhizomes, washed and mycorrhizal root colonisation was determined after staining in 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989). Root colonisation was assessed using a modified intersection method (McGonigle el al. 1990). Approx. 200 intersections per sample were scored for the absence or presence of AM hyphae, vesicles and/or arbuscules.

Each rhizosphere soil sample was sieved (<5 mm) and subsamples from three randomly selected plants of each population were air-dried, passed through a 2-mm sieve and analysed for pH, cation exchange capacity (CEC) and nutrient and metal concentrations. Available concentrations of P were assessed spectrophotometrically (Unicam UV4-100, UK) after 1 M sodium bicarbonate extraction (Olsen and Sommers 1982). Available concentrations of Ca, Mg, K, Na and Ni, Co, Cr were determined by atomic absorption spectrometer (Unicam 9200X, UK) after 1 M ammonium acetate extraction (pH 7.0) and 0.005 M DTPA extraction (Lindsay and Norvell 1978), respectively. N content and organic C content were determined by a combustion method (CHN Carlo Erba NC2500 analyser, Italy). The remaining rhizosphere soil originating from the same population was mixed thoroughly and stored at 4°C until used for the inoculation in a subsequent pot experiment (see below).

The pot experiment

Mature achenes of *K. arvensis* were collected from the eight populations (Table 1) in the summer and

Table 1 *Knautia arvensis* populations included in the study

Substrate type	Ploidy level	Population code	Site	Geographic co-ordinates	Altitude (m asl)	Site description
Serpentine	2x	S1	Borovsko (E. Bohemia, CZ)	49°40′57.7″N, 15°07′49.7″E	400	Margins of open pine forest
		S2	Staré Ransko (E. Bohemia, CZ)	49°39′04.9″N, 15°48′57.3″E	640	Margins of coniferous forest
	4x	S3	Pluhův Bor (W. Bohemia, CZ)	50°03′01.3″N, 12°46′24.3″E	710	Margins of open pine forest
		S4	Křížky (W. Bohemia, CZ)	50°03′54.2″N, 12°45′03.6″E	790	Rock outcrops, semidry meadow
Non-serpentine	2x	NS1	Tvarožná Lhota (S. Moravia, CZ)	48°51′43.6″N, 17°23′23.3″E	290	Mesophilous meadow
		NS2	Lajdovci (W. Slovakia)	48°28′29.8″N, 17°38′59.2″E	230	Dry meadow
	4x	NS3	Aš (W. Bohemia, CZ)	50°13′12.9″N, 12°13′19.2″E	670	Abandoned meadow
		NS4	Chanovice (SW. Bohemia, CZ)	49°24′39.0″N, 13°43′55.5″E	530	Dry meadow

autumn 2008. The achenes were surface-sterilised (5% NaClO, 10 min) and pre-germinated in Petri dishes. The emerged seedlings were transplanted into plastic multipot trays (15-ml cells) filled with a sterile mixture of garden soil and sand (1:2, v/v). After 2 weeks, even-sized seedlings were transplanted into experimental pots. The seedlings from each population were grown in their native substrates originating from the rhizosphere of field-sampled *K. arvensis* plants. The experimental plants were either left uninoculated or were inoculated with native AM fungi. The uninoculated plants were grown in 375 ml of the respective γ-sterilised (25 kGy) substrate. The pots assigned to the inoculated treatment were filled with 300 ml of the sterile and 75 ml of non-sterile native substrate; the latter was applied in a layer approx. 3 cm below the plants. The uninoculated plants were treated with 10 ml of a filtrate from the corresponding non-sterile substrate to balance the composition of the microbial communities in the inoculated and uninoculated treatments. The filtrates were prepared by filtration of a suspension of the non-sterile soil (1:10, w/v) through filter paper (pore size, 15 µm) to remove AM propagules. The number of replicates in the uninoculated and inoculated treatments depended on the availability of the seedlings and was seven (popns. NS1, NS2, NS4) or eight (popns. S3, S4, NS3), except in the case of the populations S1 and S2; for each of the latter, only four replicates were established due to the low germination rate of the achenes. The plants were grown in a greenhouse under natural sunlight and supplementary 12-h artificial illumination (metal halide lamps, 400 W, Philips HPI-T Plus) providing a photosynthetic photon flux density of at least 160 µmol m^{-2} s^{-1} at the plant level. The plants were cultivated from mid-November 2008 to mid-February 2009 and were irrigated daily to field capacity.

The plants were harvested after 12 weeks of cultivation. The concentrations of chlorophylls *a* and *b* and the concentration of total carotenoids were determined spectrophotometrically after dimethylformamide (DMF) extraction, according to the method of Porra et al. (1989). The leaf tissue of a known fresh weight (~0.1 g) was collected from the youngest fully developed leaf pair, placed in 20 ml of DMF and left in the dark at 8°C for 7 days until the leaves were bleached. The absorbance of the extract was measured at 480, 647 and 664 nm using a spectrophotometer (Hach DR4000U, USA). The amounts of chlorophylls and carotenoids were calculated according to Wellburn (1994). After measuring the rosette diameter, the shoots were cut off and the total leaf area was assessed using an area meter (LI-3100; LI-COR, USA). The root

systems were washed and mycorrhizal colonisation determined as described above. Dry weights of shoots and roots were recorded after drying at 65°C, taking into account the subsamples taken for mycorrhizal colonisation assessment. Shoots were ground, digested and analysed for P, Ca, Mg and Ni concentrations as above. Ni concentrations in the biomass of non-serpentine plants were negligible and were therefore not analysed.

Data analysis

To summarise the major patterns of variation in the environmental soil data (three samples of rhizosphere soil per site, each characterised by 13 chemical parameters; see Table 2), a principal component analysis (PCA) was performed on the untransformed and centred data using CANOCO 4.5 software (Biometris, The Netherlands). In a correlation biplot of the PCA, positively correlated variables have sharp angles between their biplot arrows whereas negatively correlated variables point in opposite directions; perpendicular arrows indicate no correlation between the respective variables. The length of the arrow describes the relative importance of the variable for the displayed ordination.

All other analyses were performed using Statistica 9.1 software (StatSoft, USA). A nested ANOVA model (GLM procedure) was adopted, with the population as a random factor (nested within substrate type×ploidy level combination) and the substrate type, ploidy level and AM inoculation as fixed factors. Data were checked for normality and logarithmically transformed when necessary. Data on the percentage of mycorrhizal root colonisation were arcsine transformed. Inoculated and uninoculated plants belonging to the same *K. arvensis* population were compared using one-way ANOVA. The mycorrhizal growth dependence (MGD) was calculated according to Smith et al. (2003) as a percent increase in shoot dry weight (DW) of an individual mycorrhizal plant over the mean weight of non-mycorrhizal plants [MGD=100 × (shoot DW of an individual mycorrhizal plant – average shoot DW of non-mycorrhizal plants)/shoot DW of an individual mycorrhizal plant]. All post-hoc comparisons were made using the Tukey HSD test ($P<0.05$). Linear regression analyses were conducted to test possible relationships between the level of mycorrhizal colonisation and soil chemical parameters, MGD and nutrient uptake, respectively.

Results

The field survey

All *K. arvensis* plants sampled in the field were colonised by AM fungi; the mean percentage of colonised roots ranged from 56 to 92% depending on the population (Fig. 1a). Mycorrhizal colonisation was significantly influenced by both the substrate type ($P=0.022$) and ploidy level ($P=0.050$), but their interaction was not significant. The serpentine plants showed a lower mycorrhizal colonisation (72±3%; mean±SEM) than their non-serpentine counterparts (88±1%). Lower colonisation was also observed in the roots of tetraploid plants (74±3%) than in the roots of diploids (85±2%).

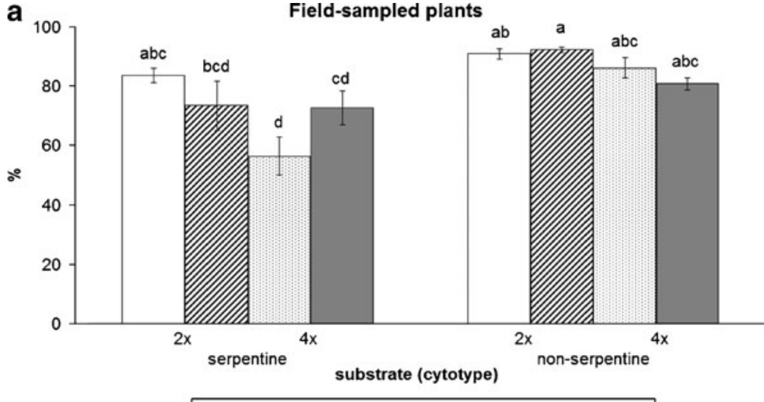
Figure 2 shows the relationships between the chemical characteristics of the rhizosphere samples used to describe the soils at the eight localities, together with the contributions of the individual chemical characteristics for the displayed ordination. The serpentine and non-serpentine soil samples formed distinct and well-separated groups. Whereas some intra-site variation was found for the serpentine localities, all three soil samples from a given non-serpentine locality were highly uniform. A certain differentiation was also observed within each substrate type; in particular, the non-serpentine diploid and tetraploid populations formed separate clusters. Serpentine sites were characterised by considerably higher Mg concentrations (2,224±247 vs. 303±52 mg kg^{-1} in non-serpentine sites), lower Ca/Mg ratios (0.7±0.1 vs. 10.2±1.6) and higher Ni (283±53 vs. 3±1 mg kg^{-1}) and Co concentrations (6.1±1.4 vs. 0.3±0.1 mg kg^{-1}) (Table 2). In addition, the serpentine localities also had generally lower pH, higher N and organic C content, lower K and higher Na concentration. The CEC and P, Ca and Cr concentrations did not significantly differ between the serpentine and non-serpentine sites. The lack of statistically significant difference in soil Ca concentrations was caused by exceptionally low Ca concentration in the NS3 soil (301 mg kg^{-1} compared to a mean of 4,592 mg kg^{-1} for the other NS sites). Some soil characteristics also differed between the sites inhabited by the different cytotypes (Table 2). The tetraploids occupied sites with significantly lower pH and CEC values. Linear regression analysis revealed a positive correlation between mycorrhizal root colonisation and soil pH ($r=0.414$, $P=0.045$), Ca concentration ($r=0.425$,

Table 2 The characteristics of the rhizosphere soil of the field-sampled *K. arvensis* plants as affected by population (nested within substrate×ploidy level combination), substrate type (*S* serpentine vs. *NS* non-serpentine), ploidy level (*2x* diploid vs. *4x* tetraploid) and their interactions

Population code		pH_{KCl}	CEC $mmol_c kg^{-1}$	N %	C_{org} %	P $mg\ kg^{-1}$	Ca $mg\ kg^{1}$	Mg $mg\ kg^{-1}$	Ca/Mg	K $mg\ kg^{-1}$	Na $mg\ kg^{-1}$	Ni $mg\ kg^{-1}$	Co $mg\ kg^{-1}$	Cr $mg\ kg^{-1}$
S1		5.6±0.2	291±30	0.66±0.09	9.6±1.9	3.0±1.0	1,689±362	2,478±191	0.7±0.1	93±8	16.3±1.2	543±51	12.2±3.0	0.13±0.03
S2		5.4±0.2	331±49	0.56±0.04	9.5±1.4	3.4±0.7	1,602±36	3,005±599	0.6±0.1	127±24	15.2±1.3	155±26	6.2±1.6	0.38±0.08
S3		4.8±0.4	180±48	0.37±0.02	5.1±0.3	3.1±1.0	814±129	1,657±514	0.6±0.2	82±15	19.5±0.8	170±73	3.5±1.7	0.27±0.03
S4		4.8±0.03	213±28	0.71±0.06	8.9±0.7	7.8±2.7	1,284±135	1,760±276	0.8±0.1	129±13	16.7±1.2	264±73	2.6±0.4	0.17±0.04
NS1		6.8±0.1	372±8	0.43±0.03	5.0±0.4	8.3±2.4	6,623±197	366±25	18.3±1.6	427±55	13.8±0.6	7.1±1.7	0.5±0.2	0.15±0.02
NS2		7.1±0.1	307±3	0.23±0.03	2.3±0.5	10.3±4.0	5,163±68	521±49	10.1±1.1	242±75	15.9±1.0	2.4±0.5	0.3±0.1	0.15±0.01
NS3		4.2±0.1	23±4	0.19±0.01	2.0±0.2	2.0±0.2	301±70	59±7	6.9±0.7	124±28	10.2±0.2	2.1±0.1	0.1±0.04	0.15±0.02
NS4		4.7±0.1	126±11	0.28±0.002	3.1±0.1	12.7±1.4	1,989±187	267±24	7.5±0.1	160±51	14.0±0.7	1.9±0.4	<0.05	0.14±0.04
Factor	*df*	significance												
Population	4	ns	ns	***	***	**	***	***	**	ns	*	**	ns	***
Substrate type	1	* (S↓)	ns	* (S↑)	* (S↑)	ns	ns	** (S↑)	*** (S↓)	* (S↓)	* (S↑)	*** (S↑)	** (S↑)	ns
Ploidy level	1	*** (2x↑)	** (2x↑)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Substrate×ploidy level	1	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

The data represent the means±SEM (*n*=3). For significant effects of single factors, the direction of change is indicated by arrows (e.g., S↑ denotes higher values in the S than in the NS substrate)

***$P<0.001$, **$P<0.01$, *$P<0.05$; *ns* non-significant effect



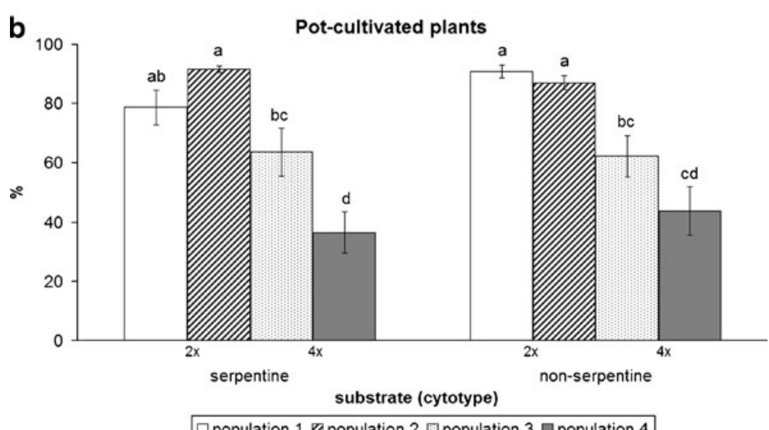


Fig. 1 Mycorrhizal colonisation of *Knautia arvensis* roots. **a** Field-sampled plants (n=10); **b** pot-cultivated plants (n=7–8, except for serpentine populations 1 and 2 where n=4). *Columns marked by different letters* are significantly different according to the Tukey HSD test (P<0.05)

P=0.038), K concentration (r=0.481, P=0.018) and Ca/Mg ratio (r=0.444, P=0.030). A negative correlation was found between root colonisation and the soil Na concentration (r=−0.465, P=0.025), while no significant correlation with Ni concentration was observed.

The pot experiment

All inoculated plants were colonised by AM fungi and no mycorrhizal structures were found in the roots of uninoculated plants. Root colonisation was significantly influenced by population (P=0.010) and ploidy level (P=0.014), with a mean colonisation of 88±2% in diploids and 51±4% in tetraploids (Fig. 1b). Mycorrhizal colonisation was not affected by either the substrate type (serpentine vs. non-serpentine) or ploidy level×substrate interaction.

Most plant growth parameters were significantly affected by the identity of the population (Table 3). The data on rosette diameter and leaf area were highly correlated with shoot DW (r=0.872 and 0.961, respectively, both at P<0.001) and therefore only the latter one is presented. AM inoculation generally enhanced plant growth. When inoculated and uninoculated plants from the same population were compared, a significant positive growth effect of the AM fungi was revealed in four of the eight populations, two serpentine (the diploid S2 and tetraploid S3) and two non-serpentine (the diploid NS2 and tetraploid NS3; Fig. 3). The most pronounced response to AM inoculation was found in the NS3 and S2 populations, in which almost nine- and sevenfold increases in shoot biomass, respectively, were observed compared to uninoculated plants. The mycorrhizal growth dependence ranged from −5.4 to 83.5% (Fig. 4) and was significantly influenced only by the population (P<0.0001). Linear regression analysis revealed a significant positive relationship between the MGD and the percentage of mycorrhizal colonisation in the serpentine (r=0.530, P=0.008), but not in the non-serpentine, populations.

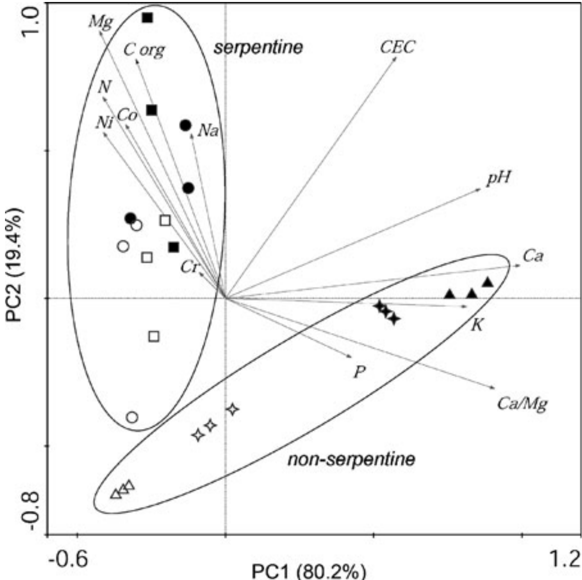


Fig. 2 PCA ordination plot showing the relationships between the rhizosphere soil samples collected at eight *K. arvensis* localities in relation to measured soil variables. Samples originating from the same population are marked with the same symbol (*solid circle*, S1; *solid square*, S2; *empty circle*, S3; *empty square*, S4; *solid triangle*, NS1; *solid star*, NS2; *empty triangle*, NS3; *empty star*, NS4). *Solid symbols* denote diploid populations; *empty symbols* denote tetraploid populations. Samples from the serpentine and non-serpentine populations are *encircled*

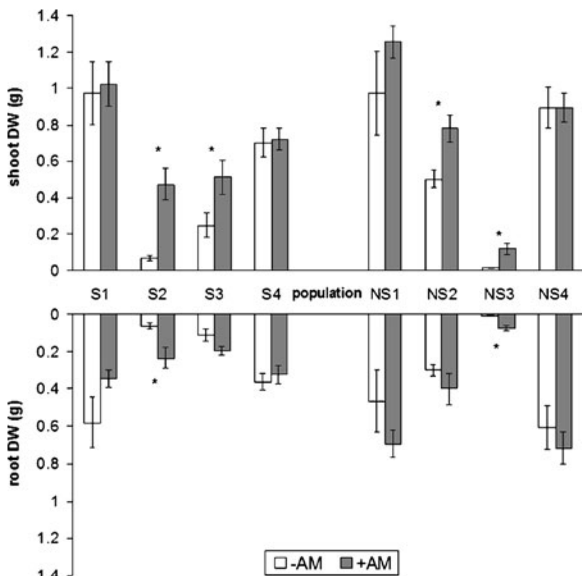


Fig. 3 Shoot and root dry weight (DW) of uninoculated and inoculated plants originating from different *K. arvensis* populations (*S1–S4* serpentine populations; *NS1–NS4* non-serpentine populations). *Columns* represent means±SEM (n=7–8, except serpentine populations 1 and 2 where n=4). *Asterisks* indicate significant differences (P<0.05) between uninoculated and inoculated plants belonging to the same population

The root–shoot ratio significantly decreased in response to AM inoculation in serpentine populations (from 0.57±0.04 to 0.43±0.03) and in the diploids (from 0.62±0.05 to 0.49±0.04), while the non-serpentine populations and the tetraploids were unresponsive in this respect (Table 3). The root–shoot

Table 3 The effects of population (nested within substrate× ploidy level combination), substrate type (*S* serpentine vs. *NS* non-serpentine), ploidy level (*2x* diploid vs. *4x* tetraploid), AM inoculation (presence vs. absence) and their interactions on the growth, photosynthetic pigments concentrations and shoot nutrient concentrations of *K. arvensis* plants according to nested ANOVA

Population code	AM	Shoot DW	Root DW	Root–shoot ratio	Chl a+b	Carotenoids	P	Ca	Mg	Ca/Mg	Ni[a]
Population	4	***	***	ns	***	***	***	**	***	***	**
Substrate type	1	ns	ns	ns	ns	ns	ns	** (S↓)	** (S↑)	* (S↓)	-
Ploidy level	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AM inoculation	1	*** (↑)	* (↑)	ns	* (↑)	ns	** (↑)	* (↓)	ns	* (↓)	** (↓)
Substrate×ploidy level	1	ns	ns	*	ns	ns	ns	ns	ns	ns	-
Substrate×AM	1	ns	ns	**	ns	*	ns	**	**	ns	-
Ploidy level×AM	1	ns	ns	*	ns	ns	ns	***	*	*	**
Substrate×ploidy×AM	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	-

***P<0.001, **P<0.01, *P<0.05; *ns* non-significant effect; for significant effects of single factors, the direction of change is indicated by arrows in parentheses

[a] Degrees of freedom for Ni concentration: population 2, inoculation 1, ploidy level 1, ploidy level×inoculation 1

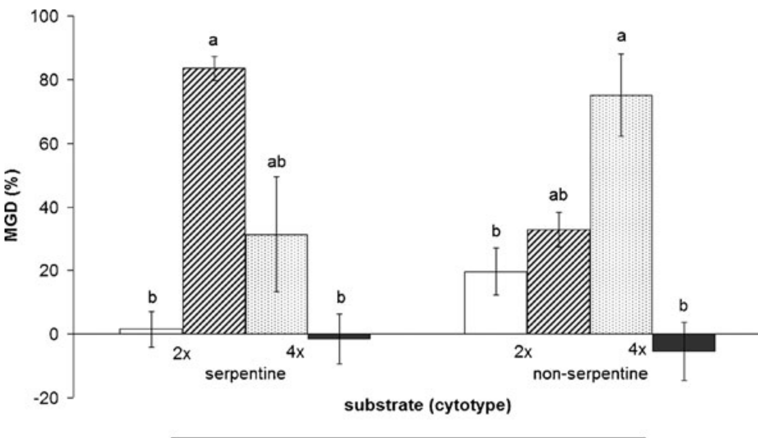


Fig. 4 Mycorrhizal growth dependence (calculated based on shoot dry weight) as affected by population, substrate type and ploidy level. *Columns* represent means±SEM (n=7–8, except for serpentine populations 1 and 2 where n=4). *Columns with different letters* are significantly different according to the Tukey HSD test (P<0.05)

ratio was also significantly affected by the ploidy level × substrate type interaction; serpentine tetraploids allocated more biomass to the shoots than non-serpentine ones (0.70±0.04 vs. 0.47±0.02), while no such difference was observed between diploids of different origin.

Shoot DW correlated positively with both total chlorophyll and carotenoid concentrations (r=0.501 and 0.400, respectively, both at P<0.001). Inoculation with AM fungi generally resulted in an increase in the total chlorophyll concentration (Table 3), with values of 2.49±0.06 and 2.26±0.08 μg mg^{-1} of fresh weight in inoculated and uninoculated plants, respectively. The interaction between AM inoculation and the substrate significantly influenced the concentration of carotenoids (Table 3). Serpentine plants had increased carotenoid concentrations when mycorrhizal (0.48±0.02 vs. 0.41± 0.02 μg mg^{-1}), whereas their non-serpentine counterparts were unresponsive in this respect.

Not surprisingly, nutrient uptake differed between populations and substrates (Table 3). Compared to non-serpentine plants, serpentine plants showed lower Ca concentrations (3,112±196 vs. 7,695±322 mg kg^{-1}), higher Mg concentrations (9,359±289 vs. 3,801± 153 mg kg^{-1}) and lower Ca/Mg ratios (0.33±0.01 vs. 2.26±0.16). There was a significant negative correlation (P<0.001) between shoot DW and Ca/Mg ratio (and Ca concentration) in the shoot biomass, both in the serpentine (r=−0.507) and non-serpentine populations (r=−0.496). In the non-serpentine populations, shoot DW also correlated positively with shoot Mg concentration (r=0.384, P=0.006). In the serpentine populations, a negative relationship was observed between shoot DW and shoot Ni concentration of uninoculated plants (r=−0.667, P<0.001); no such correlation was observed in inoculated plants.

In general, AM inoculation significantly increased P uptake (Table 3). The shoot P concentration correlated positively with the percentage of root colonisation (r=0.666, P<0.001). The effect of AM fungi on Ca uptake depended on the substrate, as evidenced by a significant interaction between AM inoculation and substrate type (Table 3). In serpentine plants, AM inoculation resulted in reduced shoot Ca concentrations, while no consistent effect was observed in the non-serpentine populations (Table 4). Also, the Mg uptake was significantly influenced by the interaction between the substrate and inoculation, indicating different effects of AM fungi in populations of different origin. In the non-serpentine populations, a trend towards increased shoot Mg concentrations due to AM inoculation was observed. The opposite effect of AM inoculation was recorded in the serpentine population S2 growing in the substrate with the highest Mg availability. The inoculated plants of this population also showed significantly lower shoot Ni concentrations (Table 4).

The ploidy level per se did not influence plant nutrient uptake. However, the effect of AM inoculation on Mg, Ca and Ni uptake and the Ca/Mg ratio depended on the cytotype, as seen from the significant interaction between ploidy level and AM inoculation (Table 3). In diploids, AM inoculation decreased the concentrations of Ca and Ni, as well as the Ca/Mg ratio, whereas no effects were observed in the tetraploids.

Table 4 The concentrations of P, Ca, Mg and Ni in the shoots of greenhouse-cultivated *K. arvensis* plants as affected by population (nested within substrate×ploidy level combination), substrate type (*S* serpentine vs. *NS* non-serpentine), ploidy level (*2x* diploid vs. *4x* tetraploid), AM inoculation (presence vs. absence) and their interactions. The data represent the means± SEM (n=7–8, except populations S1 and S2 where n=4)

Population code	AM	P (mg kg^{-1})		Ca (mg kg^{-1})		Mg (mg kg^{-1})		Ca/Mg		Ni[a] (mg kg^{-1})	
S1	–	2,418±238	**	3,657±951	ns	9,535±1139	ns	0.37±0.05	ns	36.8±4.4	ns
	+	3,359±47		2,825±278		10,225±652		0.28±0.02		35.7±4.6	
S2	–	4,058±498	ns	6,528±849	**	13,172±933	**	0.50±0.08	*	67.6±7.9	**
	+	3,494±596		2,640±339		8,867±649		0.29±0.02		28.0±5.0	
S3	–	2,022±109	*	2,848±160	ns	7,487±376	ns	0.39±0.03	ns	50.4±3.5	ns
	+	2,545±166		2,676±163		8,094±426		0.33±0.02		51.3±3.9	
S4	–	2,197±125	ns	2,822±186	ns	9,874±518	ns	0.29±0.01	ns	38.9±2.5	ns
	+	2,365±107		2,500±122		9,356±419		0.27±0.01		38.1±2.2	
NS1	–	2,349±134	**	8,601±335	ns	3,073±148	*	2.82±0.14	ns	n.d.	
	+	2,821±60		8,932±439		3,543±142		2.56±0.20		n.d.	
NS2	–	2,221±68	*	9,968±302	**	4,732±211	ns	2.12±0.10	*	n.d.	
	+	2,887±236		7,393±499		4,287±209		1.74±0.12		n.d.	
NS3	–	2,054±282	*	7,687±3556	ns	1,754±448	ns	3.98±0.83	ns	n.d.	
	+	1,227±142		9,766 ±1423		2,330±332		4.26±0.38		n.d.	
NS4	–	2,486±123	***	5,171±652	ns	4,176±233	*	1.26±0.17	ns	n.d.	
	+	3,291±129		5,688±447		4,824±152		1.18±0.09		n.d.	

Asterisks indicate significant differences between inoculated and uninoculated plants belonging to the same plant population: ***P<0.001, **P<0.01, *P<0.05; *ns* non-significant effect

[a] Due to low Ni concentrations in the non-serpentine soils, the shoot Ni concentrations were only determined in the shoots of the serpentine populations (*n.d.* not determined)

Discussion

In this study, we investigated the importance of AM symbiosis for plant performance under the adverse conditions of serpentine soils. The key findings of our research are as follows: (1) serpentine populations of *K. arvensis* showed lower mycorrhizal colonisation in the field than non-serpentine populations; (2) the mycorrhizal growth response was independent of the substrate type (serpentine vs. non-serpentine) and ploidy level of the host plant (diploid vs. tetraploid); and (3) substrate type and ploidy level influenced the response of *K. arvensis* plants to AM inoculation in terms of biomass allocation and nutrient uptake.

The lower mycorrhizal colonisation in the roots of the serpentine *K. arvensis* plants compared to their non-serpentine counterparts was likely caused by the specific chemistry of the serpentine soils. This is indicated by a positive correlation between the percentage of colonised roots and some chemical soil parameters, including pH, calcium and potassium concentrations and Ca/Mg ratio. Indeed, negative effects of the complex conditions of serpentine soils on AM colonisation were recently also reported by Ji et al. (2010). Our results do not allow assessment of the relative contributions of the individual components of the serpentine syndrome to the observed poorer fungal performance, but it is likely that the low Ca concentration and Ca/Mg ratio played a substantial role, as has previously been documented in plants (Brady et al. 2005). In contrast to Amir et al. (2007), we found no significant relationship between the concentration of available Ni in soil and mycorrhizal colonisation. Nonetheless, causal links between particular serpentine conditions and mycorrhizal colonisation can only be drawn from experiments that manipulate the single stress factors typical of serpentine soils. Although the negative effects of low Ca concentration (Hepper and Oshea 1984; Gustafson and Casper 2004), high Mg concentration (Jarstfer et al. 1998; Malcová et al. 2002), elevated Ni concentration (Vivas et al. 2006; Doherty et al. 2008) and low tissue Ca/Mg ratios (Jarstfer et al. 1998) on the development of AM fungi have been reported, no study has attempted to quantify the relative impact of these factors on serpentine AM fungi. In

contrast to our field results, the lack of a difference in mycorrhizal colonisation between substrate types in our pot experiment suggests that cultivation conditions (particularly, regular irrigation) attenuated the influence of the serpentine syndrome.

Diploid *Knautia* plants had a higher AM root colonisation than tetraploid plants, both in the field and in the pot experiment. Although an effect of ploidy level per se on the development of mycorrhizal colonisation cannot be excluded (see Jun and Allen 1991), the observed ploidy-specific differences were more likely caused by the different chemical characteristics of the native soils of the diploid and tetraploid plants. The higher pH values (5.4–7.1) at sites where diploids were collected were likely more favourable for the development of AM than the lower pH values (4.2–4.8) of the soils inhabited by the tetraploids. Although different species and isolates of AM fungi differ in their pH optima, pH values below 5 have repeatedly been shown to be detrimental to root colonisation, hyphal growth and sporulation (Abbott and Robson 1985; van Aarle et al. 2002).

No evidence was found to support the hypothesis of higher mycorrhizal growth dependence of serpentine *K. arvensis* ecotypes. A positive mycorrhizal growth response was observed in half of the serpentine and half of the non-serpentine populations; the remaining populations showed no significant changes in plant growth in response to AM fungi. Among the serpentine populations, the two positively responding populations, S2 and S3, originated from soils with the lowest Ca/Mg ratios, resulting from a very high soil Mg concentration in S2 and an extremely low Ca concentration in S3. In addition, both populations came from the sites with relatively low P availability. In contrast, no relationship was found between mycorrhizal growth response and Ni availability. When non-serpentine populations were compared, a strong mycorrhizal growth response was recorded for population NS3, growing in the most nutrient-deficient soil. Considering the fact that the benefit provided by AM fungi, in terms of both plant biomass and phosphorus uptake, was positively correlated with the percentage of mycorrhizal colonisation, we assume that the availability of phosphorus was the decisive factor determining the extent of the benefit for a particular plant population. In serpentine soils, however, the benefit from AM seemed to be partly determined by the soil Ca/Mg ratio and the absolute Ca and Mg concentrations.

To our knowledge, only one other study has compared plant growth response to AM inoculation in serpentine and non-serpentine soils. Ji et al. (2010) cultivated two C_4 grasses, *Sorghastrum nutans* and *Schizachyrium scoparium*, in their native sterilised soils (serpentine or non-serpentine) and followed their growth response to inoculation with AM fungi that were native or non-native to a given plant–soil system. For both grasses, mycorrhizal responsiveness was lower in the serpentine plant–soil system; this is consistent with our results and does not support the idea that symbiosis with AM fungi is of higher importance in serpentine soils. Nevertheless, we are aware that the conditions of our greenhouse experiment, namely the lack of water stress, were different from the in situ situation of serpentine sites, possibly resulting in underestimation of the importance of AM symbiosis. We can also hypothesise that other AM-mediated benefits than growth promotion (e.g., drought tolerance, root pathogen resistance or accumulation of defensive secondary compounds) might be more important for the performance and survival of a host plant under serpentine conditions. Finally, the outcome of the experiment might have been affected by differences in composition of AM fungal communities between the substrates.

Although mycorrhizal growth response did not differ significantly between serpentine versus non-serpentine and diploid versus tetraploid populations of *K. arvensis*, we observed differences between these populations in AM-mediated effects on biomass allocation. Serpentine populations and diploid populations allocated more biomass to the shoots, while non-serpentine populations and tetraploid populations were unresponsive in this regard. A lower relative investment of mycorrhizal plants in root systems is likely related to a partial substitution of the role of the roots in nutrient uptake (mainly P) by the extraradical mycelium, as reduced root/shoot ratios were also observed in response to P fertilisation (Hetrick 1991; Allsopp and Stock 1993). Therefore, a relationship between plant dependence on AM symbiosis and the occurrence/extent of AM-mediated reduction of the root/shoot ratio has been suggested (Hetrick 1991). According to this hypothesis, both serpentine and diploid plants showed higher mycorrhizal responsiveness not only in terms of biomass allocation but also nutrient uptake. In the former ones, this was also true for carotenoid content.

Numerous studies have shown differences in Mg and Ca uptake by serpentine and non-serpentine populations

of the same plant species when grown in a serpentine substrate (e.g., Johnston and Proctor 1981; O'Dell et al. 2006; Sambatti and Rice 2007). Higher Ca and lower Mg uptake, resulting in significantly higher shoot Ca/Mg ratios, have frequently been recorded for serpentine plants, suggesting that selective Ca accumulation and Mg exclusion and/or sequestration by roots are important evolutionary adaptations of serpentine species (O'Dell et al. 2006; Kazakou et al. 2008). In the present experiment, a direct comparison of Ca and Mg uptake in serpentine and non-serpentine populations of *K. arvensis* was not possible due to their cultivation in native substrates that differed in available Ca and Mg concentrations. Nevertheless, calculation of the Ca and Mg accumulation ratios (shoot/soil concentration of the elements) revealed that the serpentine populations took up comparatively less Mg from the available soil Mg pool but did not differ from the non-serpentine ones in the proportional uptake of Ca from the soil. Actually, the population NS3 grown in its native substrate with extremely low Ca availability showed much higher proportional Ca accumulation (and higher absolute Ca concentration) in the shoot biomass than any of the serpentine populations, indicating that selective Ca uptake is a general strategy of *Knautia* plants to cope with low calcium concentrations in soil.

Surprisingly, we found a negative correlation between Ca/Mg ratio (and Ca concentration) and shoot biomass, not only in the non-serpentine but also in the serpentine populations. In non-serpentine substrates with a low Mg concentration, this negative relationship can be caused by the antagonism of Mg and Ca uptake (Marschner 2002). For the same reason, a positive correlation was expected between plant growth and shoot Ca concentration in serpentine soils with an excess of Mg. The alleviation of Mg toxicity by Ca has been convincingly demonstrated both in culture solutions and in native serpentine soil (Proctor 1971; Johnston and Proctor 1981). In contrast, other studies have reported little effect of Ca addition on the growth of different serpentine plant species (Chiarucci et al. 1999; Gustafson and Casper 2004), suggesting considerable variation both in serpentine conditions (regarding the absolute concentrations of Ca and Mg, as well as their ratios) and in the mechanisms of plant adaptation to specific serpentine chemistry. Our results indicate that Mg exclusion in combination with tolerance for low internal Ca concentrations, rather than selective Ca uptake and accumulation in shoot biomass, is the mechanism by which *K. arvensis* adapts to serpentine soil conditions.

AM had different effects on Mg uptake in the serpentine and non-serpentine populations, which might be related to different Mg availability in these substrates (Clark and Zeto 2000). As in previous reports on higher Mg uptake by AM plants at low to moderate Mg availability (Liu et al. 2000, 2002), our non-serpentine plants showed an increase in shoot Mg concentration due to AM. Also, Mg concentration was positively correlated with shoot dry weight of non-serpentine plants. In contrast, no effect or decreased Mg uptake was recorded for the inoculated serpentine plants subjected to soil Mg concentrations that were an order of magnitude higher. Unlike Mg, no relationship between Ca availability and AM effect on Ca uptake was obvious. A considerable variation in soil Ca among the non-serpentine populations might explain why a trend towards decreased shoot Ca concentration in response to AM inoculation was observed only in the serpentine populations. Although a reduced Ca uptake in response to AM inoculation has been observed in other soil types (Kothari et al. 1990; Azcón and Barea 1992), no studies have dealt with serpentine soils in this respect.

A strong negative correlation between shoot Ni and shoot dry weight was only observed in uninoculated plants. This points to the role of AM fungi in the alleviation of Ni toxicity. AM inoculation significantly decreased shoot Ni concentration in the serpentine populations. However, this effect was mainly caused by a pronounced drop in Ni concentration in the population originating from the soil with the lowest Ni availability, suggesting that Ni can be immobilised in mycorrhizal structures only up to a certain threshold concentration. The important role of Ni availability and a particular host plant–AM fungus combination can also be suggested from the results of previous studies reporting a range of AM effects on plant Ni accumulation (Turnau and Mesjasz-Przybylowicz 2003; Vivas et al. 2006; Doherty et al. 2008).

The ploidy level of the host plant did not influence mycorrhizal growth dependence; however, diploid plants were more responsive to AM inoculation in terms of the effect on the root–shoot ratio and nutrient uptake. The significantly higher pH and CEC values of the substrates inhabited by the diploids, however, hinder the separation of the role of AM fungi and soil parameters in modulating nutrient uptake by the different cytotypes. Inter-cytotype

variation in response to inoculation with AM fungi has recently been demonstrated (Sudová et al. 2010). However, because no mixed-ploidy populations of *K. arvensis* were known at the onset of our study, no clear conclusions about the ploidy-specific effects of AM fungi on plant growth and nutrient uptake can be made.

In conclusion, the present study provides the first insight into the impact of AM inoculation on the performance of *K. arvensis* in serpentine soils. Our results do not support the hypothesis that serpentine populations show higher mycorrhizal growth dependence than non-serpentine populations when grown in their native soils and inoculated with native fungal communities. These results need to be validated by other methodological approaches, including: (1) the cultivation of different serpentine and non-serpentine populations in their native substrates and inoculation with identical AM fungi (in order to avoid a potential bias due to the uneven composition of native AM communities at different sites); and (2) a reciprocal transplant pot experiment involving serpentine and non-serpentine soils, plants and AM fungi (in order to uncover the genetic and edaphic factors that determine the responses of serpentine and non-serpentine plants to AM inoculation). The impact of AM on other parameters related to plant fitness, including during drought, should also be considered to elucidate the role of mycorrhizas in plant adaptation to the adverse conditions of serpentine soils.

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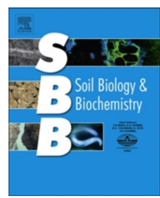

The symbiosis with arbuscular mycorrhizal fungi contributes to plant tolerance to serpentine edaphic stress

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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.

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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.

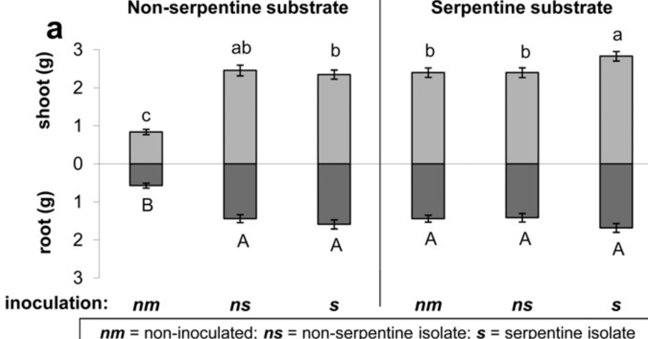
3.4. Element concentrations in plant biomass

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



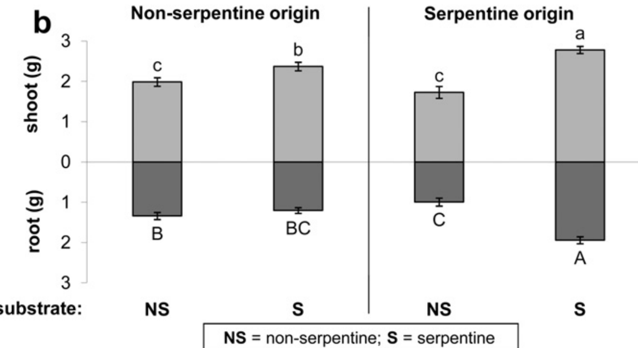


Fig. 1. Shoot and root dry biomass as affected by (a) substrate and AM inoculation and (b) plant origin and substrate. Columns represent means (±SE) of (a) 54–55 replicates; (b) 96 and 66–69 replicates for plants of non-serpentine and serpentine origin, respectively. Columns marked by different letters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

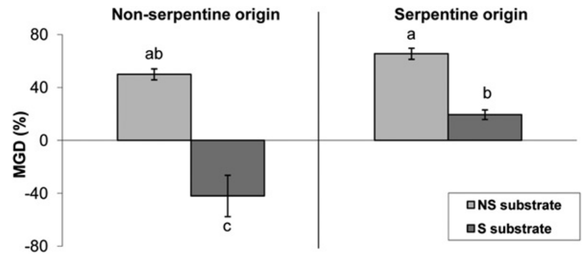
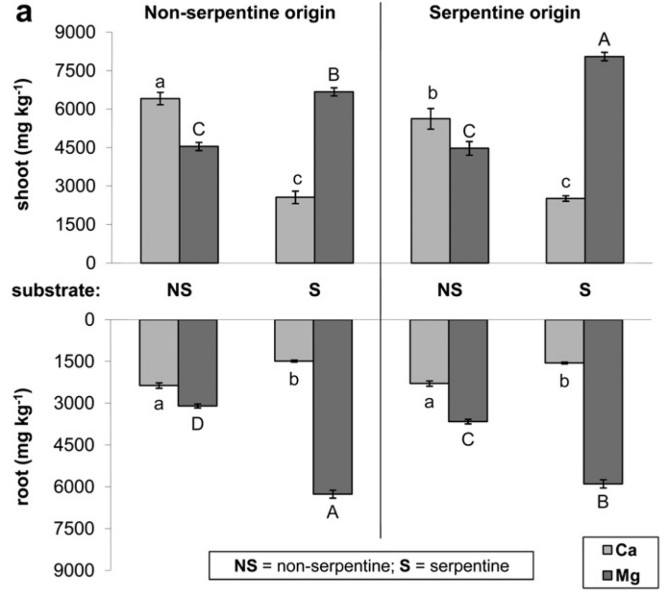


Fig. 2. Mycorrhizal growth dependence as affected by plant origin and substrate. Columns represent means (±SE) of 64 and 44–46 replicates for plants of non-serpentine and serpentine origin, respectively. Columns marked by different letters are significantly different according to Tukey HSD test ($P 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



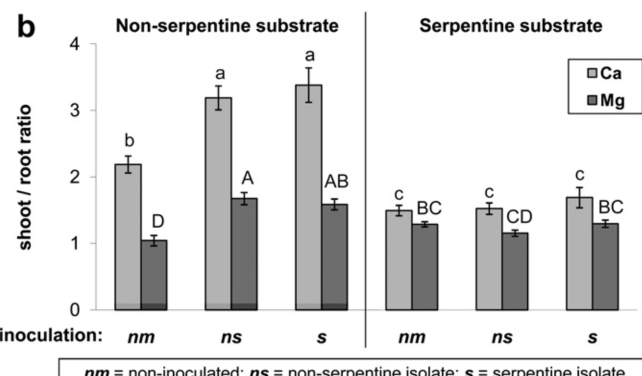


Fig. 3. (a) Calcium and magnesium concentrations in dry shoot biomass as affected by substrate and plant origin. (b) Shoot/root ratios of Ca and Mg concentrations, respectively, as affected by substrate and AM inoculation. Columns represent means (±SE) of (a) 96 and 66–69 replicates for plants of non-serpentine 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 ($P < 0.05$).

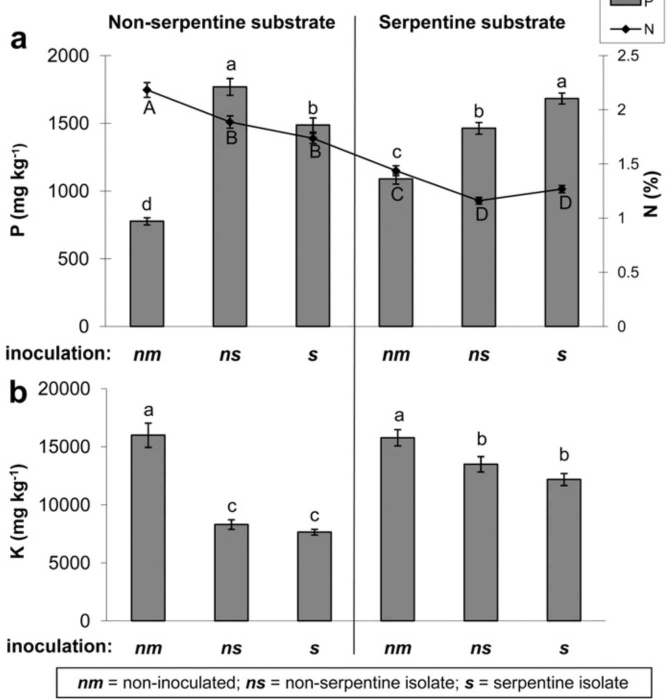


Fig. 4. Phosphorus and nitrogen (a) and potassium (b) concentrations in shoot dry biomass as affected by substrate and AM inoculation. Columns represent means (±SE) of 54–55 replicates. Columns marked by different letters (a–d for P and K concentrations; A–D for N concentrations) are significantly different 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 ± 1.7) and weak N-limitation in the S substrate (13.1 ± 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 ± 0.6) and a more intense N-limitation in the S substrate (8.1 ± 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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Supplementary data

Figure S1 Distribution of the studied *Knautia arvensis* populations within the Czech and the Slovak Republics

Figure S2 Phylogenetic tree showing the position of the isolates used for the inoculation of *K. arvensis* plants

Table S1 Root colonisation by arbuscular mycorrhizal fungi, length of extraradical mycelium and mycorrhizal growth dependence

Table S2 Plant growth parameters

Table S3 Calcium and magnesium concentrations in shoot and root biomass

Table S4 Nickel concentrations in shoot and root biomass

Table S5 Phosphorus, nitrogen and potassium concentrations, and N:P and C:N ratios in shoot biomass

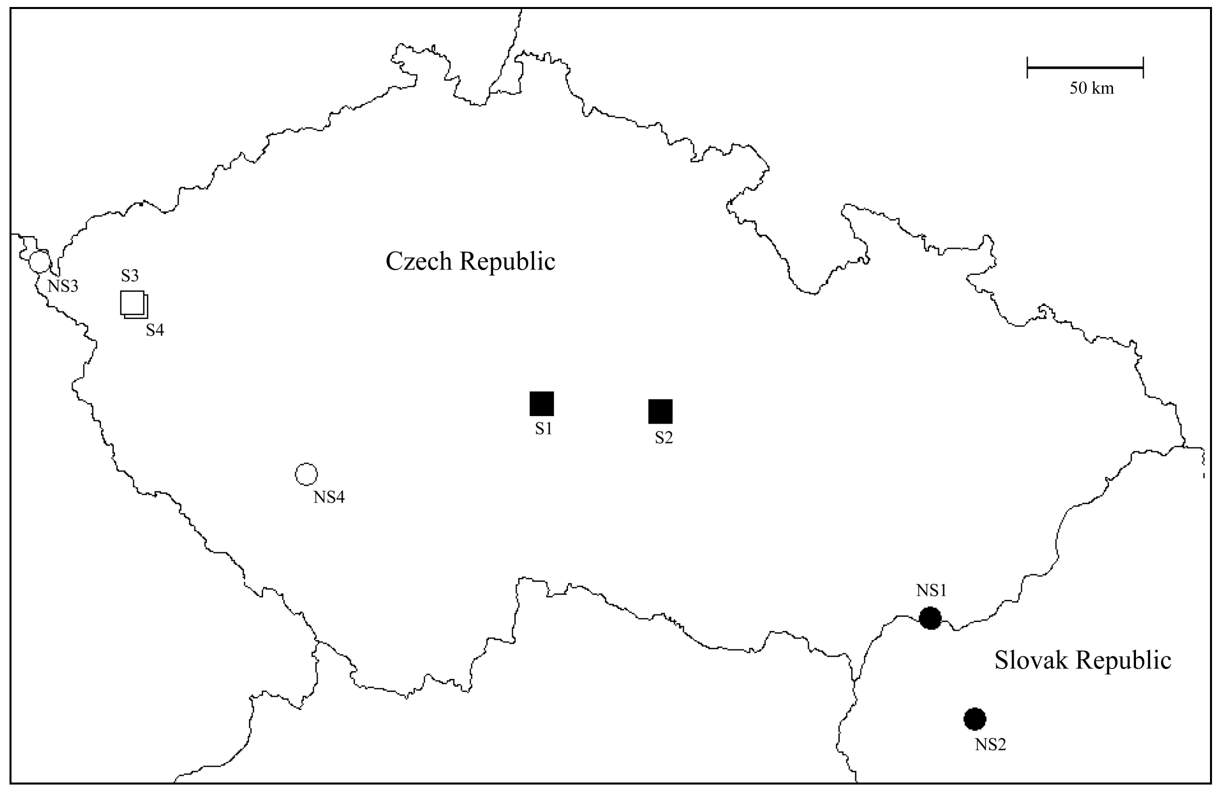

Fig. S1 Distribution of the studied *Knautia arvensis* populations within the Czech Republic and the Slovak Republic. Squares - serpentine populations; circles - non-serpentine populations; full symbols - diploids; empty symbols - tetraploids. Non-serpentine diploid and tetraploid populations show parapatric distribution while serpentine populations are restricted to isolated serpentine islands. Since introgressive hybridisation occurs frequently at tetraploid level between non-serpentine and neighbouring serpentine plants of *Knautia arvensis* (in the western part of the area) and between *K. arvensis* and *K. kitaibelii* (in the eastern part of the area), non-serpentine tetraploid populations were selected from areas harbouring genetically-pure plants (F. Kolář et al., unpublished data).

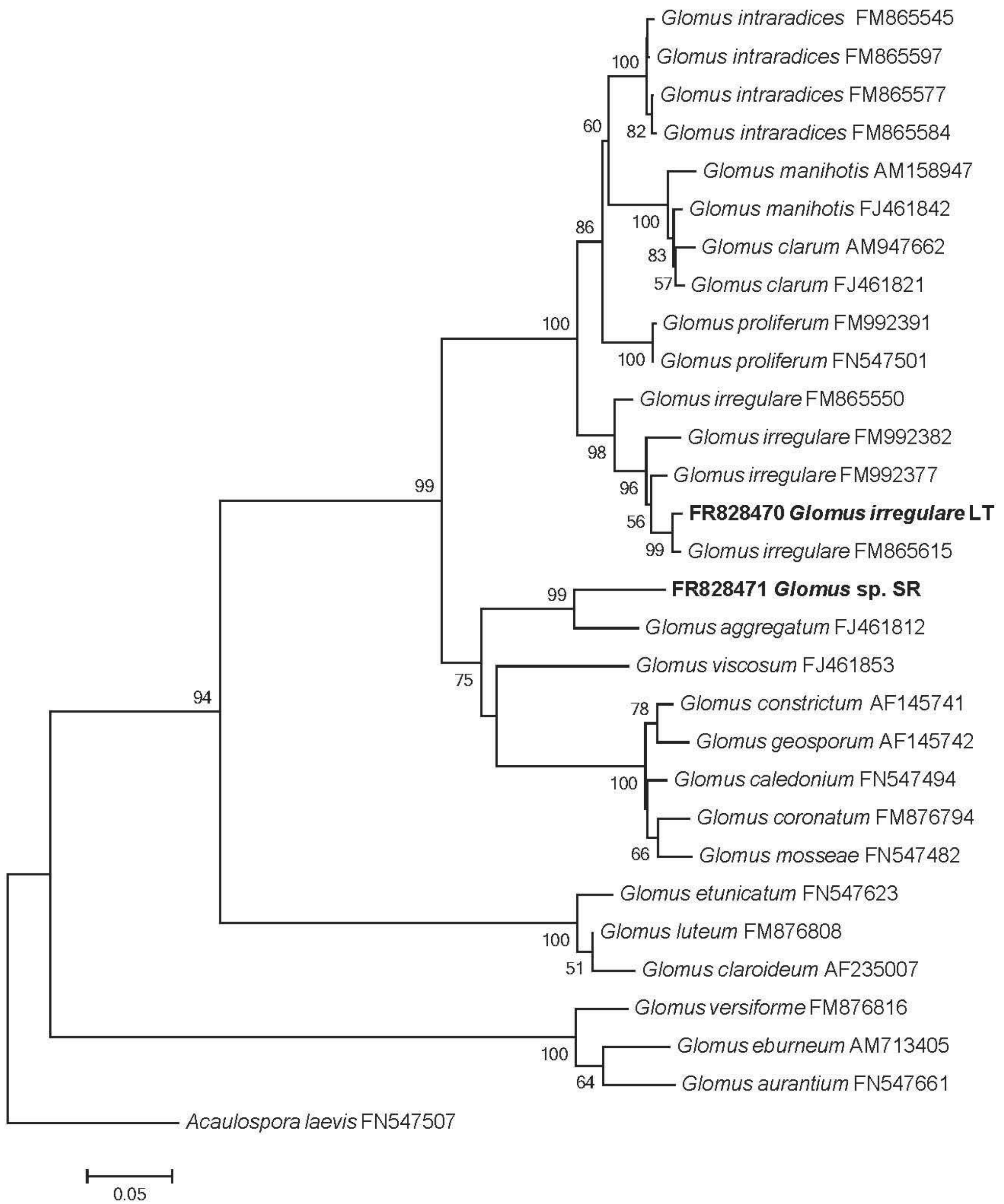

Fig. S2. Phylogenetic tree showing the position of the AMF isolates *Glomus irregulare* LT and *Glomus* sp. SR, used for inoculation of *K. arvensis* plants. The results are based on a neighbour-joining analysis of 28S (LSU) rDNA sequences (624 characters). This region was chosen for the analysis while the sequence of *Glomus aggregatum*, representing a sister clade to *Glomus* sp. SR isolate, was available only in the LSU rDNA in the GeneBank. Nevertheless, the position of both isolates in the phylogenetic tree remained the same, when the entire amplified fragment (partial 18S rDNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rDNA) was analysed. The tree was rooted using the sequence of *Acaulospora laevis*. Numbers above/below branches denote bootstrap values from 1 000 replications. The sequences of the isolates used in the present study are shown in boldface.

Table S1 Root colonisation by arbuscular mycorrhizal fungi (AMF), length of extraradical mycelium (ERM) and mycorrhizal growth dependence (MGD, calculated based on shoot dry biomass) of *Knautia arvensis* plants. The data represent means (±SE) of 16 replicates, except for *Serpentine 2x* populations where n=6–7. The following legend is used: a) population origin (non-serpentine, serpentine) and cytotype (2x = diploids, 4x = tetraploids); b) substrate type (NS = non-serpentine, S = serpentine); c) AM inoculation (*nm* = non-mycorrhizal, *ns* = non-serpentine AMF isolate, *s* = serpentine AMF isolate). The *significant effects*, calculated separately for each population type, are encoded as follows: substrate (1), AM inoculation (2), substrate × AM inoculation interaction (1×2). For significant interactions, differences among the means are marked with different letters according to Tukey HSD test at P<0.05

population	substrate	AM inoculation	AM root colonisation (%)		ERM length (m g^{-1})		MGD (%)
Non-Serpentine 2x	NS	*ns*	94.5 ± 1.4	a	2.37 ± 0.34	a	48.5 ± 6.1
		s	90.8 ± 1.4	a	1.44 ± 0.23	b	40.4 ± 8.7
	S	*ns*	84.8 ± 1.9	b	0.73 ± 0.12	b	-39.7 ± 11.9
		s	95.5 ± 0.9	a	0.71 ± 0.15	b	-14.2 ± 16.1
significant effects:			2*, 1×2***		1***, 2*, 1×2*		1***
Non-Serpentine 4x	NS	*ns*	93.0 ± 3.1	ab	1.90 ± 0.28		55.1 ± 7.6
		s	93.7 ± 1.5	ab	2.38 ± 0.29		55.8 ± 10.1
	S	*ns*	86.3 ± 2.4	b	0.39 ± 0.07		-114.4 ± 55.3
		s	96.1 ± 0.9	a	0.65 ± 0.14		0.2 ± 11.2
significant effects:			2*, 1×2***		1***		1***
Serpentine 2x	NS	*ns*	93.0 ± 1.8		1.14 ± 0.48		37.4 ± 18.3
		s	92.2 ± 4.9		0.74 ± 0.19		45.5 ± 12.8
	S	*ns*	89.3 ± 2.5		1.30 ± 0.22		16.4 ± 18.8
		s	91.3 ± 1.7		0.15 ± 0.18		32.0 ± 5.1
significant effects:			-		-		-
Serpentine 4x	NS	*ns*	94.5 ± 2.2	a	2.27 ± 0.37		74.0 ± 4.8
		s	91.3 ± 2.0	ab	1.53 ± 0.29		75.0 ± 3.6
	S	*ns*	86.7 ± 1.8	b	1.05 ± 0.12		14.0 ± 4.2
		s	94.8 ± 0.8	a	1.17 ± 0.13		20.6 ± 4.7
significant effects:			1×2**		1**		1***

*** *P*<0.001, ** *P*<0.01, * *P*<0.05, ns non-significant

Table S2 Growth parameters of *K. arvensis* plants. For abbreviations, legend and replicate number see Table S1

population	substrate	AM inoculation	leaf area (cm^2)		shoot biomass (g)		root biomass (g)		root-shoot ratio	
Non-Serpentine 2x	NS	*nm*	129.0 ± 16.8	c	1.27 ± 0.14	c	0.79 ± 0.11	b	0.60 ± 0.04	b
		ns	310.2 ± 23.0	a	2.78 ± 0.22	ab	1.56 ± 0.14	a	0.59 ± 0.05	a
		s	288.6 ± 26.1	ab	2.48 ± 0.18	b	1.62 ± 0.15	a	0.65 ± 0.05	a
	S	*nm*	288.6 ± 17.8	ab	3.24 ± 0.16	a	1.62 ± 0.11	a	0.51 ± 0.03	a
		ns	223.8 ± 12.1	b	2.51 ± 0.16	ab	1.32 ± 0.16	ab	0.53 ± 0.06	a
		s	296.8 ± 17.3	ab	3.26 ± 0.21	a	1.69 ± 0.15	a	0.52 ± 0.03	a
significant effects:			2***, 1×2***		1***, 2**, 1×2***		2**, 1×2***		1**	
Non-Serpentine 4x	NS	*nm*	86.5 ± 12.9	b	0.80 ± 0.10	b	0.68 ± 0.16	c	0.82 ± 0.08	
		ns	254.5 ± 29.0	a	2.33 ± 0.30	a	1.50 ± 0.24	ab	0.62 ± 0.06	
		s	256.6 ± 29.2	a	2.25 ± 0.18	a	1.88 ± 0.27	a	0.76 ± 0.08	
	S	*nm*	151.3 ± 15.5	b	1.67 ± 0.18	ab	0.91 ± 0.15	bc	0.53 ± 0.05	
		ns	113.8 ± 19.7	b	1.58 ± 0.25	ab	0.73 ± 0.15	bc	0.42 ± 0.04	
		s	156.4 ± 22.9	b	1.96 ± 0.20	a	0.96 ± 0.17	bc	0.46 ± 0.05	
significant effects:			1**, 2***, 1×2***		2***, 1×2***		1**, 2**, 1×2**		1***, 2*	
Serpentine 2x	NS	*nm*	83.5 ± 12.8		0.59 ± 0.12		0.41 ± 0.08		0.70 ± 0.07	
		ns	228.9 ± 58.7		1.58 ± 0.42		0.78 ± 0.22		0.47 ± 0.05	
		s	188.2 ± 45.5		1.39 ± 0.32		0.67 ± 0.19		0.49 ± 0.05	
	S	*nm*	268.7 ± 23.9		1.90 ± 0.27		1.39 ± 0.24		0.74 ± 0.09	
		ns	358.0 ± 36.1		2.61 ± 0.28		1.68 ± 0.29		0.62 ± 0.06	
		s	378.1 ± 21.4		2.84 ± 0.20		2.17 ± 0.18		0.77 ± 0.05	
significant effects:			1***, 2**		1***, 2**		1***		1**, 2*	
Serpentine 4x	NS	*nm*	61.5 ± 8.4	c	0.54 ± 0.06	c	0.30 ± 0.06	b	0.53 ± 0.06	
		ns	314.4 ± 29.8	ab	2.59 ± 0.23	ab	1.50 ± 0.18	a	0.55 ± 0.04	
		s	329.4 ± 32.0	ab	2.66 ± 0.25	ab	1.61 ± 0.20	a	0.59 ± 0.05	
	S	*nm*	272.4 ± 16.3	b	2.50 ± 0.17	b	1.81 ± 0.15	a	0.71 ± 0.03	
		ns	340.0 ± 17.6	ab	3.01 ± 0.15	ab	2.09 ± 0.17	a	0.71 ± 0.06	
		s	365.4 ± 21.2	a	3.27 ± 0.15	a	2.18 ± 0.22	a	0.67 ± 0.06	
significant effects:			1***, 2***, 1×2***		1***, 2***, 1×2***		1***, 2***, 1×2**		1**	

Table S3 Calcium and magnesium concentrations in shoot and root biomass of *K. arvensis* plants. For abbreviations, legend and replicate number see Table S1

population	substrate	AM inoculation	shoot Ca (mg kg^{-1})	root Ca (mg kg^{-1})	shoot/root Ca ratio	shoot Mg (mg kg^{-1})	root Mg (mg kg^{-1})	shoot/root Mg ratio
Non-Serpentine 2x	NS	*nm*	5 493 ± 511	3 131 ± 290	1.9 ± 0.2	2 980 ± 139 c	3 005 ± 257 b	1.1 ± 0.1 b
		ns	6 125 ± 471	2 264 ± 178	2.9 ± 0.3	4 630 ± 237 b	2 864 ± 154 b	1.8 ± 0.2 a
		s	5 950 ± 656	2 563 ± 297	2.7 ± 0.4	4 859 ± 264 b	3 279 ± 145 b	1.5 ± 0.1 ab
	S	*nm*	2 302 ± 344	1 567 ± 55	1.4 ± 0.2	6 309 ± 309 a	5 675 ± 184 a	1.1 ± 0.1 b
		ns	1 833 ± 122	1 381 ± 67	1.4 ± 0.1	6 736 ± 231 a	6 561 ± 372 a	1.1 ± 0.1 b
		s	1 946 ± 125	1 460 ± 73	1.4 ± 0.1	7 221 ± 190 a	5 712 ± 260 a	1.3 ± 0.1 ab
significant effects:			1***	1***, 2*	1***	1***, 2***, 1×2*	1***, 1×2*	1**, 2*, 1×2**
Non-Serpentine 4x	NS	*nm*	6 200 ± 415	2 584 ± 196 a	2.5 ± 0.2	3 240 ± 165 b	3 237 ± 176 c	1.0 ± 0.1 b
		ns	7 296 ± 674	1 970 ± 138 b	3.8 ± 0.3	5 567 ± 400 a	3 074 ± 183 c	1.8 ± 0.1 a
		s	7 350 ± 653	1 720 ± 102 bc	4.5 ± 0.5	5 892 ± 377 a	3 120 ± 129 c	1.9 ± 0.1 a
	S	*nm*	2 179 ± 374	1 459 ± 57 bc	1.3 ± 0.1	6 423 ± 369 a	5 585 ± 161 b	1.2 ± 0.1 b
		ns	3 694 ± 984	1 643 ± 146 bc	1.5 ± 0.2	6 721 ± 593 a	7 472 ± 368 a	1.0 ± 0.1 b
		s	3 414 ± 965	1 428 ± 107 c	2.3 ± 0.5	6 641 ± 497 a	6 682 ± 409 a	1.1 ± 0.1 b
significant effects:			1***	1***, 2**, 1×2**	1***, 2***	1***, 2**, 1×2*	1***, 2**, 1×2***	1***, 2**, 1×2***
Serpentine 2x	NS	*nm*	4 735 ± 191	2 696 ± 298 a	1.9 ± 0.2 b	2 628 ± 266	4 137 ± 466	0.7 ± 0.1
		ns	5 016 ± 636	1 597 ± 73 bc	3.4 ± 0.4 a	4 396 ± 938	3 682 ± 377	1.5 ± 0.4
		s	4 638 ± 386	2 328 ± 317 ab	2.2 ± 0.3 b	3 679 ± 625	3 446 ± 291	1.1 ± 0.3
	S	*nm*	2 111 ± 198	1 577 ± 97 bc	1.4 ± 0.1 b	8 427 ± 295	6 088 ± 462	1.4 ± 0.1
		ns	1 959 ± 137	1 557 ± 36 c	1.3 ± 0.1 b	7 939 ± 545	6 927 ± 728	1.2 ± 0.1
		s	2 086 ± 231	1 591 ± 78 bc	1.3 ± 0.1 b	8 253 ± 502	6 230 ± 458	1.4 ± 0.1
significant effects:			1***	1***, 2*, 1×2*	1***, 2**, 1×2**	1***	1***	-
Serpentine 4x	NS	*nm*	5 824 ± 1 035	2 837 ± 218 a	2.2 ± 0.3	3 938 ± 712	3 696 ± 136 c	1.1 ± 0.2
		ns	5 500 ± 857	1 993 ± 81 bc	2.8 ± 0.4	5 118 ± 302	3 543 ± 155 c	1.5 ± 0.1
		s	6 470 ± 956	2 121 ± 187 b	3.3 ± 0.5	5 416 ± 514	3 632 ± 113 c	1.5 ± 0.2
	S	*nm*	2 687 ± 177	1 497 ± 58 c	1.8 ± 0.2	7 413 ± 304	5 144 ± 172 b	1.5 ± 0.1
		ns	2 944 ± 301	1 575 ± 68 c	1.8 ± 0.1	8 214 ± 383	5 999 ± 269 a	1.4 ± 0.1
		s	2 535 ± 210	1 561 ± 80 c	1.6 ± 0.1	8 324 ± 372	5 854 ± 275 ab	1.5 ± 0.1
significant effects:			1***	1***, 2**, 1×2**	1***	1***, 2*	1***, 1×2*	-

Table S4 Nickel concentrations in shoot and root biomass of *K. arvensis* plants cultivated in the serpentine substrate. For abbreviations, legend and replicate number see Table S1

population	AM inoculation	shoot Ni (mg kg^{-1})		root Ni (mg kg^{-1})		shoot/root Ni	
Non-Serpentine 2x	*nm*	28.7 ± 2.3		166.2 ± 11.0	a	0.18 ± 0.02	
	ns	29.8 ± 2.7	ns	152.4 ± 10.2	a	0.20 ± 0.02	ns
	s	33.1 ± 2.8		113.4 ± 10.1	b	0.70 ± 0.42	
significant effects:		-		2**		-	
Non-Serpentine 4x	*nm*	27.9 ± 3.6		205.2 ± 24.9		0.17 ± 0.02	
	ns	27.7 ± 5.2	ns	227.8 ± 15.4	ns	0.14 ± 0.02	ns
	s	28.2 ± 5.1		196.9 ± 15.9		0.15 ± 0.03	
significant effects:		-		-		-	
Serpentine 2x	*nm*	35.5 ± 3.1		186.1 ± 39.2		0.21 ± 0.03	
	ns	31.4 ± 7.3	ns	188.7 ± 23.4	ns	0.17 ± 0.03	ns
	s	27.0 ± 2.9		157.6 ± 16.0		0.18 ± 0.02	
significant effects:		-		-		-	
Serpentine 4x	*nm*	27.9 ± 2.4		169.2 ± 15.6		0.20 ± 0.03	
	ns	34.7 ± 4.9	ns	161.5 ± 12.5	ns	0.25 ± 0.05	ns
	s	37.6 ± 7.3		198.5 ± 13.6		0.19 ± 0.03	
significant effects:		-		-		-	

Table S5 Phosphorus, nitrogen and potassium concentrations, and N:P and C:N ratios in shoot biomass of *K. arvensis* plants. For abbreviations, legend and replicate number see Table S1

population	substrate	AM inoculation	P (mg kg^{-1})		N (%)		N:P		C:N		K (mg kg^{-1})	
Non-Serpentine 2x	NS	*nm*	670 ± 33	d	1.79 ± 0.07	a	27.0 ± 1.9	a	26.3 ± 1.0	c	9 763 ± 495	
		ns	1 761 ± 72	a	1.89 ± 0.04	a	11.0 ± 0.5	bc	24.5 ± 0.6	c	7 714 ± 313	
		s	1 463 ± 94	b	1.73 ± 0.07	a	12.3 ± 0.9	b	27.6 ± 1.3	c	6 951 ± 387	
	S	*nm*	959 ± 42	c	1.21 ± 0.05	b	12.8 ± 0.6	b	38.3 ± 1.3	b	11 247 ± 600	
		ns	1 328 ± 60	b	1.07 ± 0.03	b	8.3 ± 0.5	c	43.6 ± 1.2	a	10 516 ± 309	
		s	1 559 ± 45	ab	1.19 ± 0.05	b	7.7 ± 0.4	c	39.5 ± 1.4	ab	10 192 ± 461	
significant effects:			2***, 1×2***		1***, 1×2*		1***, 2***, 1×2***		1***, 1×2**		1***, 2***	
Non-Serpentine 4x	NS	*nm*	749 ± 37	c	2.20 ± 0.09		30.7 ± 2.3	a	21.8 ± 1.0		16 785 ± 1 627	
		ns	1 835 ± 160	a	1.87 ± 0.16		10.5 ± 0.5	c	26.7 ± 1.6		7 797 ± 898	
		s	1 561 ± 83	ab	1.91 ± 0.10		12.3 ± 0.4	c	25.1 ± 1.3		7 473 ± 299	
	S	*nm*	917 ± 44	c	1.59 ± 0.06		17.7 ± 1.1	b	29.6 ± 1.2		15 900 ± 833	
		ns	1 393 ± 98	b	1.33 ± 0.06		10.4 ± 1.0	c	35.4 ± 1.8		12 390 ± 1 830	
		s	1 623 ± 62	ab	1.48 ± 0.08		9.3 ± 0.6	c	32.4 ± 1.6		9 776 ± 828	
significant effects:			2***, 1×2**		1***, 2*		1***, 2***, 1×2***		1***, 2**		1*, 2***	
Serpentine 2x	NS	*nm*	1 055 ± 97	b	2.67 ± 0.14		27.1 ± 4.1	a	17.7 ± 1.2		27 760 ± 3 268	a
		ns	1 933 ± 63	a	2.10 ± 0.11		10.9 ± 0.4	b	22.6 ± 1.3		12 105 ± 1 753	c
		s	1 441 ± 132	ab	1.75 ± 0.16		12.3 ± 1.0	b	28.3 ± 2.6		10 175 ± 671	c
	S	*nm*	1 525 ± 127	ab	1.82 ± 0.16		12.0 ± 0.8	b	26.7 ± 2.5		22 870 ± 1 944	ab
		ns	1 716 ± 127	a	1.22 ± 0.12		7.1 ± 0.4	b	39.3 ± 3.3		17 211 ± 498	bc
		s	1 754 ± 114	a	1.18 ± 0.03		6.9 ± 0.4	b	39.3 ± 1.2		15 931 ± 733	bc
significant effects:			2***, 1×2*		1***, 2***		1***, 2***, 1×2**		1***, 2***		2***, 1×2**	
Serpentine 4x	NS	*nm*	802 ± 42	d	2.38 ± 0.14	a	30.2 ± 2.1	a	20.4 ± 1.5	d	16 630 ± 1 552	a
		ns	1 650 ± 112	ab	1.81 ± 0.08	b	13.2 ± 2.6	b	26.3 ± 1.1	c	7 953 ± 523	b
		s	1 454 ± 121	bc	1.57 ± 0.06	bc	13.5 ± 2.6	b	29.7 ± 1.0	c	7 568 ± 448	b
	S	*nm*	1 202 ± 50	c	1.36 ± 0.07	cd	11.4 ± 0.5	b	35.2 ± 1.7	b	17 085 ± 1 199	a
		ns	1 553 ± 55	abc	1.07 ± 0.03	d	7.0 ± 0.2	b	43.0 ± 1.1	a	15 939 ± 713	a
		s	1 837 ± 94	a	1.18 ± 0.04	d	6.8 ± 0.5	b	39.3 ± 1.2	ab	14 917 ± 823	a
significant effects:			1**, 2***, 1×2**		1***, 2***, 1×2***		1***, 2***, 1×2***		1***, 2***, 1×2*		1***, 2***, 1×2***	

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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 (0.03)	5.04 (0.05)	0.62 (0.07)	9.4 (0.1)	11.3 (0.1)	394 (23)	2 231 (105)	43.0 (5.3)	5.5 (0.3)	125.4 (15.2)	28.1 (0.7)	98.3 (18.2)	3.6 (0.1)	0.32 (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

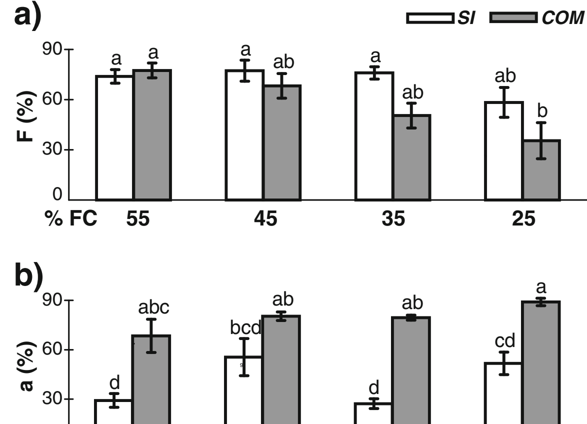


Fig. 1 **a** Mycorrhizal root colonisation (*F*) and **b** relative arbuscule abundance (*a*) in inoculated plants as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated 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

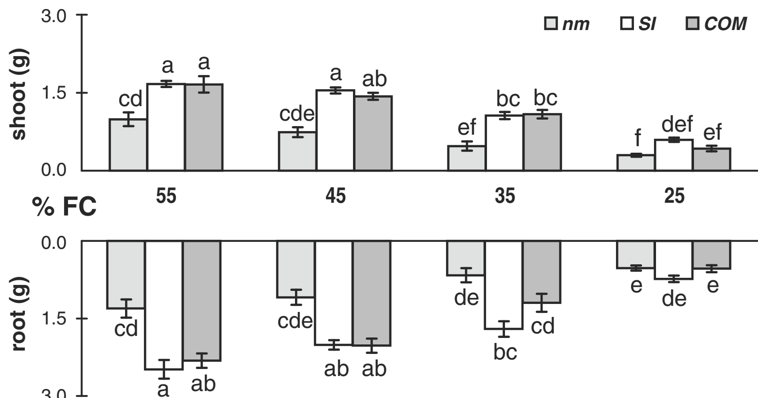


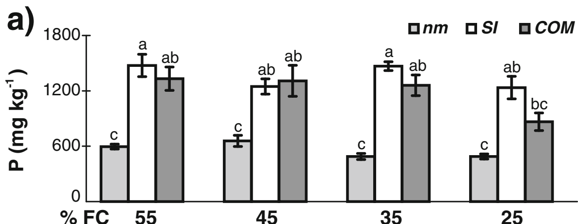
Fig. 2 Shoot and root dry weight as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns represent the means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

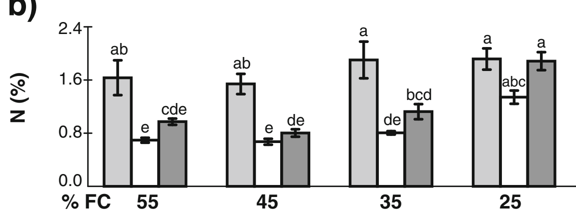
P_N and *E* values than *nm* plants; however, this effect was largely caused by a marked difference at 25 % FC (Table 2, Figs. S2a, b). *WUE* was significantly affected only by water regime × inoculation interaction (Table 2). At 55 % and 45 % FC, *M* plants showed lower *WUE* values compared to *nm* 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).





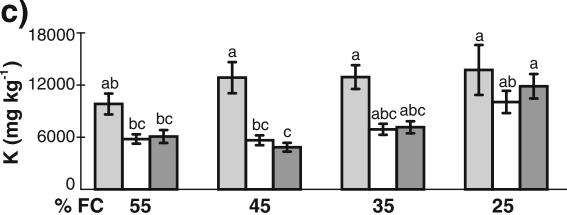
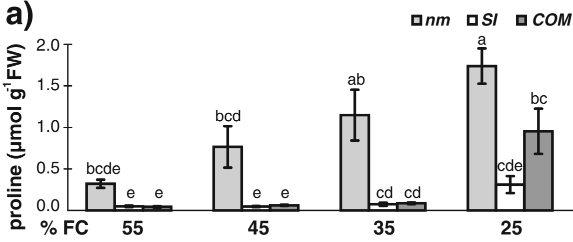


Fig. 3 **a** Phosphorus, **b** nitrogen and **c** potassium concentrations in shoot biomass as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated 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).



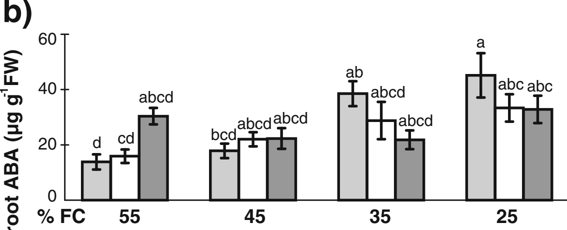


Fig. 4 Root **a** proline and **b** ABA concentrations as affected by water regime (55, 45, 35 and 25 % of field capacity, FC) and AMF inoculation treatment (*nm* – non-mycorrhizal, *SI* – plants inoculated with single *Glomus* sp. isolate, *COM* – plants inoculated with native AMF community). Columns 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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Electronic supplementary material (Plant and Soil)

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

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Figure S1 Phylogenetic tree showing the position of AMF colonising the roots of *K. arvensis*
Figure S2 Net photosynthetic rate, transpiration rate, water use efficiency and photosynthetic phosphorus-use efficiency of *K. arvensis* plants as affected by water regime and AMF inoculation treatment

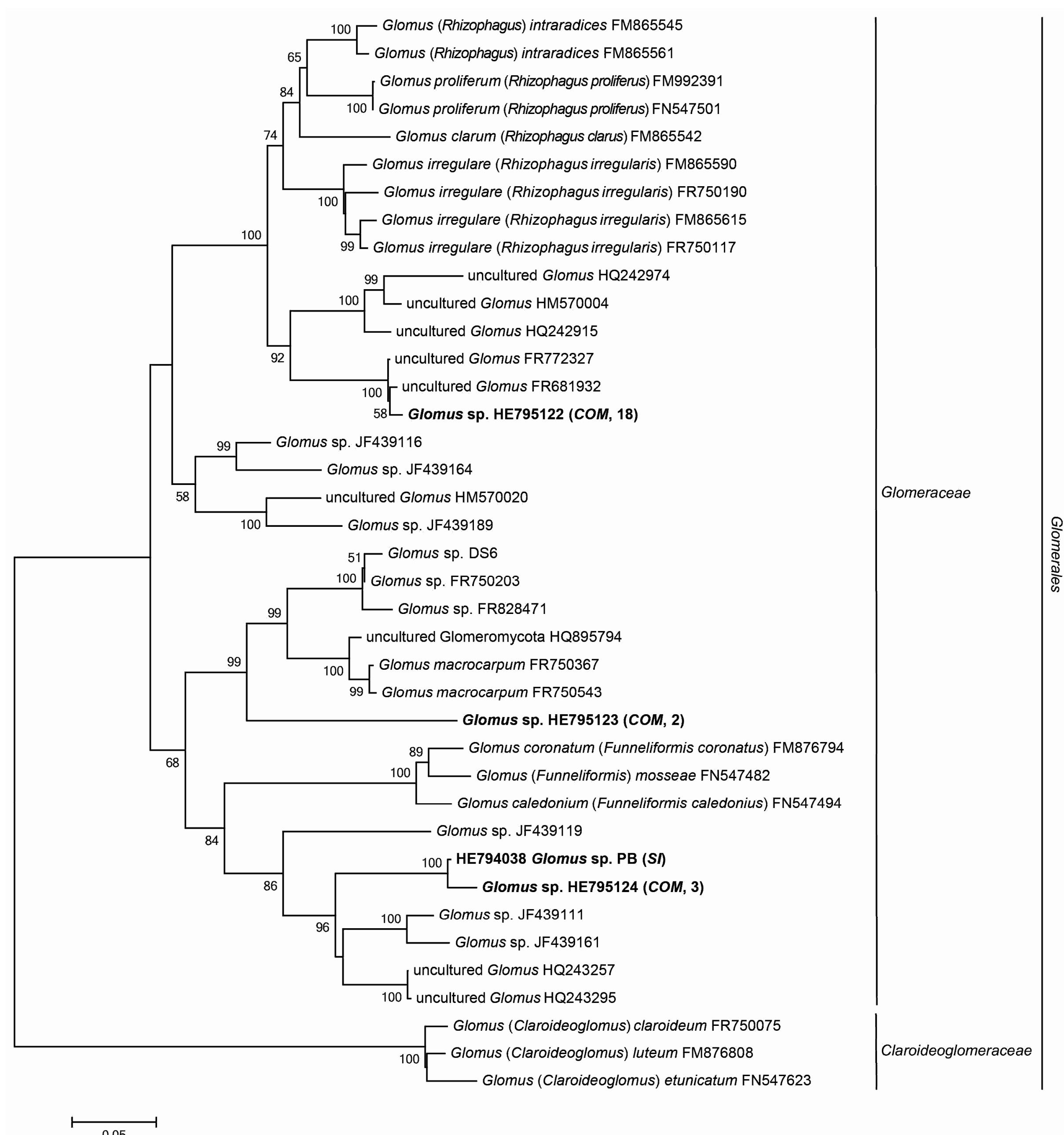
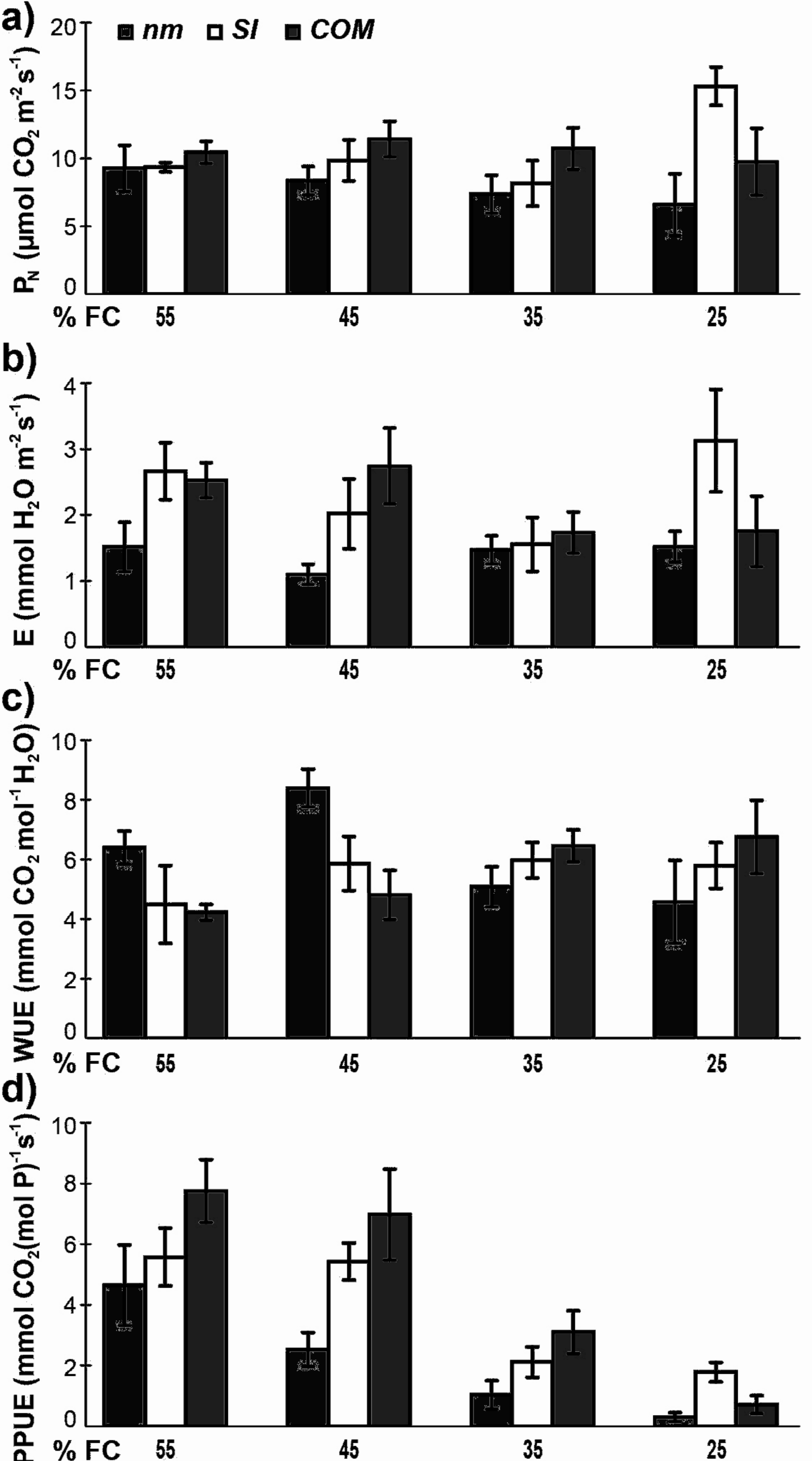


Fig. S1. Phylogenetic tree showing the position of AMF colonising the roots of *K. arvensis*. The results are based on a neighbour-joining analysis of the partial 18S rDNA, ITS1, 5.8S rRNA, ITS2 and the partial 28S rDNA sequences. The sequences of the isolate *Glomus* sp. PB (*SI* treatment) and the AMF colonising plant roots in the *COM*-treatment are shown in boldface. For each MOTU obtained in the *COM*-treatment, a representative sequence is provided, with the number of clones shown in brackets. The tree was rooted using the sequence of *Claroideoglomus* spp. Numbers above/below branches indicate the level of bootstrap support (%) from 1,000 replications; only values above 50% are given. The orders and families as well as the names of AMF taxa in brackets correspond to the most recent AMF classification according to Schüssler and Walker (2010) and Krüger et al. (2012).

Fig. S2. **(a)** Net photosynthetic rate (P_N), **(b)** transpiration rate (E), **(c)** water use efficiency (WUE) and **(d)** photosynthetic phosphorus-use efficiency (PPUE) 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



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ORIGINAL PAPER

Soil nutritional status, not inoculum identity, primarily determines the effect of arbuscular mycorrhizal fungi on the growth of *Knautia arvensis* plants

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Abstract Arbuscular mycorrhizal (AM) symbiosis is among the factors contributing to plant survival in serpentine soils characterised by unfavourable physicochemical properties. However, AM fungi show a considerable functional diversity, which is further modified by host plant identity and edaphic conditions. To determine the variability among serpentine AM fungal isolates in their effects on plant growth and nutrition, a greenhouse experiment was conducted involving two serpentine and two non-serpentine populations of *Knautia arvensis* plants grown in their native substrates. The plants were inoculated with one of the four serpentine AM fungal isolates or with a complex AM fungal community native to the respective plant population. At harvest after 6-month cultivation, intraradical fungal development was assessed, AM fungal taxa established from native fungal communities were determined and plant growth and element uptake evaluated. AM symbiosis significantly improved the performance of all the *K. arvensis* populations. The extent of mycorrhizal growth promotion was mainly governed by nutritional status of the substrate, while the effect of AM fungal identity was negligible. Inoculation with the native AM fungal communities was not more efficient than inoculation with single AM fungal isolates in any plant population. Contrary to the growth effects, a certain variation among AM fungal isolates was revealed in terms of their effects on plant nutrient uptake, especially P, Mg and Ca, with none of the AM fungi being generally superior in this respect. Regardless of AM symbiosis, *K. arvensis* populations significantly differed in their relative nutrient accumulation ratios, clearly showing the plant's ability to adapt to nutrient deficiency/excess.

Keywords Arbuscular mycorrhizal symbiosis · Magnesium · Native fungal communities · Nutrient availability · Phosphorus uptake · Serpentine soils

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Introduction

Plants inhabiting serpentine habitats have to cope with multiple edaphic stress conditions, including low Ca/Mg ratios and elevated concentrations of heavy metals, often together with macronutrient deficiencies, low content of organic matter, shallow soil profile and low water-holding capacity (for reviews, see Brady et al. 2005; O'Dell and Rajakaruna 2011). In addition to various morphological and physiological adaptations (Brady et al. 2005), many serpentine plants also rely on a symbiotic partnership with arbuscular mycorrhizal (AM) fungi (see Ji et al. 2010; Lagrange et al. 2011; Turnau and Mesjasz-Przybylowicz 2003). These obligate symbionts have been repeatedly shown to alleviate different abiotic and biotic stresses to their host plants (for reviews, see Entry et al. 2002; Pozo et al. 2010; Smith et al. 2010).

A considerable functional diversity between AM fungi, both at the interspecific and intraspecific level, has however been shown especially in terms of fungal-mediated effects

on plant P nutrition and subsequent plant growth promotion, heavy metal uptake or drought tolerance. This functional diversity results from taxon-specific differences in fungal carbon requirements and traits related to nutrient metabolism and transfer to host plants (Maherali and Klironomos 2007; Munkvold et al. 2004; van der Heijden et al. 2003). Also, AM fungi adapt to their native abiotic conditions, resulting in different levels of stress-tolerance provided to their hosts (Marulanda et al. 2007; Ruiz-Lozano et al. 1995a; Wu et al. 2007). An exploration of environmental/edaphic adaptations of AM fungi, that is a comparison of native vs. exotic strains or species in a given habitat, has been a frequently addressed issue of AM fungal ecology (see Johnson et al. 2010; Klironomos 2003; Lambert et al. 1980). At the level of an AM fungal community colonising the same root system, the concept of selection and complementarity effects (as proposed for plant biodiversity by Loreau and Hector 2001) has recently been adopted by Wagg et al. (2011). Their results indicate that both selection and complementarity interactions among AM fungal species determine the final influence of particular AM fungal mixtures on plant productivity, largely depending on the nutritional status of the soil.

In a previous reciprocal transplant experiment involving model serpentine and non-serpentine soils, *Knautia arvensis* populations and AM fungal isolates, a plant–fungal isolate combination of serpentine origin showed higher mycorrhizal growth and phosphorus uptake benefit in serpentine soil (Doubková et al. 2012). The serpentine *K. arvensis* populations did not, however, show a higher mycorrhizal growth benefit than their non-serpentine counterparts, when grown in native substrates and inoculated with native AM fungal communities (Doubková et al. 2011). The present study addressed the question of the stability of AM fungal functional diversity and variation in plant mycorrhizal responsiveness with respect to host identity and edaphic conditions, adopting the model of serpentine and non-serpentine *K. arvensis* plants. A greenhouse experiment was conducted where serpentine and non-serpentine *K. arvensis* populations were grown in their native substrates and the effects of different serpentine AM fungal isolates were compared with the effects of the respective complex native AM fungal communities. Based on results from previous studies (Doubková et al. 2011, 2012), it was hypothesised that: (1) AM fungal isolates would differ considerably in their effects on plant growth and nutrition, (2) native combinations of complex AM fungal communities, plants and substrates would give highest mycorrhizal growth and phosphorus uptake benefits and (3) mycorrhizal benefit to plants would depend mainly on the nutrient status of the substrate, irrespective of its serpentine or non-serpentine character.

Material and methods

Plant material

K. arvensis (L.) J. M. Coult. (Dipsacaceae), field scabious, is a common perennial herb, widespread in most of Europe. This mycorrhizal species (Doubková et al. 2011) involves two ploidy levels, diploid and tetraploid, both inhabiting serpentine as well as non-serpentine habitats in Central Europe (Kolář et al. 2009). This study involved two serpentine (S3, S4) and two non-serpentine (NS3, NS4) tetraploid *K. arvensis* populations from the Czech Republic (for more details, see Doubková et al. 2011). Mature achenes of *K. arvensis* were collected from the four populations in summer and autumn 2009, from approximately 50 source plants per population. The achenes were surface-sterilised (5 % NaClO, 10 min) and germinated in Petri dishes. The emerged seedlings were transplanted into plastic multipot trays (15-ml chambers) filled with a sterile mixture of low-nutrient substrate (available P 6.22 mg kg^{-1}, N 0.25 %, pH 4.8) and sand (1:2, *v*/*v*). After 2 weeks, even-sized seedlings were transplanted into 1.8-l experimental pots.

AM fungal inocula

The experiment involved four serpentine AM fungal isolates obtained from the rhizosphere of *K. arvensis* plants and AM fungal communities native to the four selected *K. arvensis* sites (referred to as COM_{S3}, COM_{S4}, COM_{NS3} and COM_{NS4}). *Glomus* sp. (EMBL database accession number HE794038), referred to as *PB*, was isolated from the S3 serpentine site. *Glomus* sp. (HE794039), referred to as *DS*, originated from the Dominova skalka site in the same serpentine area (W Bohemia, Czech Republic; 50°04′17.8″ N; 12°47′10.2″ E; 750 m a.s.l.). *Glomus* sp. (FR828471), referred to as *SR*, was isolated from the S2 serpentine site according to Doubková et al. (2011) and *Acaulospora* sp. (HE794040), referred to as *K*, was obtained from the S4 serpentine site. For the positions of the single AM fungal isolates in the phylogenetic tree, see Fig. 1.

Experimental design and establishment of the experiment

Each of the four *K. arvensis* populations was grown in its respective native substrate. The substrates were collected in the field from *K. arvensis* stands at the end of August 2009. They were excavated to the depths of approximately 30 cm, thoroughly mixed, passed through a 5-mm sieve and γ-sterilised (a total dose of 25 kGy). Chemical characteristics of the sterilised substrates together with details on the extraction methods are provided in Table 1.

For each *K. arvensis* population, six inoculation treatments were established. The number of replicates per inoculation

Fig. 1 Phylogenetic tree showing the position of the AM fungi used for *K. arvensis* inoculation in the single-isolate inoculation treatments (*Glomus* sp. *PB*, *Glomus* sp. *DS*, *Glomus* sp. *SR* and *Acaulospora* sp. *K*) and the AM fungi colonising the roots of the *COM*-plants inoculated with fungal communities native to the respective plant population (in *boldface*). The results are based on a neighbour-joining analysis of the partial 18S rDNA, ITS1, 5.8S rRNA, ITS2 and the partial 28S rDNA sequences. For each molecular taxonomic unit obtained in any of the *COM*-treatments, a representative sequence is provided, with the number of clones shown in *brackets*. The tree was rooted using the sequences of *Paraglomus* spp. *Numbers above/below branches* indicate the level of bootstrap support (%) from 1,000 replications; only values above 70 % are given. The orders and families as well as the names of AM fungal taxa in brackets correspond to the most recent AM fungal classification according to Schüssler and Walker (2010) and Krüger et al. (2012)

Glomus irregulare (*Rhizophagus irregularis*) FR750190
HE794049 *COM-NS4* (16)
HE794048 *COM-NS3* (1)
Glomus irregulare (*Rhizophagus irregularis*) FM865615
HE794047 *COM-S3* (1)
Glomus irregulare (*Rhizophagus irregularis*) FM992380
Glomus irregulare (*Rhizophagus irregularis*) FM992382
Glomus proliferum (*Rhizophagus proliferus*) FM992391
Glomus clarum (*Rhizophagus clarus*) FM865542
Glomus (*Rhizophagus*) *intraradices* FM865584
HE794046 *COM-NS4* (1)
uncultured *Glomus* HQ242915
uncultured *Glomus* HM570004
uncultured *Glomus* HQ242974
HE794045 *COM-S3* (5)
uncultured *Glomus* FR772327
HE794044 *COM-S4* (4)
uncultured *Glomus* FR681932
HE794038 *Glomus* sp. *PB*
HE794041 *COM-NS4* (2)
HE794042 *COM-S3* (14)
uncultured *Glomus* HQ243257
Glomus sp. JF439111
Glomus sp. JF439161
Glomus sp. JF439119
Glomus caledonium (*Funneliformis caledonius*) FN547494
Glomus coronatum (*Funneliformis coronatus*) FM876794
Glomus (*Funneliformis*) *mosseae* FN547482
HE794039 *Glomus* sp. *DS*
Glomus sp. FR750203
FR828471 *Glomus* sp. *SR*
uncultured Glomeromycota HQ895794
Glomus macrocarpum FR750371
Glomus macrocarpum FR750533
uncultured *Glomus* HM570020
Glomus sp. JF439189
HE794043 *COM-S4* (4)
Glomus sp. JF439162
Glomeraceae
Glomus (*Claroideoglomus*) *claroideum* FR750075
Glomus (*Claroideoglomus*) *claroideum* FR750075
Glomus (*Claroideoglomus*) *etunicatum* FN547623
Claroideoglomeraceae
Glomerales
HE794050 *COM-S4* (15)
Scutellospora spinosissima FR750149
Scutellospora calospora JF439140
Scutellospora heterogama FR750012
Scutellospora nodosa FM876834
Scutellospora gilmorei FN547604
Gigasporaceae
Glomus eburneum (*Diversispora eburnea*) AM713405
Glomus aurantium (*Diversispora aurantia*) FN547661
Glomus versiforme (*Diversispora epigaea*) FM876816
Diversisporaceae
Acaulospora laevis FN547507
Acaulospora scrobiculata FR692354
Acaulospora cavernata FM876788
Acaulospora punctata FR846385
Acaulospora delicata JF439093
HE794040 *Acaulospora* sp. *K*
Acaulospora mellea JF439091
Acaulosporaceae
Diversisporales
HE794051 *COM-NS3* (19)
Archaeospora trappei FR750038
Archaeospora schenckii FR750023
Archaeosporaceae
Ambispora appendicula FN547525
Ambispora fennica FR750157
Ambisporaceae
Paraglomus brasilianum FR750051
HE794052 *COM-NS4* (1)
Paraglomus laccatum FR750083
Paraglomeraceae
Archaeosporales

0.05

Table 1 Chemical characteristics of the γ-sterilised growth substrates

Substrate type	Site	pH_{KCl}	N[a]	C_{org}[a]	P[b]	Ca[c]	Mg[c]	K[c]	Fe[c]	Na[c]	Mn[d]	Ni[d]	Co[d]	Cr[d]
			(%)		(mg kg^{-1})									
Non-serpentine	NS3	4.1	0.20	3.4	4.2	18	6	5.5	43	7.3	15.6	0.12	0.11	<0.02
	NS4	5.0	0.34	0.6	15.7	1,000	162	24.5	289	10.6	17.3	0.54	0.27	0.07
Serpentine	S3	4.8	0.58	8.6	23.4	251	1,132	49.2	471	12.7	22.4	65.8	2.40	0.23
	S4	5.2	0.49	5.0	13.2	323	1,160	26.8	441	10.8	29.7	63.0	0.78	0.10

[a] Combustion method (CHN Carlo Erba NC2500 analyser, Italy)

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

[c] 1 M ammonium acetate-extractable, pH 7.0 (AAS Unicam 9200X, UK)

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

treatment depended on the germination rate of the respective achenes. Initially, six replicates were established for the population S4, while populations S3, NS3 and NS4 involved five replicates; a few seedlings however died soon after transplantation and therefore, the numbers of replicates dropped to five to six and four to five, respectively. The inoculation treatments involved non-inoculated plants (non-mycorrhizal, *nm*) and plants inoculated (mycorrhizal, *M*) with one of the four above single AM fungal isolates (*PB*, *DS*, *SR* and *K*) or with whole AM fungal community native to the respective site (COM_{S3}, COM_{S4}, COM_{NS3} and COM_{NS4}). Non-inoculated plants received 10 ml of the autoclaved mixture of all the inocula (121 °C twice for 25 min). AM fungal inocula were prepared by wet sieving (Gerdemann and Nicolson 1962). Single isolate inocula originated from even-aged (4-month-old) mature maize cultures with abundant sporulation and high mycorrhizal root colonisation (>70 %); the source cultures were maintained in a 1:1 mixture of original serpentine soil and sand. The *COM* inocula originated from homogenised non-sterilised substrates collected from the sites of plant origin and stored at 4–8 °C for 5 months. In a previous experiment, the application of native inocula originating from the same sites led to successful establishment of an AM association (Doubková et al. 2011). All plants in the mycorrhizal treatments received 10 ml of a suspension containing colonised root segments, extraradical mycelium and spores of the respective AM fungal isolate/community. Within each plant population, all mycorrhizal pots received 5 ml of a filtrate from each other inoculum in an effort to balance the composition of non-AM fungal microbial communities across the inoculation treatments, and non-mycorrhizal plants received microbial filtrates from all five AM fungal inocula applied in the respective population. The filtrates were prepared by filtration of substrate suspensions (1:10, *w*/*v*) through filter paper (15 μm pore size) to remove AM fungal propagules.

Plants were grown in a greenhouse under natural light and supplementary 12-h artificial illumination (metal halide lamps, 400 W, Philips HPI-T Plus) from the end of January to the end of July 2010 and were irrigated daily as required to maintain soil water content at around field capacity.

Plant harvest

All plants were harvested after a 6-month cultivation period. For each plant, mother rosette diameter and number of daughter rosettes, flowering stalks and flower heads were recorded. Shoots were then cut off and root systems were washed. A root sample of a defined weight (0.2–1.5 g fresh weight) was taken from each plant for the determination of mycorrhizal root colonisation, following staining with 0.05 % trypan blue in lactoglycerol (Koske and Gemma 1989). The percentage of mycorrhizal root colonisation was assessed under a compound microscope at 100× magnification, using a modified intersection method (McGonigle et al. 1990). In addition, a small root subsample (~100 mg fresh weight) was collected from each plant inoculated with native AM fungal community, and stored at −20 °C for subsequent determination of the identity of actively colonising fungi. Shoot and root dry weights (SDW and RDW, respectively) were then recorded after drying at 65 °C. DW of root samples taken for mycorrhizal colonisation assessment and AM fungal determination was inferred from the dry weight/fresh weight ratio of the remaining roots and total RDW was then calculated. Shoots were ground and a subsample of the ground biomass was used for determination of N concentration by 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, Ca, Mg and Ni concentrations. Ni concentrations were measured only for plants grown in the serpentine substrates with elevated Ni concentrations (see Table 1). P concentrations were analysed spectrophotometrically by the ammonium-molybdate ascorbic acid method at 630 nm (Unicam UV4-100, UK); concentrations of Ca, Mg and Ni were analysed using an atomic absorption spectrometer (AAS Unicam 9200X, UK).

Determination of native AM fungi colonising plant roots in the COM treatments

DNA was extracted from the frozen root samples using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was eluted with 75 μl of elution buffer and the DNA extracts were then used for PCR after 1:10 dilution with double-deionised water. A nested PCR was used to amplify ~1,500 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). The equimolar mixtures of SSUmAf-LSUmAr primers (0.5 μM each) were used for the first round of PCR, which was performed with 20 μl reaction mixtures, containing 1 U 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: 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 10 min final elongation at 72 °C. The PCR products were diluted 1:100 in sterile double-deionised water and used as templates in the second amplification step with 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 checked on agarose gel electrophoresis (1.0 % *w*/*v* agarose). The PCR products were then pooled per plant population into a single sample that was gel-purified with Zymoclean Gel DNA Recovery Kit (Zymo Research, USA) and cloned using the 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. Around 20 randomly selected positive clones were purified and sequenced using the same M13 vector primers in both directions by Macrogen Inc. (South Korea).

The sequences obtained were edited with FinchTV 1.4.0 (Geospiza Inc., USA) and manually checked for possible chimeras. All high-quality sequences were then submitted to INSD for BLASTn search and aligned using MAFFT version 6 together with the most similar sequences from public databases. Subsequently, a neighbour-joining analysis based on Kimura 2-parameter model (1,000 bootstrap replicates) was computed with the software MEGA5 (Tamura et al. 2011). The phylogenetic tree involving all obtained sequences was then used for delimitation of molecular taxonomical units (MOTUs). A representative sequence from each detected AM fungal taxon was submitted to EMBL database (acc. no. HE794041–HE794052) and used in phylogenetic tree construction according to the same procedure as described above (Fig. 1).

Calculated plant parameters

Mycorrhizal growth dependence (MGD) was calculated according to Smith et al. (2003) as a percentage increase in SDW of an individual mycorrhizal plant over the mean performance of non-mycorrhizal plants in the respective population [MGD = 100 × (SDW of an individual mycorrhizal plant − average SDW of non-mycorrhizal plants)/SDW of an individual mycorrhizal plant]. By analogy, mycorrhizal nutrient uptake benefits (mycorrhizal phosphorus uptake benefit, MPB; mycorrhizal nitrogen uptake benefit, MNB; mycorrhizal magnesium uptake benefit, MMgB and mycorrhizal calcium uptake benefit, MCaB) were derived from shoot nutrient concentrations in mycorrhizal relative to non-mycorrhizal plants. Mycorrhizal uptake benefits could not be evaluated for the NS3 population from which only mycorrhizal plants survived.

To compare P, N, Mg and Ca uptake levels among plant populations, nutrient accumulation ratios were calculated as: element concentration in shoot biomass/element concentration in the substrate.

Data analysis

All analyses were performed using Statistica 9.1 software (StatSoft Inc., USA). Due to the fact that non-inoculated NS3 plants did not survive, a fully factorial design was disrupted and therefore, the effects of AM fungal inoculation on plant growth and nutrition were tested in two separate analyses: (1) the effects of AM fungal inoculation per se (non-mycorrhizal vs. mycorrhizal plants) were tested for all but the NS3 population; (2) the effects of AM fungal identity (*PB* vs. *DS* vs. *SR* vs. *K* vs. *COM* inoculum) were tested for each of the plant populations. Furthermore, the inter-population differences were tested separately for non-mycorrhizal and mycorrhizal plants, due to the absence of non-mycorrhizal plants in the NS3 population.

Within the same plant population, the data were analysed using analysis of variance (ANOVA) and multiple comparisons were made using Tukey HSD multiple comparison test. Prior to analyses, the data were checked for normality and homogeneity of variance and, if necessary, they were logarithmically or arcsine transformed to meet these ANOVA assumptions. To consider potential differences between inocula in their root colonisation potential, plant growth and nutrient uptake responses to different inoculation treatments were tested both with and without the mycorrhizal root colonisation as a covariate. When comparing all plant populations, the ANOVA assumptions were not met even after transformation of data and, therefore, the non-parametric Kruskal–Wallis ANOVA followed by the post-hoc Z value test was used. Linear regression analyses were conducted to test the relationships between the mycorrhizal root colonisation, plant growth and nutrient uptake.

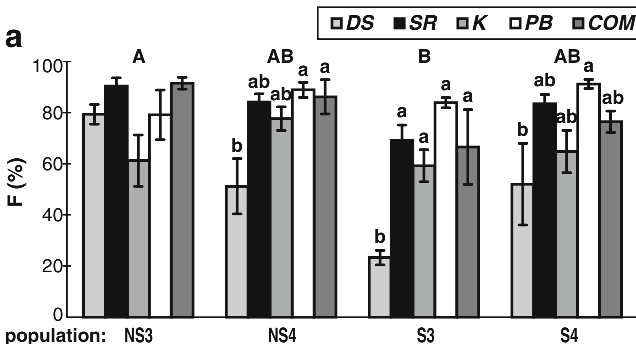
Results

Mycorrhizal root colonisation

All inoculated (*M*) plants were colonised by AM fungi, whereas no mycorrhizal structures were found in the roots of non-inoculated (*nm*) plants. Mean percentage of root colonisation differed among plant populations ($H_{3,89}$=13.47, P=0.004), with significantly higher values in the NS3 than in the S3 population (Fig. 2a). As for the differences between AM fungal inocula, the *DS* isolate showed the lowest root colonisation in all but the NS3 populations (Fig. 2a).

Composition of the native AM fungal communities

Molecular analysis of the AM fungi colonising roots of the *COM*-inoculated plants revealed relatively poor communities (Fig. 1). Only two AM fungal taxa were detected in the COM_{NS3} inoculation treatment, a member of the order Archaeosporales and *Rhizophagus irregularis*, the former being more frequent. Both COM_{S3}- and COM_{S4}-inoculated plants were colonised by three AM fungal taxa. In the COM_{S4} community, *Scutellospora* sp. was the most abundant taxon, accompanied by two *Glomeraceae* spp. lacking morphologically described counterparts in the INSD database. Analysis of the COM_{S3} community revealed the highest abundance of the *Glomeraceae* sp. belonging to the same MOTU as *Glomus* sp. *PB* isolate that was isolated from the same field site. In addition, a further unidentified *Glomeraceae* sp. and *R. irregularis* were the other members of the COM_{S3} community. The COM_{NS4}-inoculated plants were colonised by the richest AM fungal community, comprising primarily *R. irregularis*, together with two undetermined *Glomeraceae* spp. and *Paraglomus laccatum*.



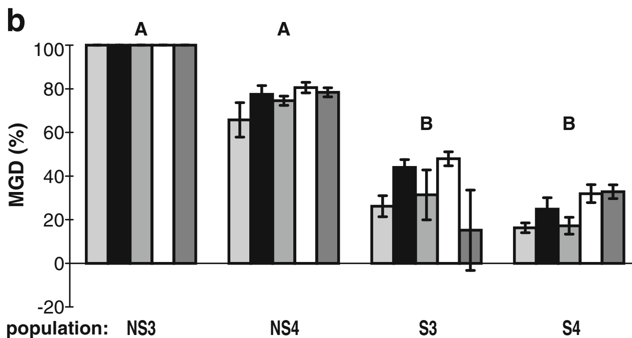


Fig. 2 **a** Frequency of mycorrhizal root colonisation (*F*) and **b** mycorrhizal growth dependence (*MGD*) of *K. arvensis* plants as affected by population (*NS3*, *NS4*, *S3* and *S4*) and AM fungal identity (*DS*, *SR*, *K* and *PB*—plants inoculated with different single AM fungal isolates; *COM*—plants inoculated with AM fungal community native to the respective plant population). The *columns* represent the means (±SE) of four to six replicates. Intra-population comparisons represent the results of one-way ANOVA, while inter-population comparisons were made using non-parametric Kruskal–Wallis ANOVA. *Lower case letters* indicate significant differences between AM fungal inocula within the same population according to Tukey HSD test (P<0.05), while *capitals* denote inter-population differences according to the *Z* value test (P<0.05); no letters are given when no significant difference was detected

Plant growth parameters and MGD

Due to a close positive correlation between mother rosette diameter and SDW (r=0.82, P<0.001), only the latter was considered in further analyses. Both SDW and RDW were significantly influenced by plant population (Table 2, Fig. 3). The number of daughter rosettes significantly differed

Table 2 The effects of plant population on *K. arvensis* growth and nutrient concentrations and accumulation ratios in shoot biomass

		nm plants	*M* plants	Mycorrhizal benefit
Rosette number		5.80 ns	61.90***	–
Shoot DW/*MGD*		12.44**	68.26***	78.76***
Root DW		9.71**	49.97***	–
Root/shoot ratio		3.28 ns	33.88***	–
P	*conc./MPB*	3.58 ns	10.35*	6.77*
	accum. ratio	11.12**	63.26***	–
N	*conc./MNB*	6.55 ns	36.65***	43.84***
	accum. ratio	10.74**	46.22***	–
Mg	*conc./MMgB*	13.35***	73.32***	53.37***
	accum. ratio	12.44***	72.71***	–
Ca	*conc./MCaB*	9.71**	55.90***	4.44 ns
	accum. ratio	11.32**	69.48***	–

For the *M* plants, also the differences in relative mycorrhizal benefit parameters (mycorrhizal growth dependence, *MGD*; mycorrhizal phosphorus uptake benefit, *MPB*; mycorrhizal nitrogen uptake benefit, *MNB*; mycorrhizal magnesium uptake benefit, *MMgB*; mycorrhizal calcium uptake benefit, *MCaB*) are presented. Due to the fact that non-mycorrhizal NS3 plants did not survive, this population was not included in the comparisons of *nm* plants or mycorrhizal benefit parameters. The *H* values and significances according to non-parametric Kruskal–Wallis ANOVA are given for all parameters

ns non-significant effect

*P<0.05; **P<0.01; ***P<0.001

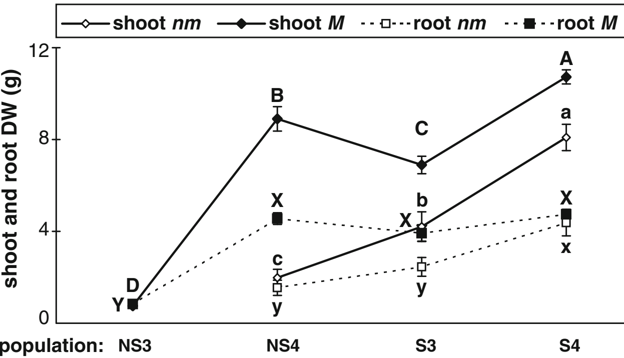


Fig. 3 Shoot and root DW of *K. arvensis* plants as affected by plant population (*S3*, *S4*, *NS3* and *NS4*) and AM fungal inoculation (presence/absence). The data represent the means (±SE) of five to six and 22–28 replicates for non-mycorrhizal (*nm*) and mycorrhizal (*M*) plants, respectively. The means within the *same line marked with different letters* (*a–d* for shoot DW, *x–y* for root DW; *lower case* for *nm* plants, *capitals* for *M* plants) are significantly different according to non-parametric Kruskal–Wallis ANOVA followed by *Z* value test ($P<0.05$)

between populations only for the mycorrhizal plants (Table 2), with markedly lower values in the NS3 population and the highest numbers in the S4 population. In all *K. arvensis* populations, *M* plants had significantly higher SDW than their *nm* counterparts, with an extreme difference recorded for the NS3 population where no *nm* individual survived (Fig. S1 in Online Resource 1). In the S3 and NS4 populations, SDW positively correlated with mycorrhizal root colonisation (r=0.56 and r=0.59, respectively, P=0.005). RDW was significantly influenced by AM fungal inoculation only in the NS4 population, with higher values for *M* than *nm* plants (Table 3). The root/shoot ratio of *M* plants was significantly affected by plant population (Table 2), with the NS3 plants investing considerably more into belowground biomass (Fig. 3). AM fungal inoculation did not significantly influence root/shoot ratio in any of the populations (Table 3); however, a negative correlation between mycorrhizal colonisation and root/shoot ratio was recorded in the S4 and NS4 populations (r=−0.53, P=0.004 and r=−0.55, P=0.010, respectively).

Table 3 The effects of AM fungal inoculation (presence vs. absence) on plant growth of and element uptake by *K. arvensis* plants

	NS4	S3	S4
Rosette number	8.30** ↑*M*	0.67 ns	1.24 ns
Shoot DW	36.41*** ↑*M*	9.13** ↑*M*	13.80*** ↑*M*
Root DW	28.96*** ↑*M*	3.42 ns	0.50 ns
Root/shoot ratio	3.92 ns	0.11 ns	2.90 ns
P concentration	3.79 ns	0.24 ns	3.49 ns
N concentration	39.11*** ↑*nm*	4.21 ns	0.12 ns
Mg concentration	27.31*** ↑*M*	7.64* ↑*M*	1.72 ns
Ca concentration	0.36 ns	0.17 ns	3.62 ns
Ni concentration	–	0.40 ns	0.14 ns

The *F* values and significances according to one-way ANOVA are given for all parameters, except for the number of rosettes evaluated by non-parametric Kruskal–Wallis ANOVA. For significant effects, the direction of change is indicated by arrows (e.g. M↑ denotes higher values for mycorrhizal plants compared to non-mycorrhizal ones)

ns non-significant effect

*$P<0.05$; **$P<0.01$; ***$P<0.001$

As for vegetative reproduction, the *M* plants of the NS4 population formed more daughter rosettes than the *nm* plants (Fig. S2 in Online Resource 1), while no effect of inoculation was recorded in either serpentine population (Table 3). The NS4 population was also the only one to show more abundant flowering. The number of both flowering stalks and flower heads was significantly higher for *M* plants than *nm* plants ($H_{1,25}$=5.60, P=0.018 and $H_{4,20}$=6.60, P=0.010, respectively); however, there was no difference between the AM fungal inocula (Fig. S2).

MGD was significantly influenced by plant population (Table 2), with markedly higher values for both non-serpentine populations compared to both serpentine ones (Fig. 2b). On the other hand, no significant differences were recorded between the AM fungal inocula in any of the populations (Fig. 2b). Although there was a positive correlation between MGD and mycorrhizal root colonisation (r=0.40, $P<0.001$), the difference between AM fungal inocula in their effects on MGD remained non-significant even when mycorrhizal root colonisation was used as a covariate in the ANOVA model.

Plant element uptake

Highly significant inter-population differences were recorded not only for shoot P, N, Mg and Ca concentrations, but also for their accumulation ratios (Fig. 4a–d, Tables 2, S1 and S2—in Online Resource 1). Generally, the NS3 population showed by far the highest accumulation ratios for all four elements.

In spite of a generally positive influence of AM fungal inoculation on plant growth, it did not significantly affect shoot P concentration in any of the populations (Table 3). MPB was significantly influenced by population (Table 2), with significantly higher values being recorded for the NS4 than the S3 plants (Fig. 5a). In both NS4 and S3 populations, MPB also differed among AM fungal inocula ($F_{4,16}$=4.05, P=0.019 and $F_{4,19}$=3.55, P=0.025, respectively), with the *PB* isolate being the most efficient in this respect (Fig. 5a). However, when mycorrhizal root colonisation was included into the analysis as a covariate, the difference between AM fungal inocula remained significant only in the NS4 population. In the NS3 population, AM fungal identity had a significant effect on shoot P concentration ($F_{4,16}$=6.64, P=0.002), with lower values recorded in the *DS*-inoculated plants (Table S1). Similarly to MGD, also

Fig. 4 Concentrations and accumulation ratios of **a** phosphorus, **b** nitrogen, **c** magnesium and **d** calcium in shoots of *K. arvensis* plants as affected by plant population (*NS3*, *NS4*, *S3* and *S4*) and AM fungal inoculation (presence/absence). The points represent the means (±SE) of five to six and 18–28 replicates for non-mycorrhizal (*nm*) and mycorrhizal (*M*) plants, respectively. The means within the *same line marked with different letters* (*a–c* for accumulation ratios, *x–z* for shoot concentrations; *lower case* for *nm* plants, *capitals* for *M* plants) are significantly different according to non-parametric Kruskal–Wallis ANOVA followed by *Z* value test ($P<0.05$); no letters are given when no significant difference was detected

Fig. 5 Relative mycorrhizal nutrient uptake benefits as affected by *K. arvensis* population and AM fungal identity: **a** mycorrhizal phosphorus-uptake benefit (*MPB*), **b** mycorrhizal nitrogen-uptake benefit (*MNB*), **c** mycorrhizal magnesium-uptake benefit (*MMgB*) and **d** mycorrhizal calcium-uptake benefit (*MCaB*). For details and abbreviations, see Fig. 2

MPB positively correlated with mycorrhizal root colonisation ($r=0.44$, $P<0.001$).

Shoot N concentration was significantly decreased by AM fungal inoculation in the NS4 population, while no significant effect was recorded in the serpentine populations (Table 3). MNB was significantly affected by plant population (Table 2), with the NS4 population showing significantly lower values than both serpentine ones (Fig. 5b). The identity of AM fungal inocula did not significantly influence MNB. In the NS3 population, however, significant differences between AM fungal inocula in their effects on shoot N concentration were recorded ($F_{4,12}=11.66$, $P<0.001$), with significantly lower values for the *COM*- and *PB*-inoculated plants (Table S1).

AM fungal inoculation significantly increased Mg uptake in the NS4 and S3 populations (Table 3). However, MMgB was highly dependent on plant population (Table 2), with NS4 plants profiting markedly more from AM fungal inoculation in terms of Mg uptake than both serpentine populations (Fig. 5c). The effect of the AM fungal identity on MMgB was significant in both NS4 and S3 populations ($F_{4,16}=3.29$, $P=0.038$ and $F_{4,19}=4.09$, $P=0.015$,

respectively), but no consistent pattern was revealed (Fig. 5c). In both populations, the differences between AM fungal inocula disappeared when the mycorrhizal root colonisation was used as a covariate. In contrast to Mg, shoot Ca concentration was not affected by AM fungal inoculation in any population (Table 3). On the other hand, AM fungal inocula significantly differed in their effects on MCaB in the S4 population ($F_{4,23}$= 3.95, P=0.014; Fig. 5d). The effect of plant population on MCaB was not significant (Table 2, Fig. 5d). AM fungal inoculation did not influence shoot Ni concentration in either serpentine *K. arvensis* population (Table 3). In the shoots of S4 plants, however, Ni concentration was significantly influenced by AM fungal identity ($F_{4,23}$=11.27, P<0.001), with the *COM*-inoculated plants showing higher values than the plants in any other inoculation treatment (Table S1).

Regarding the relationship between plant growth and nutrient uptake, SDW was negatively correlated with shoot N concentration in all plant populations except for S4 (r=−0.72 for S3, r=−0.80 for NS3 and r=−0.70 for NS4 population, all at P<0.001). In the NS4 population, SDW was positively correlated with shoot P (r=0.50, P=0.010) as well as with shoot Mg (r=0.77, P<0.001) concentration; in the S3 population, SDW was positively correlated with shoot Ca concentration (r=0.41, P=0.029).

Discussion

The present study addressed the impact of edaphic conditions and AM fungal identity on mycorrhizal responsiveness of the same host plant species by comparing the effects of different single AM fungal isolates or native AM fungal communities on two serpentine and two non-serpentine populations of *K. arvensis* in their native substrates. The main findings extend the results of our previous studies (Doubková et al. 2011, 2012) in that: (1) irrespective of AM symbiosis, *K. arvensis* populations are adapted to macronutrient deficiency/excess of their substrates; (2) the identity and complexity of AM fungal inoculum (single isolates vs. native communities) had little effect on the mycorrhizal growth response of plants, but a certain divergence among inocula was recorded in their effects on plant nutrient uptake and (3) the general benefit from AM symbiosis was governed by the nutritional status of the substrate, irrespective of its serpentine/non-serpentine character or AM fungi involved.

Inoculation with the same AM fungi improved plant growth in all four *K. arvensis* populations, but the extent of the growth-promotion effect was much higher for the selected non-serpentine than serpentine populations. Similar observations were previously reported by Ji et al. (2010) and for the same plant species by Doubková et al. (2012). However, considering more pronounced nutrient limitation in the non-serpentine populations in the present study, generalisations are not possible in this respect.

In the NS3 population, high mycorrhizal benefit was likely related to the above-mentioned nutrient deficiency of the native non-serpentine substrate, where the non-mycorrhizal plants were not even able to survive. The substantial mycorrhizal effect might partly be related to slightly higher intraradical fungal development compared to that in other populations. The increasing importance of AM symbiosis with diminishing soil-nutrient sources is generally acknowledged (Smith and Read 2008), particularly in terms of the contribution of the mycorrhizal pathway to total P uptake (for review, see Smith and Smith 2011). The mycorrhizal NS3 plants were able to counterbalance the extreme nutrient limitation by higher investment into roots as well as by a generally high nutrient uptake efficiency, as indicated by surprisingly high P, N, Mg and Ca accumulation ratios. Consequently, shoot concentrations of all four elements were comparable between both non-serpentine populations, in spite of vastly different plant-available nutrient concentrations in their native substrates.

The growth and reproductive benefit of NS4 plants from being mycorrhizal was markedly higher than in both serpentine populations. The nutritional benefit in terms of MPB was inversely related to substrate P availabilities and was higher in the NS4 substrate than in the S3 population. As indicated by a close positive correlation between plant growth and shoot Mg concentrations, the mycorrhizal effect on NS4 plants might be additionally related to an increase in Mg uptake from the native non-serpentine substrate with low Mg availability, as already shown in a previous study (Doubková et al. 2011). On the other hand, AM fungal inoculation markedly decreased plant N acquisition in the NS4 population resulting in shoot N concentrations comparable to S3 and NS3 populations, independently of N concentration in the corresponding native substrates. This finding is in accordance with results from previous experiments with the same host plant species (Doubková et al. 2012, 2013). The decrease in N concentration due to AM fungal inoculation might be caused by a biomass-dilution effect and/or by higher N demands of the AM fungi resulting in a retention of part of the absorbed N in fungal tissues (Hodge et al. 2010; Johnson 2010).

Regarding both serpentine *K. arvensis* populations, the lack of inter-population differences in mycorrhizal growth dependence and P uptake benefit was recorded, in spite of a higher P availability in the S3 substrate. Comparable plant P nutritional status of both serpentine populations provides more evidence for the plant's ability to adapt to low substrate P availability by rising the P accumulation ratio, as observed for the NS3 population (see also Doubková et al. 2011). Importantly, there was no significant difference between mycorrhizal and non-mycorrhizal plants in their shoot

P concentrations in any of the serpentine populations. However, this might be a result of simultaneous uptake via both direct plant and mycorrhizal pathways, for which the relative participation in total P uptake depends both on the identity of the plant and the fungal symbionts (Facelli et al. 2010; Pearson and Jakobsen 1993; Smith et al. 2004) and on substrate nutrient availability (Nagy et al. 2009). Nagy et al. (2009) observed a gradual suppression of the mycorrhizal pathway with increasing P supply, leading to dominance of the direct root pathway.

Apart from the exceptionally high nutrient accumulation ratios in the NS3 population, the serpentine plants had a comparable Ca accumulation ratio with the NS4 plants, without being affected by AM fungal inoculation. Although selective Ca uptake or root-to-shoot translocation by serpentine vs. non-serpentine plants has been repeatedly reported for different plant species (e.g. Asemaneh et al. 2007; O'Dell et al. 2006; Rajakaruna et al. 2003), it was not observed in previous studies on *K. arvensis* (Doubková et al. 2011, 2012) and cannot therefore be considered as an adaptive response of this plant species to low substrate Ca concentration. Regarding Mg, the higher shoot concentrations but lower accumulation ratios for the serpentine compared to non-serpentine plants is in accordance with previous findings (Doubková et al. 2011). Similarly, the observation of a more pronounced AM fungal-mediated increase in shoot Mg concentration in NS4 plants compared to either a lower or no AM fungal effect in the serpentine populations is also consistent with previous results (Doubková et al. 2011), confirming the existence of a nutrient availability-dependent effect of AM symbiosis on Mg uptake (Clark and Zeto 2000; Liu et al. 2000, 2002). In the case of Ni, the effect of AM fungal inoculation was limited to an increase in shoot Ni concentration in the *COM*-inoculation treatment in one of the serpentine substrates, which were both of comparable Ni availability. Comparison of these results with previous observations (Doubková et al. 2011, 2012) supports the view that the AM fungal effect on metal uptake largely depends not only on metal availability but also on the particular combination of plant and fungal symbionts (Leyval et al. 1997).

In spite of the reported functional diversity in the effects of different AM fungal isolates (Klironomos 2003; Munkvold et al. 2004; Ruiz-Lozano et al. 1995b), no significant differences were found among inocula in their effects on biomass production in any *K. arvensis* population, regardless of the differences in intraradical fungal development. This indicates that neither AM fungal identity nor the level of mycorrhizal root colonisation were the overriding factors in determining mycorrhizal benefits to plants under the present experimental conditions. The effects of individual AM fungi on nutrient uptake were more variable compared to the effects on plant growth, but no one AM fungus proved to be generally most efficient. Similar to other studies (Jansa et al. 2008; Smith et al. 2004), a divergence was recorded between P uptake and growth-promoting effects of particular AM fungi. In some cases, the effects seemed to be based mainly on differences in mycorrhizal root colonisation (shoot Mg in the NS4 population or both shoot P and Mg concentrations in the S3 population). In other cases, the effects caused by different AM fungal isolates were independent of the level of intraradical development (the influence on shoot P concentration in the NS4 population). The latter is in accordance with observations made by Clark et al. (1999), Newsham et al. (1995) and Ruiz-Lozano et al. (1995b), showing that the differences between single AM fungal isolates in root colonisation and their effects on shoot P concentration are not necessarily correlated.

There was no convincing evidence in the present study for a prominent role of native AM fungal communities in conferring growth and nutrient uptake benefits to their hosts in any of the *K. arvensis* populations. In contrast to the original hypothesis, the profits provided by native AM fungal communities were generally comparable with those of single isolates. Although it cannot be entirely excluded that non-AM fungal (including pathogenic) microorganisms may have been introduced to a greater extent with the *COM* inocula compared to the single isolate counterparts, the application of microbial filtrates across all inoculation treatments makes this explanation unlikely. It appears more likely that complex interactions between particular combinations of AM fungal isolates played a crucial role, in line with the concept of selection and complementarity effects (Wagg et al. 2011). However, it is also important to consider that only part of the originally coexisting AM fungi from the *COM* inocula might have become established in the experimental pots, and thus inter-fungal relations existing at the native sites might have been disrupted. In this respect, mostly AM fungal taxa representing the r-strategy have been reported to become established in plants inoculated with native substrates in pots (Sýkorová et al. 2007; Verbruggen et al. 2012).

In conclusion, the results from the present study indicate a positive role of AM symbiosis in the performance of both serpentine and non-serpentine *K. arvensis* populations in their native substrates. The effects of different AM fungal isolates as well as of native AM fungal communities on plant growth were comparable, with the extent of mycorrhizal growth promotion particularly depending on substrate nutritional status, irrespective of the serpentine/non-serpentine character. In spite of the lack of differences between AM fungal inocula at the level of plant growth promotion, a certain divergence was recorded in terms of their effects on P, Mg and Ca uptake although no individual inoculum was most efficient in promoting

plant nutrient uptake. For magnesium, a substrate availability-dependent response to AM fungal inoculation was recorded, with more pronounced relative increase of Mg uptake in Mg-poor non-serpentine substrates. With respect to the *K. arvensis* populations, they significantly varied in their relative nutrient-accumulation ratios irrespective of AM fungal inoculation, providing an illustrative example of plant adaptation to nutrient deficiency/excess. Plants subjected to extreme nutrient limitation showed markedly higher uptake efficiency of P, N, Ca and Mg, while the serpentine populations growing in Mg-rich substrates displayed relatively lower Mg accumulation than the non-serpentine ones.

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Mycorrhiza

Soil nutritional status, not inoculum identity, primarily determines the effect of arbuscular mycorrhizal fungi on the growth of *Knautia arvensis* plants

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Supplementary data

Figure S1 Shoot DW as affected by AM fungal inoculation within each population

Figure S2 Number of daughter rosettes and flower heads as affected by AM fungal inoculation in the NS4 population

Table S1 Phosphorus, nitrogen, calcium, magnesium and nickel concentrations in shoot biomass

Table S2 Phosphorus, nitrogen, calcium and magnesium accumulation ratios in shoot biomass

Fig. S1 Shoot DW of *K. arvensis* plants originating from two non-serpentine (NS3, NS4) and two serpentine (S3, S4) populations as affected by AM fungal inoculation (*nm* – non-mycorrhizal plants; *DS*, *SR*, *K*, *PB* – plants inoculated with different single AM fungal isolates; *COM* – plants inoculated with AM fungal community native to the respective plant population). The columns represent means (± SE) of 4–6 replicates

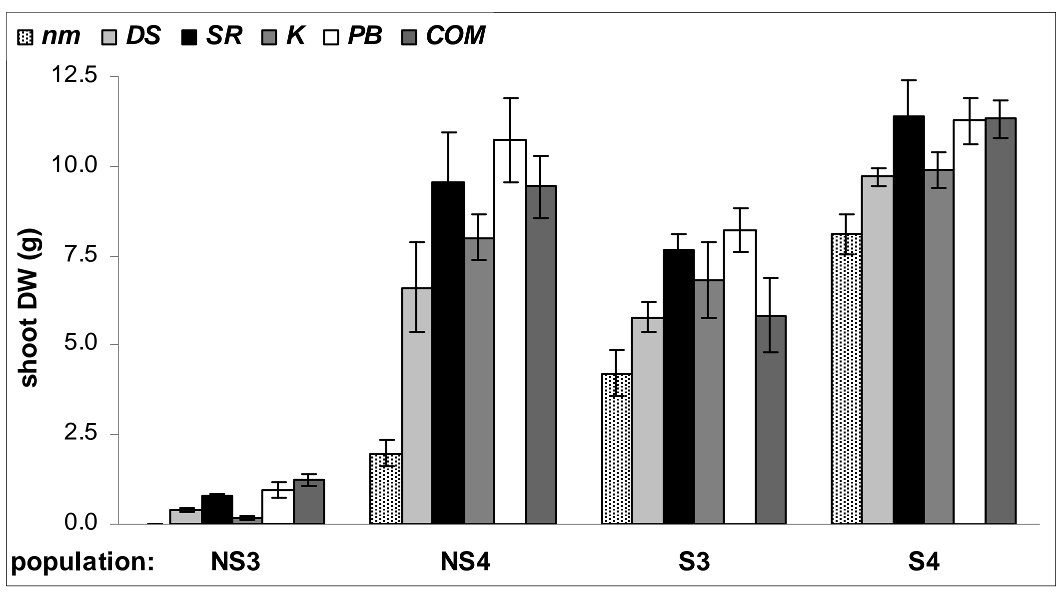

Fig. S2 Number of daughter rosettes and flower heads of *K. arvensis* plants from the NS4 population as affected by AM fungal inoculation (*nm*, non-mycorrhizal plants; *DS*, *SR*, *K*, *PB* – plants inoculated with different single AM fungal isolates; *COM* – plants inoculated with native AM fungal community). The columns represent means (± SE) of 5–6 replicates

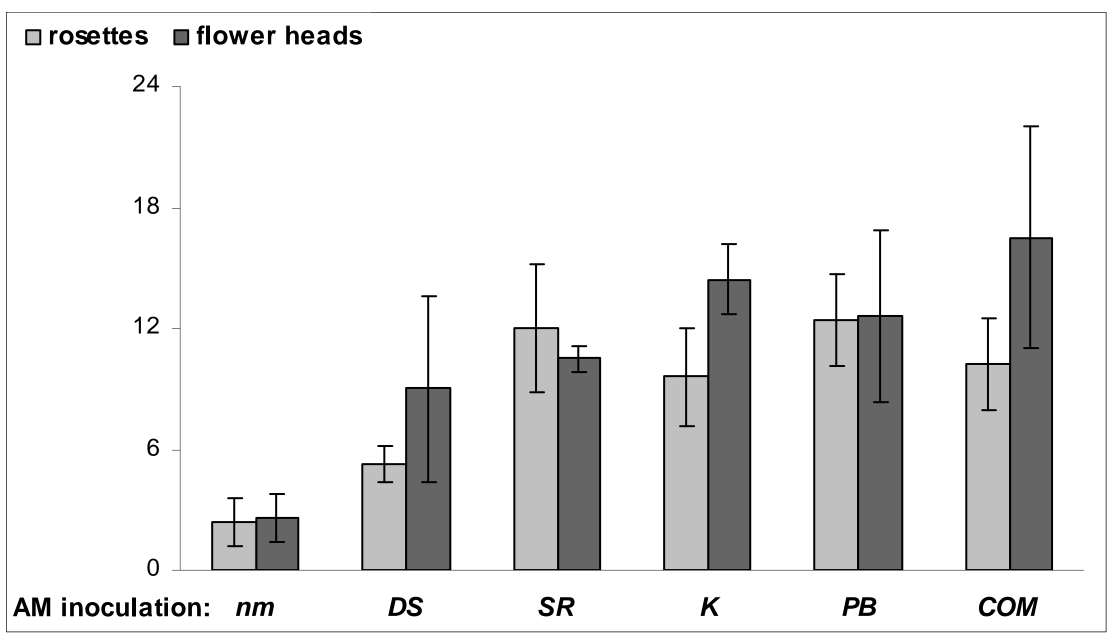

Table S1 Phosphorus, nitrogen, calcium, magnesium and nickel concentrations in shoot biomass of *K. arvensis* plants originating from two non-serpentine (NS3, NS4) and two serpentine (S3, S4) populations as affected by AM fungal inoculation (*nm* – non-mycorrhizal plants; *DS*, *SR*, *K*, *PB* – plants inoculated with different single AM fungal isolates; *COM* – plants inoculated with AM fungal community native to the respective plant population). The data represent means (± SE) of 4–6 replicates

Population	AM inoculation	P (mg kg^{-1})	N (%)	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Ni (mg kg^{-1})
NS3	*nm*	-	-	-	-	-
	DS	679 ± 183	1.7 ± 0.2	9 757 ± 4 707	2 536 ± 1 368	-
	SR	1 523 ± 145	1.7 ± 0.1	4 965 ± 472	1 524 ± 162	-
	K	1 753 ± 185	2.3 ± 0.1	3 760 ± 59	1 207 ± 162	-
	PB	1 425 ± 144	1.0 ± 0.1	4 850 ± 283	1 413 ± 140	-
	COM	902 ± 157	1.0 ± 0.1	5 276 ± 243	1 304 ± 82	-
NS4	*nm*	1 029 ± 147	3.1 ± 0.3	4 532 ± 182	2 628 ± 213	-
	DS	1 220 ± 101	2.0 ± 0.2	4 072 ± 143	4 825 ± 421	-
	SR	1 400 ± 118	1.6 ± 0.1	5 807 ± 722	6 012 ± 368	-
	K	1 057 ± 117	1.8 ± 0.1	5 592 ± 785	4 064 ± 397	-
	PB	1 742 ± 101	1.5 ± 0.1	4 703 ± 574	5 781 ± 354	-
	COM	1 241 ± 49	2.1 ± 0.2	4 762 ± 853	5 411 ± 480	-
S3	*nm*	1 360 ± 158	2.3 ± 0.3	2 391 ± 193	7 321 ± 289	68 ± 2
	DS	1 037 ± 115	1.9 ± 0.1	2 277 ± 254	7 678 ± 439	64 ± 3
	SR	1 475 ± 181	1.5 ± 0.2	2 669 ± 625	9 062 ± 398	58 ± 3
	K	1 296 ± 129	1.9 ± 0.3	2 503 ± 263	9 150 ± 366	70 ± 5
	PB	2 148 ± 395	1.8 ± 0.3	2 672 ± 324	9 942 ± 821	62 ± 5
	COM	1 608 ± 233	1.9 ± 0.3	2 897 ± 452	11 132 ± 858	69 ± 6
S4	*nm*	1 392 ± 63	2.3 ± 0.1	2 421 ± 243	11 294 ± 1 167	59 ± 7
	DS	1 464 ± 113	2.3 ± 0.1	3 974 ± 492	10 383 ± 1 140	47 ± 3
	SR	1 564 ± 83	2.4 ± 0.2	2 599 ± 147	9 525 ± 531	47 ± 2
	K	1 548 ± 61	2.3 ± 0.1	2 910 ± 165	10 996 ± 1 125	55 ± 4
	PB	1 694 ± 82	2.4 ± 0.1	2 987 ± 199	9 224 ± 593	52 ± 5
	COM	1 458 ± 55	2.1 ± 0.2	2 478 ± 242	9 891 ± 687	84 ± 6

Table S2 Phosphorus, nitrogen, calcium and magnesium accumulation ratios in shoot biomass of *K. arvensis* plants as affected by AM fungal inoculation. For details, see Table S1

Population	AM inoculation	P	N	Ca	Mg
NS3	*nm*	-	-	-	-
	DS	101.4 ± 7.9	8.4 ± 0.8	246.2 ± 25.8	178.6 ± 25.6
	SR	367.2 ± 35.0	8.5 ± 0.6	278.8 ± 26.5	262.7 ± 27.9
	K	422.6 ± 44.5	11.7 ± 0.1	211.1 ± 3.3	208.1 ± 27.9
	PB	343.7 ± 34.7	4.9 ± 0.4	272.4 ± 15.9	243.5 ± 24.1
	COM	217.5 ± 37.8	5.2 ± 0.5	296.3 ± 13.6	224.8 ± 14.1
NS4	*nm*	65.7 ± 9.4	9.3 ± 0.9	4.5 ± 0.2	16.2 ± 1.3
	DS	77.8 ± 6.5	5.8 ± 0.5	4.1 ± 0.1	29.8 ± 2.6
	SR	89.3 ± 7.5	4.6 ± 0.3	5.8 ± 0.7	37.1 ± 2.3
	K	67.4 ± 7.4	5.3 ± 0.4	5.6 ± 0.8	25.1 ± 2.5
	PB	111.2 ± 6.4	4.4 ± 0.2	4.7 ± 0.6	35.7 ± 2.2
	COM	79.2 ± 3.1	6.1 ± 0.6	4.8 ± 0.9	33.4 ± 3.0
S3	*nm*	58.3 ± 6.8	4.0 ± 0.4	9.5 ± 0.8	6.5 ± 0.3
	DS	44.4 ± 4.9	3.3 ± 0.2	9.1 ± 1.0	6.8 ± 0.4
	SR	63.2 ± 7.8	2.5 ± 0.3	10.6 ± 2.5	8.0 ± 0.4
	K	55.5 ± 5.5	3.3 ± 0.5	10.0 ± 1.0	8.1 ± 0.3
	PB	92.0 ± 16.9	3.1 ± 0.6	10.6 ± 1.3	8.8 ± 0.7
	COM	68.9 ± 10.0	3.2 ± 0.5	11.5 ± 1.8	9.8 ± 0.8
S4	*nm*	105.8 ± 4.8	4.6 ± 0.2	7.5 ± 0.8	9.7 ± 1.0
	DS	111.3 ± 8.6	4.8 ± 0.1	12.3 ± 1.5	8.9 ± 1.0
	SR	118.9 ± 6.3	5.0 ± 0.3	8.1 ± 0.5	8.2 ± 0.5
	K	117.7 ± 4.6	4.6 ± 0.2	9.0 ± 0.5	9.5 ± 1.0
	PB	128.8 ± 6.2	4.8 ± 0.3	9.3 ± 0.6	8.0 ± 0.5
	COM	110.9 ± 4.2	4.3 ± 0.4	7.7 ± 0.7	8.5 ± 0.6

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ORIGINAL PAPER

Nickel tolerance of serpentine and non-serpentine *Knautia arvensis* plants as affected by arbuscular mycorrhizal symbiosis

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Abstract Serpentine soils have naturally elevated concentrations of certain heavy metals, including nickel. This study addressed the role of plant origin (serpentine vs. non-serpentine) and symbiosis with arbuscular mycorrhizal fungi (AMF) in plant Ni tolerance. A semi-hydroponic experiment involving three levels of Ni and serpentine and non-serpentine AMF isolates and populations of a model plant species (*Knautia arvensis*) revealed considerable negative effects of elevated Ni availability on both plant and fungal performance. Plant growth response to Ni was independent of edaphic origin; however, higher Ni tolerance of serpentine plants was indicated by a smaller decline in the concentrations of photosynthetic pigments and restricted root-to-shoot Ni translocation. Serpentine plants also retained relatively more Mg in their roots, resulting in a higher shoot Ca/Mg ratio. AMF inoculation, especially with the non-serpentine isolate, further aggravated Ni toxicity to host plants. Therefore, AMF do not appear to be involved in Ni tolerance of serpentine *K. arvensis* plants.

Keywords Arbuscular mycorrhizal fungi · Calcium · Magnesium · Nickel toxicity · Semi-hydroponics · Tolerance index

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Introduction

Although serpentine soils represent a highly variable group in terms of their chemical composition, they share several characteristic features such as low calcium-to-magnesium ratio, elevated and potentially phytotoxic concentrations of heavy metals (especially nickel, cobalt, chromium or manganese) and often general macronutrient deficiency (for reviews, see, e.g. Kazakou et al. 2008; O'Dell and Rajakaruna 2011). Among heavy metals typical of serpentine soils, Ni is of particular importance due to its high plant-available concentrations, considerable plant toxicity and relatively high rate of root-to-shoot translocation (Seregin and Kozhevnikova 2006; Kazakou et al. 2008). Although Ni ranks among essential micronutrients, the amount required for normal plant growth is very low (Marschner 2002; Chen et al. 2009). The toxicity of Ni generally consists of interference with other essential metal ions and induction of oxidative stress, resulting in restricted cell division and expansion, disruption of photosynthesis, inhibition of root growth and branching, leaf chlorosis, necrosis and wilting, modification in uptake of other cations, etc. (Chen et al. 2009; Nagajyoti et al. 2010).

Plants inhabiting serpentine habitats have adopted different mechanisms of avoidance or tolerance of elevated heavy metal availability such as exclusion, compartmentalisation in certain tissues, tolerance to toxic effects or even hyperaccumulation (Kazakou et al. 2008; O'Dell and Rajakaruna 2011). With regard to Ni, hyperaccumulation (i.e. shoot concentration higher than 1,000 mg Ni kg^{-1} of dry biomass) is probably the most studied strategy. Nevertheless, the majority of plant species inhabiting serpentine soils are rather tolerant to elevated Ni availability, or they limit Ni uptake and translocation to the aboveground biomass (Kazakou et al. 2008; O'Dell and Rajakaruna 2011), which also applies to our model species, *Knautia arvensis* (Doubková et al. 2012). Previous studies comparing the response of non-hyperaccumulating serpentine

and non-serpentine plants to high Ni availability (see, e.g. Nagy and Proctor 1997; Taylor and Levy 2002; Nyberg Berglund et al. 2003) produced highly diverse results, likely related to different soil Ni concentrations at the sites of plant origin.

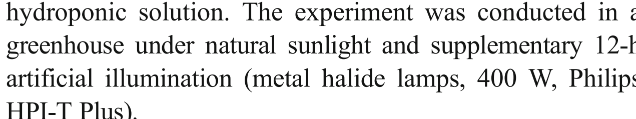
In general, a considerable role in plant adaptation to adverse soil conditions belongs to arbuscular mycorrhizal fungi (AMF) from the phylum Glomeromycota that enter into a symbiosis with roots of most vascular plants (Smith and Read 2008). The involvement of these obligatory symbiotic fungi in plant nutrient uptake and drought stress alleviation in serpentine soils has been previously documented, though the extent of AMF-mediated benefits seems to depend on particular soil–plant–AMF combinations (e.g. Castelli and Casper 2003; Ji et al. 2010; Doubková et al. 2012, 2013). Considering specifically the AMF interaction with Ni, it has received relatively little attention compared with other heavy metals such as cadmium, zinc or lead. Both positive and negative effects of AMF inoculation on plant Ni tolerance have been documented in this respect, often associated with an AMF-mediated decrease (Vivas et al. 2006; Orlowska et al. 2011; Amir et al. 2013) or increase (Turnau and Mesjasz-Przybylowicz 2003; Lagrange et al. 2011) in Ni uptake.

A series of pot experiments involving serpentine and non-serpentine populations of *K. arvensis*, and their native AMF and soils, has recently provided ambiguous results concerning AMF involvement in plant Ni uptake and translocation (Doubková et al. 2011, 2012, 2013). In the present study, a semi-hydroponic system was adopted enabling to separate Ni toxicity from the other aspects of serpentine soil chemistry. The following hypotheses were addressed: (1) *K. arvensis* population and AMF isolate of serpentine origin will show a higher tolerance to elevated Ni concentrations than their non-serpentine counterparts; (2) with rising Ni concentration in solution, the extent of mycorrhizal growth promotion will increase for plants inoculated with serpentine AMF; and (3) plant Ni uptake and translocation will be affected both by plant origin and AMF inoculation, with the serpentine plant–AMF complex showing the most effective barrier to root-to-shoot Ni translocation.

Material and methods

Cultivation system and experimental design

The experiment was conducted under semi-hydroponic conditions in a cultivation system adopted and modified from Gryndler et al. (1992). Plants were grown in plastic tubes (20 cm long, 5 cm wide) sealed with geotextile at the bottom and filled with 300 mL of sterilised (121 °C twice for 25 min) quartz sand (particle size fraction 0.3–1.6 mm). Sets of six tubes were inserted into plastic flower boxes filled with 2 L of



hydroponic solution. The experiment was conducted in a greenhouse under natural sunlight and supplementary 12-h artificial illumination (metal halide lamps, 400 W, Philips HPI-T Plus).

The experiment consisted of 18 treatments resulting from a combination of (1) two *K. arvensis* populations (serpentine and non-serpentine), (2) three AMF inoculation treatments (non-inoculated plants, plants inoculated with a serpentine AMF isolate and plants inoculated with a non-serpentine AMF isolate) and (3) three Ni concentrations in the hydroponic solution (0, 50 and 100 μM Ni). Each treatment involved six replicates (i.e. 108 plants in total were grown in 18 flower boxes), with three individuals of both plant populations being combined in the same flower box.

Plant material

Field scabious, *K. arvensis* (L.) J. M. Coult. (Dipsacaceae), is a perennial rosette-forming herb, entering into symbiosis with AMF (Doubková et al. 2011) and encompassing both serpentine and non-serpentine populations in Central Europe (Kaplan 1998; Kolář et al. 2009). The present semi-hydroponic study involved two *K. arvensis* populations from the Czech Republic. The serpentine population (referred to as S) originating from a semi-dry meadow in W Bohemia (Křížky, 50°03′54.2″N, 12°45′03.6″E) thrives naturally in soil with a DTPA-extractable Ni concentration of ~260 mg kg^{-1} and a Ca/Mg ratio of 0.8. In contrast, the non-serpentine population (NS) from a dry meadow in SW Bohemia (Chanovice, 49°24′39.0″N, 13°43′55.5″E) grows in soil with a low DTPA-extractable Ni concentration (<2 mg kg^{-1}) and a Ca/Mg ratio of 7.5. For more details on both *K. arvensis* populations, please see Doubková et al. (2011).

Mature *K. arvensis* achenes were collected during the 2010 summer season, from approximately 50 plants per population. 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 sterilised (121 °C twice for 25 min) mixture of sand and low-nutrient non-serpentine soil (2:1, *v*/*v*). After 4 weeks, seedlings uniform in size were selected and transplanted into a semi-hydroponic cultivation system. The plants that did not survive the initial post-transplantation period were not replanted except for the first week (101 plants were included in the final analyses).

AMF inoculation

The inoculation treatments involved (1) non-inoculated plants (referred to as *nm*, non-mycorrhizal), (2) plants inoculated with a serpentine AMF isolate *Glomus* sp. SR (EMBL database accession number FR828471; referred to as *s*) and (3) plants inoculated with a non-serpentine AMF isolate *Glomus irregulare* LT (FR828470; referred to as *ns*). The *s* isolate has

been permanently maintained in a mixture of serpentine soil and inert substrate (1:1, *v*/*v*) to keep a selection pressure of serpentine conditions. Both *s* and *ns* AMF isolates belong to the *Glomus* group A as defined by Schwarzott et al. (2001) and are representatives of fast-sporulating r-strategists. These two isolates do not originate from the sites of *K. arvensis* populations involved in the present study, but they correspond to inoculation treatments used in a previous study (Doubková et al. 2012) where their different tolerances to serpentine conditions as well as different promotion effects on plant growth and P uptake were demonstrated in the serpentine substrate.

Non-inoculated plants were treated with 10 mL of autoclaved mixed inoculum (121 °C twice for 25 min). All inoculated plants were treated with 10 mL of a suspension containing colonised root segments, extraradical mycelium and spores of either AMF isolate. The inocula were prepared by wet sieving (Gerdemann and Nicolson 1962) from mature maize cultures with abundant sporulation and high root colonisation. Prior to the experiment, a bioassay was conducted to examine mycorrhizal inoculation potential of both AMF source cultures. After a 6-week growth of inoculated maize plants (six replicates per isolate) in a mixture of sand and low-nutrient non-serpentine soil (2:1, *v*/*v*), mean (±SE) values of AMF colonisation reached 67±7 % and 75±4 % for the s and ns isolate, respectively.

Finally, microbial filtrates from the inocula were applied in an attempt to balance initial non-AMF microbial communities across inoculation treatments. The nm plants were thus supplied with 5 mL of microbial filtrate from each of the *s* and *ns* inocula, and the mycorrhizal treatments received 10 mL of the filtrate from the complementary inoculum. Microbial filtrates were prepared via the filtration of soil suspensions (1:10, *w*/*v*) through a filter paper (pore size 15 μm) to exclude AMF propagules.

Nutrient solutions

Based on a preliminary experiment testing different nutrient solutions for their effects on *K. arvensis* and AMF performance in a model semi-hydroponic system, a modified formulation by Jarstfer et al. (1998) with ten times higher phosphorus concentration was used in the present study. The composition of the basic nutrient solution was as follows: 1.5 mM $Ca(NO_3)_2$ $4H_2O$, 1.5 mM KNO_3, 0.6 mM $MgSO_4$ $7H_2O$, 0.03 mM KH_2PO_4, 0.045 mM NaCl, 13.88 μM H_3BO_3, 0.02 μM $Na_2MoO_4{\cdot}2H_2O$, 2.74 μM $MnCl_2{\cdot}4H_2O$, 0.23 μM $ZnSO_4{\cdot}7H_2O$, 0.96 μM $CuSO_4{\cdot}5H_2O$, 30 μM FeNaEDTA and 0.15 mM MES buffer.

For the first 2 weeks, all flower boxes were supplied only with 2 L of a half-strength basic solution to allow plant and fungal establishment. After this initial period, three different Ni treatments were started. Besides the control treatment (0 μM Ni), the plants were treated either with 50 or 100 μM Ni (applied as $Ni(NO_3)_2{\cdot}6H_2O$ to the basic nutrient solution). These Ni concentrations were selected based on a hydroponic study involving the same plant species (Kolář et al. 2013) and on a preliminary experiment showing a pronounced inhibitive effect of 150 μM Ni on both *K. arvensis* growth and AMF colonisation (unpublished data). Prior to application, the pH value of all the solutions was adjusted to 5.5. The solutions were replaced three times a week to avoid their undue acidification and nutrient depletion.

Plant harvest

After 12 weeks of different Ni treatments, all plants were harvested. A subsample of leaf tissue of a known fresh weight (FW; 50–200 mg) was taken from the youngest fully developed leaf pair of each plant for the photosynthetic pigment assessment. Their concentrations were determined spectrophotometrically after *N*,*N′*-dimethylformamide extraction (Porra et al. 1989). The absorbance of extracts was measured at 480, 647 and 664 nm using a spectrophotometer (DR4000U, Hach, USA), and the concentrations of chlorophyll *a* and *b* and carotenoids were calculated (Wellburn 1994).

Shoots were then cut off and total leaf area assessed using an area metre (LI-3100, LI-COR, USA). The whole root systems were washed, and a root subsample of a known FW was taken from each plant for determination of AMF colonisation. Dry weight (DW) of remaining shoots and roots was recorded after drying for 24 h at 65 °C. DW of all the shoot and root subsamples was inferred from the DW/FW ratio of the remaining shoots and roots, respectively, and both total shoot and root DW were then calculated. Both shoot and root biomass were ground, digested in 65 % HNO_3 and 30 % H_2O_2 and analysed for P, Ca, Mg and Ni concentrations. P concentrations were determined spectrophotometrically by the ammonium-molybdate ascorbic acid method at a wavelength of 630 nm (Unicam UV4-100, UK); the remaining elements were analysed using an atomic absorption spectrometer (AAS Unicam 9200X, UK). Root samples for the determination of AMF colonisation were stained in 0.05 % trypan blue in lactoglycerol (Koske and Gemma 1989) and mounted on slides. The presence of AMF structures (hyphae, arbuscules or vesicles) was then evaluated under a compound microscope at × 100 magnification, using a modified intersection method (McGonigle et al. 1990).

Data analysis

Tolerance indices (TI) to elevated Ni availability were calculated as the relative change in plant total DW (TI_{DW}) and AMF colonisation (TI_{col}), respectively, of a Ni-treated individual to the mean performance of the control (i.e. 0 μm Ni) in the respective population × AMF inoculation treatment. This

relativisation allowed a direct comparison of Ni tolerance of different plant populations (TI_{DW}) and AMF isolates (TI_{col}), independently of the differences in their intrinsic growth rates.

All data were analysed with the Statistica 9.1 software (StatSoft Inc., USA). The correlations between different plant growth parameters or concentrations of different photosynthetic pigments were first explored to reduce the number of variables. Effects of the main factors were then tested using a nested ANOVA model (general linear model (GLM) procedures). Prior to the analyses, all variables were checked for normality and homogeneity of variance. If necessary, they were transformed using logarithmic (log_{10}), square root (sqrt) or arcsine functions. The GLM model involved flower box (nested within Ni treatment × AMF inoculation combination) as a random factor and Ni treatment, AMF inoculation and plant population as fixed factors. Post-hoc comparisons for all significant effects ($P<0.05$) were made using the Tukey HSD test. The effects of main factors and their interactions on all plant and mycorrhizal parameters are summarised in Table 1.

Results

AMF colonisation

All inoculated plants were colonised by AMF, whereas no mycorrhizal structures were found in the roots of non-inoculated plants. AMF colonisation significantly decreased with rising Ni concentration in solution (Table 1). In the control treatment, 82 % of the root length was, on average, colonised by AMF compared to only 58 % and 43 % colonised at 50 μM Ni and 100 μM Ni, respectively. This decline was more gradual in the NS than in the S plants (significant Ni treatment × population interaction; Table S1 in Online Resource 1). The *s* isolate showed generally lower values of AMF colonisation (40±5 %) than the *ns* isolate (84 ±2 %). The effect of Ni treatment × AMF inoculation interaction on AMF colonisation was not significant; nevertheless, the fungal tolerance index (TI_{col}) showed significantly higher values for the *ns* than *s* isolate (Fig. 1). TI_{col} decreased with

Table 1 The effects of Ni treatment (C - control 0 μM Ni, 50 μM Ni, 100 μM Ni), AMF inoculation (*nm* non-mycorrhizal plants, *s* plants inoculated with serpentine isolate, *ns* plants inoculated with non-serpentine isolate), plant population (*S* serpentine, *NS* non-serpentine) and their interactions on *K. arvensis* growth and element concentrations, mycorrhizal parameters and both plant and fungal tolerance indices (TI_{DW} based on total plant dry weight, TI_{col} based on AMF colonisation)

	Ni treatment (1) (*df*=2)		AMF inoculation (2) (*df*=2)		Plant population (3) (*df*=1)		1 × 2 (*df*=4)	1 × 3 (*df*=2)	2 × 3 (*df*=2)	1 × 2 × 3 (*df*=4)
Shoot biomass[a]	4.9*	C>50=100	1.4 ns		0.9 ns		2.7 ns	0.3 ns	1.7 ns	1.5 ns
Root biomass[a]	34.5***	C>50>100	1.4 ns		9.6**	↓S	1.9 ns	1.7 ns	2.2 ns	1.3 ns
TI_{DW}[a,e]	2.0 ns		10.8*	nm>s>ns	1.5 ns		<0.1 ns	0.2 ns	60 **	1.9 ns
Total chlorophyll	45.9***	C>50>100	0.3 ns		9.0**	↑S	1.5 ns	12.1***	1.3 ns	1.6 ns
Carotenoids	47.2***	C>50>100	0.3 ns		9.5**	↑S	1.4 ns	13.8***	1.4 ns	1.7 ns
AMF colonisation[b,d]	8.7*	C>50>100	26.9**	↓s	<0.1 ns		0.9 ns	3.5*	0.2 ns	0.5 ns
TI_{col}[b,d,e]	17.7*	50>100	116.7***	↓s	5.1*	↓S	5.5 ns	2.5 ns	<0.1 ns	2.5 ns
Shoot P[a]	55.6***	C>50=100	2.4 ns		11.6**	↓S	6.1*	1.0 ns	3.3*	4.9**
Root P[a]	197.8***	C>50>100	88.7***	ns> nm=s	0.4 ns		13.0**	1.7 ns	1.0 ns	2.8*
Shoot Ni[a,e]	7.1*	50<100	0.4 ns		0.7 ns		0.5 ns	0.8 ns	4.2 ns	0.7 ns
Root Ni[a,e]	229.1***	50<100	3.0 ns		23.6***	↑S	7.0 ns	1.1 ns	0.2 ns	0.9 ns
Shoot Ca	2.8 ns		0.5 ns		3.7 ns		0.7 ns	5.1**	6.6**	3.2*
Root Ca[a]	9.2**	C<50=100	1.4 ns		1.2 ns		0.5 ns	0.8 ns	1.2 ns	2.3 ns
Shoot Mg[a]	0.8 ns		1.1 ns		75.4***	↓S	0.5 ns	5.7**	7.0**	1.2 ns
Root Mg[a]	10.2**	C>50=100	0.9 ns		7.1**	↑S	1.7 ns	0.9 ns	1.1 ns	0.9 ns

For significant effects of single factors, the direction of change is indicated by arrows and mathematical symbols (e.g. ↑S denotes higher values for S population than NS population; C>50=100 denotes higher values in control than in both 50 and 100 μM Ni solution). *df* error=75.The data in columns represent *F* values with significance level

ns non-significant effect

***$P<0.001$, **$P<0.01$, *$P<0.05$.

[a] Data transformed using log_{10} function

[b] Data transformed using arcsine function

[c] Data transformed using sqrt function

[d] Only inoculated plants considered (*df*=1 for 2 and 2 × 3; *df* error=53)

[e] Only 50 and 100 μM Ni treatments considered (*df*=1 for 1 and 1 × 3; *df* error=53)

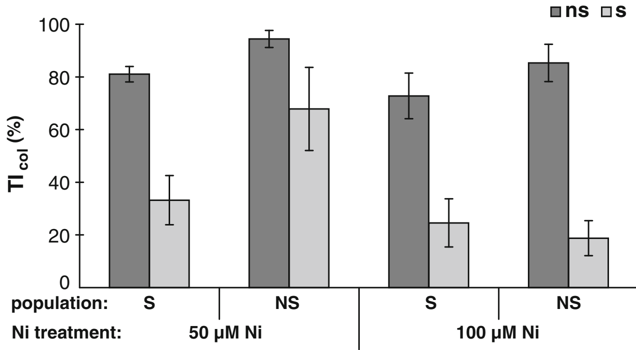


Fig. 1 Fungal Ni tolerance index (TI_{col}) calculated based on AMF colonisation of inoculated *K. arvensis* plants as affected by Ni treatment (50 μM Ni, 100 μM Ni), plant population (*S* serpentine, *NS* non-serpentine) and AMF inoculation (*s* serpentine isolate, *ns* non-serpentine isolate). The data represent the means (±SE) of five to six replicates

rising Ni concentration in solution, and generally lower values were recorded for S plants compared to the NS plants.

Plant growth and concentrations of photosynthetic pigments

Due to a highly positive correlation between shoot DW and leaf area ($r=0.91$, $P<0.001$), the latter is not presented. Both above- and belowground growth of plants significantly decreased with rising Ni concentration in solution (Table 1), but the detrimental effect on root growth was more pronounced (Fig. 2). The application of 100 μM Ni reduced shoot biomass by 43 %, while root biomass was 60 % lower than in the control. Accordingly, the root/shoot ratio gradually decreased from the control to 100 μM Ni treatment ($F=12.85$, $P=0.003$). In the Ni-treated plants, both branching and lateral root growth were strongly inhibited and root tips became dark brown (see Fig. S1). The S plants showed generally significantly lower root DW than the NS plants (and also a lower root/shoot ratio; $F=8.39$, $P=0.005$), while no interpopulation difference was found in terms of shoot DW (Table 1, Fig. 2).

The plant tolerance index TI_{DW} was significantly higher for *nm* plants compared to those in both mycorrhizal treatments, and lower values were recorded for the *ns*-inoculated than *s*-inoculated plants (80±6 % for *nm*, 37±2 % for *ns*, 65±5 % for *s*). When AMF colonisation was included in the GLM model as a covariate, the *ns*- and *s*-inoculated plants however had comparable TI_{DW} values. The difference between both inoculation treatments was more pronounced in the NS than S plants, regardless of whether AMF colonisation was used as covariate (significant population × AMF inoculation interaction; Tables 1 and S2).

Concentrations of photosynthetic pigments were significantly influenced by Ni treatment, plant population and their interaction. Due to a close positive correlation between chlorophyll *a* and *b* ($r=0.98$, $P<0.001$), only total chlorophyll concentrations are further presented. Concentrations of both chlorophyll and carotenoids gradually decreased with rising Ni concentration in solution (Table 1). As indicated by the highly significant Ni treatment × population interaction, the drop in both the chlorophyll and carotenoid concentrations in response to Ni application was more pronounced in the NS than S plants (Fig. 3). In the S plants treated with 100 μM Ni, chlorophyll and carotenoid concentrations reached 73 and 78 %, respectively, of the levels recorded in the control, while in the NS plants, they were only 47 and 53 %. The carotenoids/chlorophyll ratio was higher at 100 μM Ni than in both 50 μM Ni and control treatments ($F=10.81$, $P=0.005$), and also in the NS compared to S plants ($F=5.61$, $P=0.020$) (see Fig. 3).

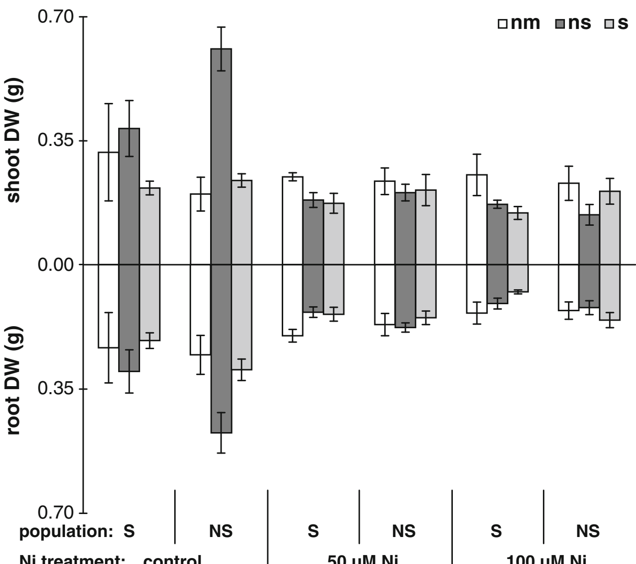


Fig. 2 Shoot and root dry weight (DW) of *K. arvensis* plants as affected by Ni treatment (control 0 μM Ni, 50 μM Ni, 100 μM Ni), plant population (*S* serpentine, *NS* non-serpentine) and AMF inoculation (*nm* non-mycorrhizal, *s* serpentine isolate, *ns* non-serpentine isolate). The data represent the means (±SE) of five to six replicates

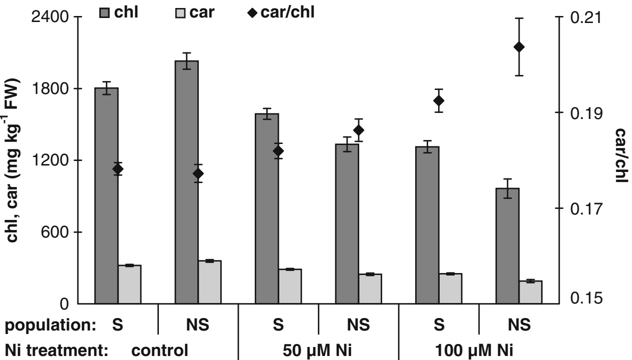


Fig. 3 Concentrations of photosynthetic pigments (*chl* chlorophyll *a*+*b*, *car* carotenoids) and their ratio (car/chl) in fresh weight (FW) of leaf tissues of *K. arvensis* plants as affected by Ni treatment and plant population. The data represent the means (±SE) of 15–18 replicates. For abbreviations and legend, see Fig. 2

Plant element uptake

Both shoot and root P concentrations (Table 1; Fig. S2), as well as P contents (shoot P: $F=21.12$, $P<0.001$; root P: $F=68.45$, $P<0.001$), significantly decreased with Ni addition. Shoot P concentrations (Table 1) and both shoot and root P contents (shoot P: $F=5.70$, $P=0.019$; root P: $F=7.50$, $P=0.008$) were significantly higher in the NS compared to S plants. AMF inoculation did not influence either shoot P concentrations or contents, but the *ns*-inoculated plants showed significantly higher root P concentrations (Table 1) and contents ($F=11.30$, $P=0.005$) than plants in other inoculation treatments. However, the significant Ni treatment × AMF inoculation, AMF inoculation × population and triple interactions indicate that the AMF effect on P uptake depended both on plant population and Ni treatment. Briefly, the *ns*-inoculated NS plants in the control treatment showed significantly higher shoot and root P concentrations and contents than plants in most of the other treatments (Fig. S2).

Both shoot and root Ni concentrations increased with rising Ni concentration in solution (Fig. 4). However, shoot as well as root Ni contents were comparable in both Ni treatments due to Ni-induced plant growth depression (data not presented). Ni application influenced also the uptake of other nutrients, as shown by higher Ca and lower Mg concentrations in the roots of Ni-treated plants, which resulted in a higher Ca/Mg ratio ($F=18.74$, $P=0.001$) compared to the control plants.

With regard to interpopulation differences, the S plants showed significantly higher root Ni concentrations but lower shoot/root Ni ratios ($F=13.18$, $P<0.001$; see also Fig. 4). S plants also showed significantly lower shoot but higher root Mg concentrations compared to the NS plants (Table 1). In addition, marginally significant ($P=0.059$) difference was

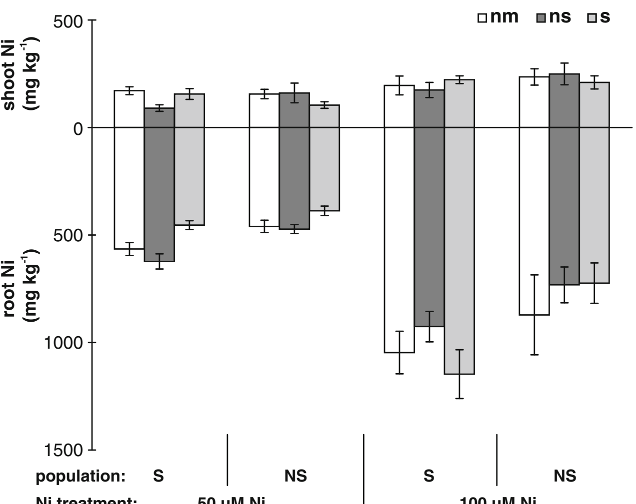
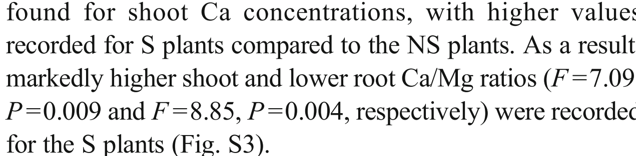


Fig. 4 Nickel concentrations in shoot and root dry biomass of *K. arvensis* plants as affected by Ni treatment, plant population and AMF inoculation. For abbreviations, legend and replicate number, see Fig. 2



found for shoot Ca concentrations, with higher values recorded for S plants compared to the NS plants. As a result, markedly higher shoot and lower root Ca/Mg ratios ($F=7.09$, $P=0.009$ and $F=8.85$, $P=0.004$, respectively) were recorded for the S plants (Fig. S3).

As for the AMF effects, inoculation with the *s* isolate significantly increased shoot Ca concentrations in the S plants, but no difference among inoculation treatments was recorded in the NS plants (AMF inoculation × population interaction). Nevertheless, shoot Ca concentrations were significantly influenced by both Ni treatment × population and triple interactions, thus indicating complex relationships between plant and fungal identity and cultivation conditions (Table S3). Within the S population, higher shoot Mg concentrations were recorded at 50 µM Ni compared to the 100 µM Ni treatment and for *s*-inoculated compared to the *nm*- and *ns*-inoculated plants; whereas for the NS plants, Mg concentrations differed neither between Ni nor AMF inoculation treatments (Ni treatment × population and Ni treatment × AMF inoculation interactions). In the S population, plants inoculated with the *s* isolate showed significantly higher shoot/root Ni ratio than the *ns*-inoculated plants, while no differences between the inocula were recorded for the NS plants (AMF inoculation × population interaction).

Discussion

The effects of edaphic origin (serpentine vs. non-serpentine) and AMF inoculation on Ni tolerance of *K. arvensis* plants were addressed in a semi-hydroponic experiment. Although hydroponics is an artificial cultivation system, it represents a widely used tool to evaluate effects of both nutrients and heavy metals on plant growth. In the present study, semi-hydroponics was employed to separate Ni toxicity to plant–AMF association from other components of serpentine syndrome, which was not possible using conventional soil-based cultivation. A similar approach was recently adopted by Amir et al. (2013) to test the effects of two AMF isolates on plant Ni tolerance, and notably, their results matched well with those from serpentine soil. In comparison, the benefit to *K. arvensis* plants of being mycorrhizal was lower under semi-hydroponic control (Ni-free) conditions than in our previous soil-based experiment (Doubková et al. 2012), where both growth and P uptake were promoted in plants inoculated with the same AMF isolates. These differences might be related to higher P availability in the semi-hydroponic cultivation system compared to generally low P mobility in soils, resulting in low plant-available P concentrations (Schachtman et al. 1998). In conditions of non-limiting P supply, *K. arvensis* plants might have suppressed AMF involvement in P uptake (Nagy et al. 2009). Nevertheless, Amir et al. (2013) reported pronounced AMF-mediated increase in plant growth and P uptake even

under ten times higher P concentration in Ni-free nutrient solutions.

Nickel addition had a pronounced negative effect on *K. arvensis* growth and concentrations of photosynthetic pigments as well as on AMF colonisation. A detrimental effect of elevated Ni availability on both plant and fungal performance, even of those of serpentine origin, has previously been reported (Doherty et al. 2008; Ghasemi and Ghaderian 2009; Orlowska et al. 2011). Ni accumulation in plant roots significantly inhibited their development and led to tissue necrosis, which is considered one of the typical symptoms of Ni phytotoxicity and an important indicator of plant Ni tolerance level (e.g. Nyberg Berglund et al. 2003; Soudek et al. 2011). The Ni-induced root damage concurrently impaired plant P uptake. In addition, competition between divalent cations might have played a role in the modified plant nutrition (Marschner 2002), resulting in significantly lower Mg concentrations in the roots of Ni-treated plants on one hand and increased Ca concentrations on the other. The latter possibly indicates higher demands of Ni-treated plants for calcium, which plays an essential role in the division and extension of root cells (Marschner 2002). The involvement of Ca in the alleviation of Ni phytotoxicity has previously been documented (Seregin and Kozhevnikova 2006; Wang et al. 2010).

Regarding Ni toxicity to AMF, the isolate of serpentine origin was surprisingly more sensitive to Ni than the non-serpentine isolate, in terms of decrease in root colonisation. In this respect, a wide spectrum of response of serpentine and non-serpentine AMF to elevated Ni concentrations has been reported (Doherty et al. 2008; Orlowska et al. 2011; Amir et al. 2013). In contrast to root colonisation level or spore production, which is affected by carbon allocation from the host plant, spore germination might provide a better indication of Ni tolerance of the AMF isolates. Based on a spore germination bioassay, Amir et al. (2008) proved a clearly higher Ni tolerance for serpentine AMF isolates. Nevertheless, they also reported induction of Ni tolerance in AMF of non-serpentine origin when temporarily exposed to Ni, which provides a potential explanation of high Ni tolerance of our non-serpentine isolate. It cannot however be excluded that the observed differences between the two AMF isolates used in the present study were partly caused by their different taxonomical position. To provide unambiguous evidence on differentiation in Ni tolerance, a comparison of serpentine and non-serpentine AMF isolates of identical species would be necessary. With regard to *K. arvensis* interpopulation differences in Ni effects on AMF colonisation, a more pronounced decline in the serpentine plants might be related to higher Ni and Mg accumulation in their roots; an inhibitory impact on AMF development has been reported for elevated concentrations of both Ni (Vivas et al. 2006; Amir et al. 2008) and Mg (Jarstfer et al. 1998; Malcová et al. 2002) concentrations.

Although *K. arvensis* plants of different edaphic origins did not differ in their Ni tolerance in terms of growth, the serpentine plants were able to maintain relatively higher concentrations of photosynthetic pigments in their leaves than the non-serpentine plants under elevated Ni availability. The higher chlorophyll concentrations can be considered as evidence of a better physiological state of the Ni-exposed serpentine plants, while the higher concentrations of carotenoids with antioxidative effects indicate an intense plant-defensive response to Ni toxicity (Krupa et al. 1996; Drazkiewicz and Baszynski 2010). Also, the carotenoids-to-chlorophyll ratio, which is broadly used as an indicative measure of plant physiological status and as a stress biomarker (see, e.g. Penuelas and Filella 1998; Martínez-Peñalver et al. 2011), suggests that Ni-induced oxidative stress and related damage were higher in the non-serpentine *K. arvensis* plants compared to the serpentine plants. Generally, hydroponic studies testing the effects of high Ni concentrations on serpentine vs. non-serpentine plants (in terms of shoot and/or root growth) have provided contrasting results, including clearly higher Ni tolerance of serpentine populations (e.g. Nagy and Proctor 1997; Nyberg Berglund et al. 2003) as well as a comparable degree either of Ni tolerance (e.g. Nagy and Proctor 1997) or Ni-induced plant growth reduction (Ghasemi and Ghaderian 2009) in populations of both origins, similar to our results.

With regard to element uptake, the serpentine *K. arvensis* plants restricted more efficiently root-to-shoot translocation of Ni and Mg and were characterised by markedly higher shoot Ca/Mg ratio. These traits are generally suggested as important components of serpentine edaphic tolerance (Kazakou et al. 2008; O'Dell and Rajakaruna 2011). Concerning the restricted Ni translocation in serpentine *K. arvensis* plants, although no attempt was made to further localise Ni in root tissues, retention of Ni in the root cortex (especially in the apoplast) might be involved, as previously reported for hyperaccumulating plants (Mesjasz-Przybylowicz et al. 2007; Moradi et al. 2010). Considering the high Ca/Mg ratio (2.5) of the nutrient solutions used, which exceeded the typical ratio of serpentine soils (lower than 1.0), the recorded Mg retention in roots of serpentine plants is in accordance with data for serpentine *K. arvensis* plants grown in a non-serpentine substrate in a previous reciprocal transplant experiment (Doubková et al. 2012). Both studies thus support the fact that serpentine *K. arvensis* plants respond to actual soil Mg availability by modifications in their Mg translocation pattern, lacking any intrinsic adaptation in this respect.

Contrary to our working hypothesis, inoculation with either AMF not only did not increase plant Ni tolerance, but also even aggravated Ni-induced plant growth depression. Such a negative mycorrhizal impact has also been previously recorded by Doherty et al. (2008) for Ni-exposed plants inoculated with a serpentine AMF isolate/community. In comparison, of the two AMF isolates involved in the present study,

inoculation with the serpentine fungi resulted in a less pronounced growth inhibition. However, it might have been a consequence of lower root colonisation in Ni-enriched treatments, signifying a relatively lower carbon cost to the host plant. Inoculation of serpentine plants with the serpentine AMF isolate led to a higher root-to-shoot Ni translocation, but it also increased shoot Ca concentrations. This might have helped to alleviate a potential Ni phytotoxicity to leaf tissues and thus to maintain aboveground growth (Gabbrielli and Pandolfini 1984; Chaney et al. 2008), possibly through stabilisation of photosystem II and improvement of photosynthetic parameters as has been suggested for both Ni and other metal ions (Ouzounidou et al. 1995; Maksymiec and Baszynski 1999; Wan et al. 2011).

In conclusion, the main findings from the present study can be summarised as follows: (1) Ni addition had a strong negative effect on the performance of both *K. arvensis* plants and AMF isolates. (2) Plant growth response to Ni exposure was independent of edaphic origin; however, a lower Ni tolerance of the non-serpentine plants was indicated by a stronger decline in their photosynthetic pigment concentrations. (3) Serpentine plants retained relatively more Ni and Mg in their roots and showed higher Ca/Mg ratio in leaf tissues than their non-serpentine counterparts. (4) AMF inoculation further enhanced the inhibitory effect of Ni on plant growth.

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Mycorrhiza

Nickel tolerance of serpentine and non-serpentine *Knautia arvensis* plants as affected by arbuscular mycorrhizal symbiosis

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Supplementary data

Figure S1 The appearance of non-serpentine and serpentine *Knautia arvensis* plants after two-month cultivation in semi-hydroponic system under different treatments
Figure S2 Phosphorus concentrations in shoot and root biomass of *K. arvensis* plants as affected by Ni treatment, plant population and AMF inoculation
Figure S3 Ratio of calcium to magnesium concentrations in shoot and root biomass of *K. arvensis* plants as affected by Ni treatment and plant population

Table S1 AMF colonisation of *K. arvensis* roots
Table S2 Plant nickel tolerance index calculated based on *K. arvensis* total dry weights
Table S3 Calcium and magnesium concentrations in shoot and root biomass of *K. arvensis* plants

Online supplementary material

Fig. S1 The appearance of non-serpentine (A) and serpentine (B) *Knautia arvensis* plants after two-month cultivation in semi-hydroponic system under different treatments

A	Non-mycorrhizal	Non-serpentine AMF isolate	Serpentine AMF isolate
0 μM Ni			
50 μM Ni			
100 μM Ni			

B	**Non-mycorrhizal**	**Non-serpentine AMF isolate**	**Serpentine AMF isolate**
0 µM Ni			
50 µM Ni			
100 µM Ni			

Fig. S2 Phosphorus concentrations in shoot and root biomass of *K. arvensis* plants as affected by Ni treatment (control – 0 µM Ni, 50 µM Ni, 100 µM Ni), plant population (S – serpentine, NS – non-serpentine) and AMF inoculation (*nm* – non-mycorrhizal plants; *s* – plants inoculated with serpentine isolate; *ns* – plants inoculated with non-serpentine isolate). The data represent the means (± SE) of five to six replicates

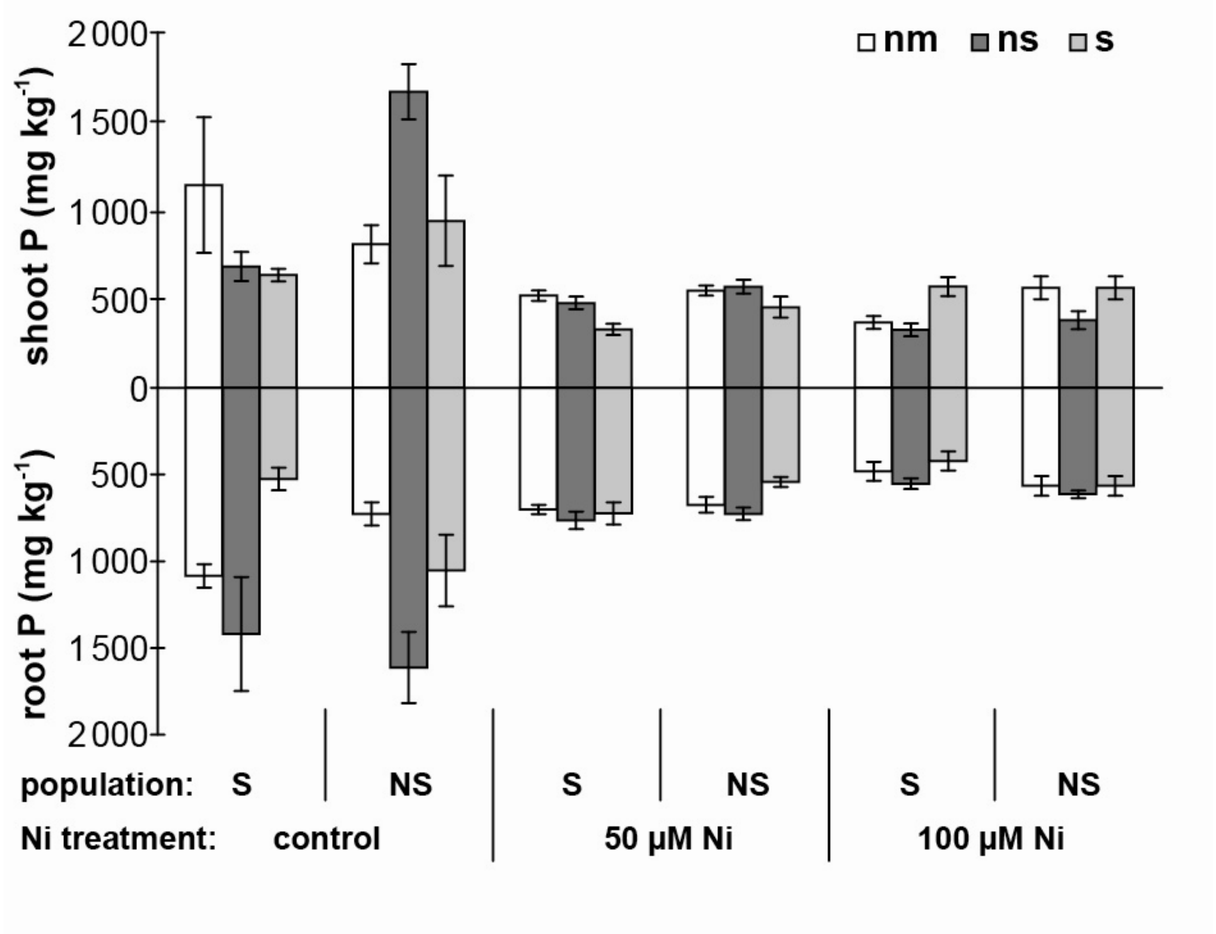

Fig. S3 Ratio of calcium to magnesium concentrations in shoot and root biomass of *K. arvensis* plants as affected by Ni treatment and plant population. The data represent the means (± SE) of 15–18 replicates. For abbreviations and legend see Fig. S2

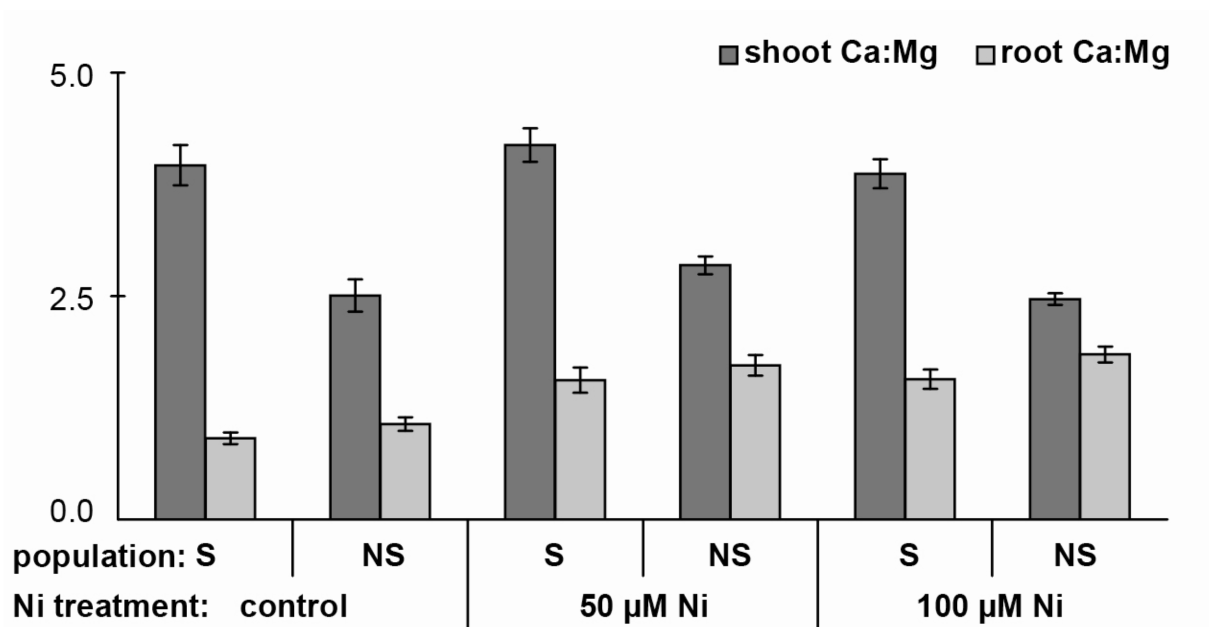

Table S1 AMF colonisation of *K. arvensis* roots. The data represent means (±SE) of five to six replicates. The following legend is used: Ni treatment (control = 0 μM Ni; 50 μM Ni; 100 μM Ni); plant population (NS – non-serpentine; S – serpentine); AMF inoculation (*s* – plants inoculated with serpentine isolate; *ns* – plants inoculated with non-serpentine isolate)

Ni treatment	Plant population	AMF inoculation	AMF colonisation (%)
Control	NS	*ns*	91 ± 2
		s	64 ± 16
	S	*ns*	97 ± 1
		s	75 ± 11
50 μM Ni	NS	*ns*	86 ± 3
		s	43 ± 10
	S	*ns*	78 ± 3
		s	25 ± 7
100 μM Ni	NS	*ns*	78 ± 6
		s	12 ± 4
	S	*ns*	70 ± 8
		s	18 ± 7

Table S2 Plant nickel tolerance index (TI_{DW}) calculated based on *K. arvensis* total dry weights. The data represent means (±SE) of five to six replicates. The following legend is used: Ni treatment (50 μM Ni; 100 μM Ni); plant population (NS – non-serpentine; S – serpentine); AMF inoculation (*nm* – non-mycorrhizal plants; *s* – plants inoculated with serpentine isolate; *ns* – plants inoculated with non-serpentine isolate)

Ni treatment	Plant population	AMF inoculation	TI_{DW} (%)
50 μM Ni	NS	*nm*	89 ± 13
		ns	35 ± 3
		s	68 ± 12
	S	*nm*	81 ± 5
		ns	46 ± 5
		s	73 ± 9
100 μM Ni	NS	*nm*	79 ± 15
		ns	24 ± 4
		s	68 ± 10
	S	*nm*	71 ± 16
		ns	41 ± 3
		s	52 ± 5

Table S3 Calcium and magnesium concentrations in shoot and root biomass of *K. arvensis* plants. For abbreviations, legend and replicate number see Table S1

Ni treatment	Plant population	AMF inoculation	Shoot Ca (mg kg^{-1})	Root Ca (mg kg^{-1})	Shoot Mg (mg kg^{-1})	Root Mg (mg kg^{-1})
Control	NS	*nm*	6 335 ± 461	2 722 ± 207	1 976 ± 309	2 430 ± 246
		ns	4 699 ± 171	2 896 ± 461	2 382 ± 199	2 903 ± 329
		s	5 828 ± 334	2 848 ± 129	2 347 ± 223	2 580 ± 269
	S	*nm*	4 936 ± 504	2 407 ± 284	1 282 ± 20	3 274 ± 163
		ns	5 371 ± 512	3 203 ± 408	1 450 ± 120	2 812 ± 357
		s	6 873 ± 461	1 947 ± 128	1 710 ± 150	2 303 ± 232
50 µM Ni	NS	*nm*	5 249 ± 426	3 644 ± 191	2 003 ± 205	1 975 ± 147
		ns	5 920 ± 409	3 257 ± 220	1 970 ± 120	1 791 ± 127
		s	5 419 ± 555	2 869 ± 206	1 741 ± 110	2 169 ± 220
	S	*nm*	6 939 ± 445	2 899 ± 135	1 506 ± 137	2 296 ± 130
		ns	6 482 ± 575	3 370 ± 335	1 545 ± 145	1 970 ± 137
		s	6 944 ± 529	3 165 ± 246	1 974 ± 197	2 144 ± 188
100 µM Ni	NS	*nm*	5 129 ± 221	3 041 ± 479	1 941 ± 124	2 221 ± 234
		ns	6 023 ± 488	3 487 ± 288	2 490 ± 204	1 666 ± 92
		s	4 520 ± 267	3 182 ± 149	1 839 ± 87	1 833 ± 116
	S	*nm*	4 567 ± 293	3 461 ± 359	1 396 ± 156	2 431 ± 89
		ns	4 821 ± 550	3 456 ± 342	1 126 ± 72	2 217 ± 136
		s	5 930 ± 301	3 695 ± 189	1 499 ± 85	2 318 ± 229

Niche partitioning in arbuscular mycorrhizal communities in temperate grasslands: a lesson from adjacent serpentine and non-serpentine habitats

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Abstract

Arbuscular mycorrhizal fungi (AMF, phylum Glomeromycota), as obligate symbionts in roots of most vascular plants, represent an important soil microbial group playing a fundamental role in many terrestrial ecosystems. We explored the effects of deterministic (soil characteristics, host plant life stage, neighbouring plant communities) and stochastic (geographical distances) processes on AMF colonisation, richness and community composition in roots of *Knautia arvensis* (Dipsacaceae) plants from three serpentine grasslands and adjacent non-serpentine sites. Methodically, the study was based on the amplification on rDNA by a combination of AMF-specific primers, followed by 454-sequencing of the ITS region. In total, we detected 81 molecular taxonomical units (MOTUs) belonging to the Glomeromycota. A positive correlation between soil pH and AMF MOTUs richness was detected, together with niche differentiation of AMF MOTUs along the pH gradient from 3.5 to 5.8. Contrary, K and Cr soil concentration had a negative influence on AMF MOTUs richness. Interestingly, we detected a strong relation between neighbouring plant community composition and AMF MOTUs richness. Although spatial distance also contributed to structuring AMF communities in *K. arvensis* roots, environmental parameters were key factors in this respect. The complex of serpentine conditions and available soil Ni concentration in particular proved to play an important role in shaping the composition of AMF communities. The structure of AMF communities was also dependent on host plant life stage (vegetative *vs* flowering). Serpentine character of the site also negatively influenced AMF root colonisation, similarly as higher Fe concentration. In conclusion, our study supports the dominance of deterministic factors in structuring AMF communities in heterogeneous environment composed of an edaphic mosaic of serpentine and non-serpentine soils.

Introduction

Biodiversity is a key factor driving ecosystem processes (Hooper et al., 2005). Understanding the mechanisms structuring the diversity and community assemblages is therefore crucial for predicting ecosystem responses to environmental changes. In general, natural communities are shaped by deterministic (niche) processes, which involve both abiotic and biotic factors, and neutral (stochastic) processes (such as dispersal limitation) that altogether influence species distribution and abundance (Hubbell, 2001; Leibold and McPeek, 2006). Deterministic processes (e.g., soil characteristics, climate, symbiotic interactions, and competition) assemble communities in a defined manner as a result of competition for available niches in the ecosystem (McGill, 2003).

33 Contrary, neutral theory assumes that species assemblages are random, and neutrality is defined
34 as the ecological equivalence among all individuals of all species at one trophic level (Hubbell,
35 2001). The significance of both deterministic and neutral processes in structuring microbial
36 communities has been demonstrated in most previous studies (Ramette and Tiedje, 2007; van
37 der Gast et al., 2008; Martiny et al., 2011; Stegen et al., 2012).

38 Arbuscular mycorrhizal fungi (AMF) are an important soil microbial group, which plays
39 a fundamental role in many terrestrial ecosystems. As obligate symbionts of plant roots, AMF
40 mediate nutrient flow from the soil to the host plant, in exchange for photosynthetically assimilated
41 carbon (Smith and Read, 2008). Although the majority of recognized AMF species (Schüssler's
42 Glomeromycota phylogeny, http://schuessler.userweb.mwn.de/amphylo/) are believed to be
43 non-host specific (Smith and Read, 2008), they clearly differ in their life-traits (Maherali and
44 Klironomos, 2007, 2012; Powell et al., 2009) as well as in their effects on plant performance and
45 physiology (Fitter et al., 2005; Hoeksema et al., 2010). Therefore, understanding of the processes
46 underlying AMF community structure represents one of the major goals of ecosystem ecology.

47 Similarly to other microbial groups, AMF communities have been found to be structured
48 by deterministic as well as stochastic processes (Dumbrell et al., 2010), although their relative
49 contribution may vary between different spatial scales and/or environments (Dumbrell et al.,
50 2010; Lekberg et al., 2011; Hazard et al., 2013). Based on their comprehensive literature survey,
51 Caruso et al. (2012b) assumed that the effect of limited dispersal is usually overwhelmed
52 by non-neutral processes in disturbed or extremely heterogeneous environment, while the role
53 of stochasticity increases in relatively homogeneous environment.

54 Soil structure and chemistry (particularly pH and macronutrient availability) seem to be key
55 deterministic factors governing the richness and composition of AMF communities (Fitzsimons
56 et al., 2008; Dumbrell et al., 2010; Lekberg et al., 2011; Verbruggen et al., 2012). Notably,
57 a decline in AMF richness and changes in species composition have been seen in anthropogenic,
58 degraded or heavy metal contaminated soils (e.g., Del Val et al., 1999; Egerton-Warburton and
59 Allen, 2000; Vallino et al., 2006; Zarei et al., 2010; Hassan et al., 2011). Because of the tight
60 coupling of AMF life cycles with their host plants, AMF communities are also shaped by the
61 identity of host plant species (e.g., Helgason et al., 2002; Vandenkoornhuyse et al., 2002;
62 Sýkorová et al., 2007b; Davison et al., 2011) as well as by neighbouring plant communities
63 (Mummey et al., 2005; Hawkes et al., 2006; Hausmann and Hawkes, 2009).

64 Serpentine habitats, which are scattered around the world and cover ~ 1% of land surface, 65 provide a unique opportunity to untangle the mechanisms driving the structure of plant and 66 microbial communities in a harsh environment. Similarly to man-made or heavy metal polluted 67 sites, serpentine soils impose severe edaphic conditions on their inhabitants, particularly 68 adverse calcium-to-magnesium ratio and elevated heavy metal concentrations (for reviews, see 69 Alexander et al., 2006; Brady et al., 2005; Kazakou et al., 2008; O´Dell and Rajakaruna, 2011). 70 However, naturally metalliferous sites are under strong and long-term selective pressures, which 71 facilitate the evolution of adaptations to cope with the edaphic stress (see also Schechter and 72 Bruns, 2012). A higher importance of niche-based processes on the distribution of fungal 73 species might therefore be expected in island-like serpentine sites compared to disturbed 74 systems of anthropogenic origin or less heterogeneous natural habitats.

75 In the present study, we explored the mechanisms driving the diversity and structure of 76 AMF assemblages in an area encompassing a mosaic of different soil types. *Knautia arvensis* 77 (L.) J. M. Coult. (Dipsacaceae), which inhabits both adjacent serpentine (S) and non-serpentine 78 (NS) sites, was selected as a model host plant, and the following questions were addressed: 79 1) What is the relative contribution of deterministic (soil characteristics, including pH, nutrient 80 and heavy metal concentrations, life stage of host plant, and neighbouring plant communities) 81 *vs* stochastic (spatial distance) processes to structuring AMF communities at a local scale? 82 2) Which deterministic factors most influence the species assembly and richness of AMF 83 communities, and in what manner?

84

85 **Materials and Methods**

86 *Study area and sampling*

87 The study was carried out in the Slavkovský les Mts in the western part of the Czech Republic, 88 where serpentine and non-serpentine soils form an edaphic mosaic over relatively small spatial 89 scale (diameter of serpentine patches ranging from ~90 m to ~1.5 km). Based on previous analyses 90 of Ca, Mg and Ni concentrations in soil samples from a high number of sites, we chose three 91 pairs of adjacent serpentine and non-serpentine grasslands inhabited by *K. arvensis* (Fig. S1). 92 This species has been shown to enter into symbiosis with AMF both in serpentine and non- 93 serpentine soils (Doubková et al., 2011).

94 At each of the six sites, we collected 11 or 12 *K. arvensis* plants from an area of 15–25 m^2 95 (depending on population density), 70 samples in total. Immediately prior to plant excavation,

96 volumetric rhizosphere soil moisture (%) was determined by three independent measurements
97 using the Theta Probe ML2x (Moisture Meter HH2, Delta-T Devices Ltd., Cambridge, UK). The
98 root system of each plant was excavated together with rhizosphere soil (blocks approx. 10 × 10 cm,
99 15 cm deep). All accompanying plant species in sample soil blocks were recorded based on the
100 presence of their above-ground parts in order to assess the role of neighbouring plant communities
101 in structuring AMF communities. All excavated samples were transported into the laboratory
102 within a few hours of collection and stored at 4°C until processed, within two days of sampling
103 time. Rhizosphere soil was carefully separated from each *K. arvensis* root system by hand
104 shaking. Soil was then thoroughly mixed, dried, sieved (mesh size 2 mm), and subsequently
105 analysed for selected properties (Table S1; mean ± SE values of Ca:Mg ratios were 4.72 ± 0.47
106 and 0.36 ± 0.03 at NS and S sites, respectively). The shoots of excavated *K. arvensis* plants
107 were cut off and plant life stage (vegetative *vs* flowering) was recorded. Separated root systems
108 were washed free of soil, dried with a paper towel and divided into two parts. The first part was
109 subjected to evaluation of mycorrhizal root colonisation after staining with 0.05% trypan blue in
110 lactoglycerol (Koske and Gemma, 1989). The percentage of mycorrhizal root colonisation (the
111 presence of arbuscules, vesicles, and/or hyphae) was assessed under a compound microscope
112 at 100× magnification, using a modified intersection method (McGonigle et al., 1990). The other
113 part of root system was divided in two aliquots (~100 mg fresh weight) and stored at -80°C until
114 used for downstream DNA analyses.
115
116 *Molecular analyses*
117 The frozen root samples (two replicates per plant) were homogenized using the Tissue Lyser II
118 (Qiagen, Hilden, Germany). DNA was extracted with the aid of the DNeasy Plant Mini Kit
119 (Qiagen) according to the manufacturer's instructions, with a final elution with 75 µl of AE buffer.
120 Isolated DNA was 10× diluted with ddH_2O and used as a template for subsequent PCR reactions.
121 Two independent PCR reactions were run from each root sample to avoid PCR bias (i.e.,
122 products of four independent PCR reactions were obtained per individual plant). In the first
123 step, the AMF specific primers SSUmAf-LSUmAr were used to amplify partial SSU, whole ITS
124 and partial LSU regions of the rDNA (Krüger et al., 2009). The mix for the 1st PCR reaction
125 included 1× Taq buffer with KCl/without $MgCl_2$ (Thermo Scientific), 0.2 mM of each of dNTPs,
126 2 mM $MgCl_2$, 0.5 µM of each primer, 40 µg of BSA (Thermo Scientific), 0.5 µL mix of 4% Pfu
127 (2.5 U/µL; Thermo Scientific) and 96% Taq (5 U/µL; Thermo Scientific) DNA polymerases,

128 26 μL of sterile ddH_2O and 2.5 μL of the template in a final volume of 50 μL. Cycling conditions
129 were 5 min at 95°C, followed by 38 cycles of 30 sec at 95°C, 90 sec at 60°C and 2 min at 72°C.
130 The program was concluded by a final extension phase of 10 min at 72°C. The obtained PCR
131 products were pooled per individual plant (total volume 200 μL), purified using the QIAquick
132 PCR Purification Kit (Qiagen), eluted into 30 μL of ddH_2O and used as a template for the 2nd PCR
133 with eukaryotic primers ITS1-ITS4 (White et al., 1990). The mix for the 2nd PCR reactions
134 included 1× Taq buffer with KCl/without $MgCl_2$, 1.5 μL of DMSO PCR Reagent, 0.2 mM of each
135 of dNTPs, 2 mM $MgCl_2$, 0.2 μM of each primer, 1.5 μL mix of 4% Pfu (2.5 U/μL; Thermo
136 Scientific) and 96% Taq (5 U/μL; Thermo Scientific) DNA polymerases, 33 μL of sterile ddH_2O
137 and 2 μL of the template in a final volume of 50 μL. Cycling conditions were 5 min at 95°C,
138 followed by 20 cycles of 60 s at 95°C, 60 s at 55°C and 60 s at 72°C. The program was concluded
139 by a final extension phase of 10 min at 72°C. The obtained PCR products were used as a template
140 for a PCR reaction with tag-encoded 454-Titanium pyrosequencing primers (ITS1–ITS4), which
141 also included Titanium A or B adaptors (Baldrian et al., 2012). The reaction mix was the same
142 as described for the 2nd PCR except for a lower concentration (0.1 μM each) of the fusion primers.
143 Cycling conditions were identical as for the 2nd PCR except for higher annealing temperature
144 (62°C) and only 15 cycles. The PCR products were then purified using Agencourt AMPure XP
145 Beads (Beckman Coulter, Beverly, MA, USA) with NEBNext Sizing Buffer (New England Bio-
146 Labs, Ipswich, MA, USA) and eluted with 10 μL of 1× TE buffer. DNA concentration was
147 quantified with the Qubit® 2.0 Fluorometer using the Qubit dsDNA HS Assay Kit (Life
148 Technologies, Carlsbad, CA, USA), and all samples were then equimolarly mixed. To eliminate
149 the risk of a higher proportion of short reads, the mixed sample was gel purified using the
150 Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA), followed by additional
151 purification by AMPure Beads and QIAquick PCR Purification Kit, as described above. The
152 number of DNA copies in the resultant sample was quantified by the Library Quantification Kit
153 (Kapa Biosystems, Woburn, MA, USA), and their optimal amount was subjected to emulsion
154 PCR and subsequent sequencing on GS Junior platform (Roche, Basel, Switzerland).

155

156 *Sequence processing and phylogenetic analyses*

157 The obtained pyrosequencing reads were analysed using the Mothur 1.26.0. All flows with more
158 than one error either in tag or primer sequences were excluded and the remaining flows were
159 subjected to reduction of pyrosequencing noise (denoising) and removal of all sequences shorter

160 than 380 bp (Schloss et al., 2009). We further collapsed all homopolymers to the maximum number
161 of five using the CLOTU pipeline (Kumar et al., 2011). The sequences were subsequently
162 clustered using CD-HIT (Li and Godzik, 2006) at 97% similarity level using the PlutoF pipeline
163 (Tedersoo et al., 2010). The obtained consensus sequences together with singletons were subjected
164 to another clustering step at the same similarity level. All singletons from the second clustering
165 were excluded from further analyses and the obtained consensus sequences were considered as
166 representatives of distinct molecular operational taxonomic units (MOTUs). Chimeric sequences
167 identified in the dataset using *de-novo* chimera checking in UCHIME (Edgar et al., 2011) or
168 manual blasting of ITS1 and ITS2 fragments against the NCBI database were deleted. All MOTUs
169 were then assigned to Glomeromycota families according to the closest blast hits of their
170 consensus sequences. We decided to use phylogenetic analyses for delimitation of AMF taxa
171 because there is no widely accepted barcoding gap for the ITS rDNA region in the
172 Glomeromycota (Stockinger et al., 2010). Six alignments comprising the obtained consensus
173 sequences and the phylogenetic reference dataset of AMF rDNA sequences published by
174 Krüger et al. (2012) were prepared separately for each of the following AMF families: the
175 Acaulosporaceae, Claroideoglomeraceae, Diversisporaceae, Gigasporaceae and Glomeraceae,
176 except for the Archaeosporaceae that were aligned together with the Ambisporaceae, using
177 MAFFT 6.6 http://mafft.cbrc.jp/alignment/server/index.html), and subjected to phylogenetic
178 analysis. Phylogenetic trees were obtained by distance analysis using the neighbour joining
179 algorithm in MEGA5 (Tamura et al., 2011) with 1,000 bootstrap replicates, the Kimura two-
180 parameter model and gamma shape parameter of 0.5.

181

182 *Statistical analyses*

183 The individual-based rarefaction curves were calculated using the EstimateS 8.20 (Colwell, 2006)
184 to compare differences in accumulating MOTUs richness among individual plants. To determine
185 the effect of sample size (number of sequences per sample) on AMF MOTUs richness, we
186 calculated residuals of MOTUs richness using the resid function of linear correlation between
187 the sequence numbers and MOTUs richness. Very strong correlation was found between the
188 observed MOTUs richness and residuals of the richness (Fig. S2; $R^2 = 0.872$; $p < 0.001$),
189 therefore AMF MOTUs richness variable was used in subsequent statistical analysis.

190 Bray-Curtis and Euclidean dissimilarity measures were used to construct community (plant
191 species presence/absence and fungal abundance) and environmental (soil characteristics,

geographical distance) distance matrices, respectively. For categorical environmental variables (i.e., plant life stage, soil type, and locality), Gower's dissimilarity metric was calculated in the "cluster" package of R (R Core Development Team, 2008). Soil element concentrations together with pH values, cation exchange capacity (CEC), Ca:Mg ratio and soil moisture were log-transformed prior to analyses to obtain normal distribution. Relationships among plant communities were investigated using principal component analysis (PCA). The first two PCA axes (PC1 and PC2) explaining 45% and 19% of the total variation, respectively, were used in model selection.

The general least-squares (GLS) model was built to identify the main predictors of mycorrhizal root colonisation and AMF taxa richness, based on the following parameters: locality, soil type (serpentine *vs* non-serpentine), number of accompanying plant species, first two PCA axes of plant community composition and soil characteristics (moisture, pH_{H2O}, CEC, C_{org}, C_{carbon}, P, N, K, Ca, Mg, Ca:Mg, Fe, Mn, Ni, Cr, and Co). The best model was selected according to the corrected Akaike information criterion (AIC). Multicollinearity between predictor variables was checked by calculating the variance inflation factor (VIF); variables with VIF > 10 were excluded prior to model selection. Moran's I tests was used to explore the effect of spatial autocorrelation in univariate (plant species richness and colonisation). In addition, it was used to test for autocorrelation between AMF MOTUs richness and plant community composition. A generalized additive modelling (GAM) available in the "mgcv" package was employed to explore the potential non-linear relationships between the indicator species and main predictors of AMF community composition. This approach allows better fitting of non-linear relationships owing to its nonparametric nature.

MOTU abundances across samples were standardized using the Hellinger transformation, which enables the use of linear-based ordinations (e.g., RDA) for non-linear data (Legendre and Gallagher, 2001). A Constrained Analysis of Principal Coordinates (CAP), which is a modified RDA analysis compatible with non-Euclidean distances, was performed in the "vegan" package in R (Oksanen et al., 2012). Prior to CAP, an automatic stepwise model building was conducted to select a set of non-redundant predictors of AMF community composition, based on permutation-based P-values and AIC criterion as implemented in the "ordistep" function of "vegan". In addition, partial Mantel test was used to determine the effects of environmental variables, neighbouring plant communities and geographical distances on structuring the composition of AMF communities.

Indicator plant species (having the greatest effect on the AMF richness) were selected using the "indval" function implemented in the "labdsv" package (Roberts, 2014). Here, the samples are

224 clustered according to environmental variables and the most abundant plant species in each cluster
225 are then identified; the significance of indicator values is evaluated by permutation test.
226
227 **Results**
228 *Mycorrhizal root colonisation*
229 All sampled *K. arvensis* plants had their roots colonised by AMF, with mycorrhizal colonisation
230 ranging from 19% to 100%. The best model (AICc = 12.33) selected two main explanatory
231 variables for the level of mycorrhizal colonisation (Fig. 1a). Specifically, the colonisation was most
232 affected by serpentine *vs* non-serpentine character of the site and available soil Fe concentration.
233 Higher mycorrhizal root colonisation was observed in plants from NS than from S habitats
234 (Fig. S3a; $p = 0.02$) and from localities with lower Fe concentration (Fig. S3b; $p = 0.007$).
235
236 *AMF richness*
237 Overall, 135,618 sequences of the ITS rDNA region were obtained from 454-sequencing. After
238 denoising, quality check and first clustering (97% similarity level), we received 177 contigs,
239 142 of which were singletons. Second clustering (including the singletons from the first clustering)
240 and subsequent exclusion of singletons from the second clustering yielded 176 MOTUs, which
241 were subjected to chimera checking, resulting in 158 MOTUs with affinities to the Glomeromycota.
242 Phylogenetic analyses of consensus sequences of these MOTUs revealed 81 AMF MOTUs,
243 51 of which belonged to the Glomeraceae (Fig. 2), eight to the Acaulosporaceae (Fig. S4a), six
244 to the Archaeosporaceae (Fig. S4b), five to the Claroideoglomeraceae (Fig. S4c), four to each
245 Diversisporaceae (Fig. S4d) and Gigasporaceae (Fig. S4e), two to the Ambisporaceae (Fig. S4b),
246 and one to the Paraglomeraceae. The final data set encompassed 41,526 sequences longer than
247 380 bp. Species accumulation curves showed that sampling of AMF taxa was relatively
248 complete in more than 70% of investigated plant individuals from each site (Fig. S5).
249 AMF richness varied from four to 26 taxa per individual host plant, with average of
250 15 MOTUs. The best model (AICc = 381.96) selected five main explanatory variables (pH, K,
251 Fe and Cr concentrations, and PC1) for the AMF richness (Fig. 1b). However, the effect of
252 Fe concentration itself was non-significant ($p = 0.086$), contrary to other variables. Both Cr
253 (Fig. 3a; $R^2 = 0.259$; $p < 0.001$) and K (Fig. 3b; $R^2 = 0.128$; $p = 0.001$) concentrations showed
254 negative correlations with AMF richness. On the contrary, AMF richness increased linearly
255 along the pH gradient (Fig. 3c; $R^2 = 0.295$; $p < 0.001$). In addition, AMF richness was

256 significantly influenced by neighbouring plant communities (PC1; $R^2 = 0.212$; $p < 0.001$; also
257 supported by the results of the Moran's I test: Moran's I = 0.203; $p < 0.001$). Four plant species
258 were identified as having the greatest effect on AMF richness: *Veronica chamaedrys* (n = 19;
259 p = 0.001) and *Hypericum maculatum* (n = 12; p = 0.023) were associated with rich AMF
260 communities whereas the opposite was true for *Avenella flexuosa* (n = 53; p = 0.006) and
261 *Galium saxatile* (n = 13; p = 0.012). Although we detected significant relationships between
262 neighbouring plant communities and AMF richness, no such correlation existed between AMF
263 richness and neighbouring plant species richness.

264

265 *Composition of AMF communities*

266 The structure of AMF communities significantly correlated with environmental factors (Mantel-
267 r = 0.278, $p < 0.001$), geographic position (Mantel-r = 0.175, $p < 0.001$), and marginally
268 significantly also with neighbouring plant communities (Mantel-r = 0.08, p = 0.056).

269 Step-wise selection of predictors identified locality, pH, plant life stage, soil type, and
270 concentration of Ni as the best explanatory factors affecting the composition of AMF communities
271 (Fig. 1c). All variables were significant and collectively explained nearly 40% of the total
272 variance (Table 1). For all selected factors but locality, we identified MOTUs responsible for
273 the observed pattern (Table 2). Most of these MOTUs did not cluster with any named AMF species.
274 Exceptions were GLOM11 (n = 11), clustering with sequences of *Glomus macrocarpum,* that
275 showed pH optimum around 5.6 ($p < 0.01$) and preferred NS sites with lower Ni concentration
276 ($p < 0.05$ in both cases), and GLOM52 (n = 65), exhibiting close affinity to *Rhizophagus*
277 *irregularis*, that was more often recorded in the roots of plants in blossom ($p < 0.01$). Most
278 MOTUs in which ecological sorting along the studied parameters was observed belonged
279 to abundant taxa, although the occurrence of the most abundant one (GLOM9 detected in 18
280 samples) was not affected by any of the environmental variables.

281

282 **Discussion**

283 In recent years, 454-sequencing enabled us to delve deeper into the structuring of natural AMF
284 communities (e.g. Öpik et al., 2009; Dumbrell et al., 2011; Lekberg et al., 2012; Hiiesalu et al.,
285 2014). However, the resulting picture may be biased by the choice of a target rDNA region
286 as well as by differences between individual AMF-specific primers in terms of their specificity
287 and discrimination of certain AMF lineages (Gamper et al., 2009; Kõljalg et al., 2013; Kohout

288 et al., 2014). To overcome these problems, Krüger et al. (2009) designed a combination of
289 AMF-specific primers which does not discriminate any known AMF lineages and has a high
290 species resolution power (Stockinger et al., 2010). To our knowledge, the present study is the first
291 to adopt the Krüger's et al. (2009) primers for 454-based investigation of AMF communities,
292 in combination with amplification and pyrosequencing of the ITS region of rDNA.

293 In total, we detected 81 MOTUs belonging to the Glomeromycota, which is one of the highest
294 AMF richness revealed at a local scale by 454-sequencing (for comparison, see Öpik et al., 2009;
295 Davison et al., 2012; Lekberg et al., 2012 and 2013; Hiiesalu et al., 2014; Saks et al., 2014). The
296 main differences between the present and the above-cited studies are in the target rDNA region
297 and in the sequence clustering approach/algorithm. Despite some disadvantages of the ITS region,
298 including a high divergence between different AMF families and the lack of a universal barcoding
299 threshold for the Glomeromycota (which, however, applies also to other rDNA regions), our
300 clustering approach followed by phylogenetic analyses using the Glomeromycota reference
301 dataset (Krüger et al., 2012) together with our previous work (Kohout et al., 2014) demonstrated
302 the value of the ITS region for 454-sequencing analysis of AMF communities.

303 Although the adjacent serpentine and non-serpentine habitats represent relatively sharp
304 environmental gradient, our findings suggest that the community assemblage of AMF were also
305 partly driven by neutral processes (Caruso et al., 2012a). Other studies focused on AMF
306 communities on serpentine soils on the other hand did not show any importance of neutral
307 processes (Schechter and Bruns, 2008, 2012). In general, studies focused on the effects of
308 deterministic and neutral processes on the structuring of AMF communities showed either
309 significance of both of them (Lekberg et al., 2007; Dumbrell et al., 2010; van der Gast et al., 2011)
310 or dominance of deterministic processes only (Lekberg et al., 2011; Hazard et al., 2013). It is
311 highly questionable if there is some common pattern in the effects of deterministic and stochastic
312 processes on AMF communities as has been shown on different scales for soil bacteria (Martiny
313 et al., 2011) or microbial communities in general (Hanson et al., 2012). Importantly, studies
314 showing the effect of stochastic processes might have overlooked important environmental
315 variables possibly responsible for the observed community changes.

316 Although geographical distance contributed to structuring of AMF communities in our study,
317 environmental factors were far more important. Interestingly, environmental factors responsible
318 for changes in mycorrhizal root colonisation, AMF richness and community composition
319 differed considerably. There were only two factors with multiple effects: soil pH governed both

AMF richness and community composition, while soil type (S *vs* NS) influenced mycorrhizal root colonisation and AMF community composition. The importance of soil pH for shaping AMF communities has been reported in many studies (e.g. Fitzsimons et al., 2008; Helgason and Fitter, 2009; Dumbrell et al., 2010). The pH effect on AMF communities might dominate over significant environmental gradients like in geothermal soils (Lekberg et al., 2011) and serpentine habitats (our study). The observed ecological sorting of some AMF taxa according to soil acidity is consistent with published reports of species-specific performance of AMF along the pH gradient (Vosátka et al., 1999; van Aarle et al., 2002). Our data also support previously stated preferences of species from the Acaulosporaceae family for habitats with lower pH (Oehl et al., 2010). On the other hand, species belonging to the Glomeraceae thrived under a relatively wide range of pH values in our study. In addition to species composition, pH is also known to influence the total richness of AMF (Toljander et al., 2008). We documented positive linear correlation between AMF richness and soil pH along a gradient from 3.5 to 5.8 in temperate grassland. The linear shape of the correlation suggests that pH values around or slightly above 6 promote AMF richness in the studied ecosystem.

Contrary to pH, AMF richness decreased with increasing concentrations of soil potassium and chromium. Our observations are among the first to display negative effects of these elements on AMF diversity (Khan, 2001). However, because neither potassium nor chromium triggered changes in AMF community composition, we can hypothesize that the decrease in AMF diversity may at least partly be related to negative effects of these elements on plant fitness, leading to a lower ability of host plant to support more AMF species. Chromium as a heavy metal has a direct detrimental impact on plant physiology (Babula et al., 2008; Hayat et al., 2012). Explanation of the negative effect of potassium is more challenging, however, considering high soil K concentrations at *K. arvensis* sites, plants might have suffered from imbalance in cation uptake caused by the excess of K^+ at the expense of other cations such as Ca^{2+} (Troyanos et al., 2000; Egilla et al., 2001; Marschner, 2002).

Lower mycorrhizal root colonisation observed in neighbouring serpentine *vs* non-serpentine sites inhabited by *K. arvensis* corresponds to the results of our previous study conducted on the same host plant species on a larger spatial scale (Doubková et al., 2011). Mycorrhizal root colonisation also decreased with increasing Fe availability, which may possibly stem from oxidative stress induced by high Fe concentrations, given its involvement in redox reactions (Meharg, 2003; Smith and Read, 2008). Another difference between closely-spaced serpentine

352 and non-serpentine sites in the Slavkovský les Mts concerned the composition of AMF
353 communities. The same conclusion was drawn by Schechter and Bruns (2008, 2012) who
354 argued that soil chemistry was the major factor shaping AMF communities in Californian
355 serpentines. On the contrary, Fitzsimons and Miller (2010) did not find any effect of serpentine
356 habitats on AMF communities, possibly due to different size and connectivity of serpentine
357 areas studied, in line with the theory of island biogeography (MacArthur and Wilson, 1967).
358 It has been suggested that smaller and more spatially isolated serpentine patches in Portugal
359 (Fitzsimons and Miller, 2010) are less likely to support unique AMF communities or endemic
360 plant species, compared to larger serpentine islands in California (Schechter and Bruns, 2008).
361 However, our data did not favour this explanation because serpentine sites in the Slavkovský les
362 Mts (and in Central Europe in general) are small and highly fragmented. Among the studied soil
363 parameters, Ni concentration had the major effect on the composition of AMF communities,
364 which is in agreement with the reported differences in Ni tolerance among different AMF
365 species/isolates in terms of spore germination and mycorrhizal root colonisation (Amir et al.,
366 2008, 2013; Doubková and Sudová, 2014).

367 As obligate plant symbionts, AMF communities largely depend on communities of their
368 host plants. However, there is no consistent pattern between diversities of plants and AMF, as
369 both positive (van der Heijden et al., 1998; Meadow and Zabinski, 2012) and negative (Johnson
370 et al., 2004) relationships were observed. In our study, an assessment of vegetation immediately
371 accompanying the sampled *K. arvensis* plants (based on the presence of above-ground biomass)
372 revealed no correlation between diversities of AMF and plants. However, we cannot exclude the
373 existence of such a relationship belowground because recent findings suggested much stronger
374 link between AMF diversity and belowground plant diversity, given generally higher plant
375 richness in soil due to the presence of dormant species, clonal plants with extensive rhizome
376 networks and/or ephemerals (Hiiesalu et al., 2014). Despite the lack of correlation between
377 plant-AMF diversities in our study, some plant species seemed to influence (either positively
378 or negatively) the diversity of AMF. Although this observation can hardly be taken as causal
379 evidence, such plants may possibly serve as indicators of AMF diversity in temperate grasslands.
380 Similar relationships between the occurrence of particular host plant species and AMF diversity
381 have previously been reported by König et al. (2010) for managed plots of lowland grassland.

382 Besides neighbouring plant communities, life stage of a host plant (vegetative *vs* flowering)
383 has also been reported to significantly influence the composition of soil microbial communities

384 (Inceoglu et al., 2011; van Overbeek and van Elsas, 2008). The effect of plant growth cycle
385 on AMF communities was recently observed in *Pisum sativum* – roots of juvenile plants were
386 colonized by communities dominated by *Funneliformis mosseae* (formerly *Glomus mosseae*),
387 while *Paraglomus* sp. increased its abundance in roots of senescent individuals (Yu et al., 2012).
388 However, the cited study was conducted in pots where the role of life stage of the host plant in
389 shaping AMF communities might have been confounded by other factors such as disturbance
390 (Sýkorová et al., 2007a). We found that AMF taxa clustering in phylogenetic analyses with
391 *Rhizophagus irregularis* (i.e., a widespread species with a broad ecological niche) were
392 significantly more frequent in fungal communities associated with plants at flowering (compared
393 to the vegetative) stage. As nutritional requirements may increase during the flowering due
394 to (re)allocation especially of P to generative organs (e.g., Bucciarelli et al., 2006; Peng and Li,
395 2005), AMF species with higher efficiency in P uptake as *Rhizophagus irregularis* (Jansa et al.,
396 2005; Lendenmann et al., 2011) might be favoured at this stage.

397 To conclude, our study addressing the role of stochastic and deterministic processes in
398 structuring AMF communities on a local scale supported the dominance of the latter factors in
399 a heterogeneous environment involving different soil types. In addition to correlations between
400 selected environmental variables and AMF richness, we also revealed niche differentiation among
401 AMF MOTUs along gradients of pH and Ni concentration. Our findings also contributed to the
402 ongoing debate about relationships between plant and AMF diversities, and provided further
403 insight into the effects of plant life stage on natural AMF communities. Last but not least, our
404 data suggested an important role of serpentine conditions in structuring AMF communities.

405

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Tables and Figures

Table 1. Results of Adonis analysis of putative factors affecting communities of arbuscular mycorrhizal fungi (AMF) in the roots of *K. arvensis*

	F model	R^2	p-value
Site	11.06	0.213	0.001
Soil pH	7.50	0.072	0.001
Host plant life stage (flowering *vs* vegetative)	3.64	0.034	0.001
Soil type (serpentine *vs* non-serpentine)	2.69	0.026	0.006
Soil Ni concentration	4.77	0.046	0.001
Residuals		0.607	

Table 2. Relationships between environmental factors (soil pH_{H20}, soil Ni concentration, soil type, host plant life stage) and the occurrence of particular AMF molecular taxonomic units (MOTUs) in the roots of *K. arvensis* as based on indicator species analyses. For pH, approximate optimum values are given for each MOTU, while decrease/increase in their abundance in response to increasing Ni concentration is indicated by arrows. Preferential associations of MOTUs with a particular life stage (F – flowering, V – vegetative) of the host plant and soil type (S – serpentine, NS – non-serpentine) are also indicated.

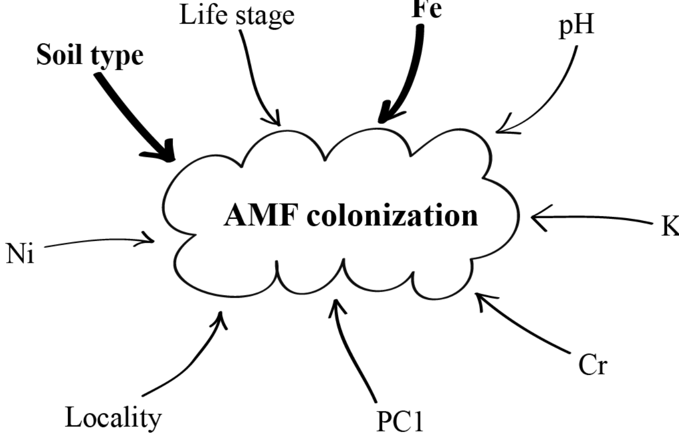
		n[1]	pH_{H20}	Ni conc.	Life stage	Soil type
Acaulospora	**MOTU4**	11	< 4.58 **	↓ **		NS **
	MOTU7	21			V *	
	MOTU8	22		↓ **		NS **
Claroideoglomus	**MOTU4**	10		↓ *		NS **
Glomus	**MOTU2**	40	> 6.41 *	↑ **		S **
	MOTU6	10	> 6.41 *	↑ **		S **
	MOTU8	10	~ 5.3 *	↑ ***		S ***
	MOTU11	11	~ 5.6 **	↑ *		S *
	MOTU16	63		↓ *		NS *
	MOTU17	27	< 4.58 *		V *	
	MOTU33	42	> 6.41 ***	↑ *	F *	S *
	MOTU36	29	> 6.41 ***			
	MOTU39	30	~ 5.7 ***			
	MOTU40	24	~ 4.8 ***	↓ ***		NS ***
	MOTU44	58	< 4.58 *		V **	
	MOTU45	18	< 4.58 ***	↓ ***		NS ***
	MOTU49	43			F **	
	MOTU50	39			F *	
	MOTU52	65			F **	

[1] number of plants showing occurrence (max. no. is 70)

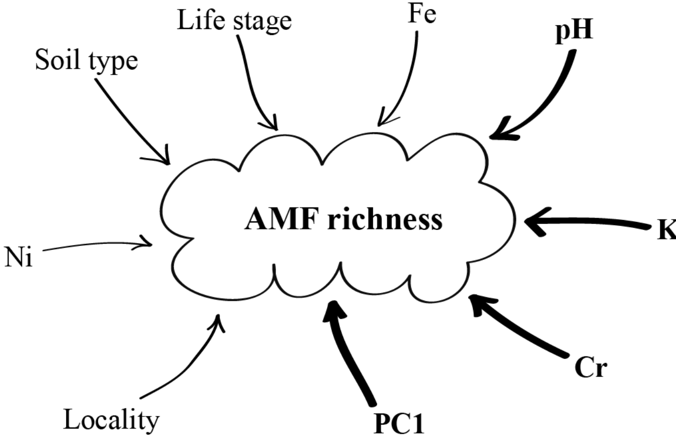
Significance levels: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Fig. 1 Schematic representation of the effects of different environmental factors on (a) AMF colonisation, (b) AMF richness, and (c) composition of AMF communities (factors with significant effects in bold). Life stage (flowering *vs* vegetative), Soil type (serpentine *vs* non-serpentine), PC1 – the first axis from the principal component analysis of relationships among plant communities (for further details, see Results)

a)



b)



c)

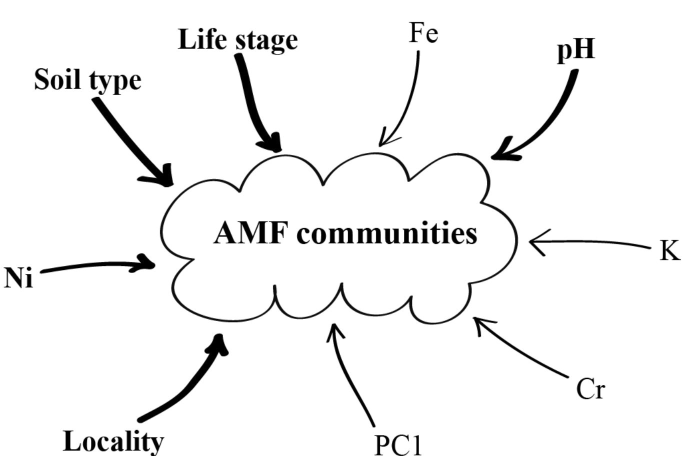


Fig. 2 Phylogenetic tree of the family Glomeraceae, based on a neighbour-joining analysis of partial SSU, ITS and partial LSU regions of rDNA. The numbers above or below branches denote neighbour-joining bootstrap values from 1,000 replications. Consensus sequences obtained in this study are highlighted in bold.

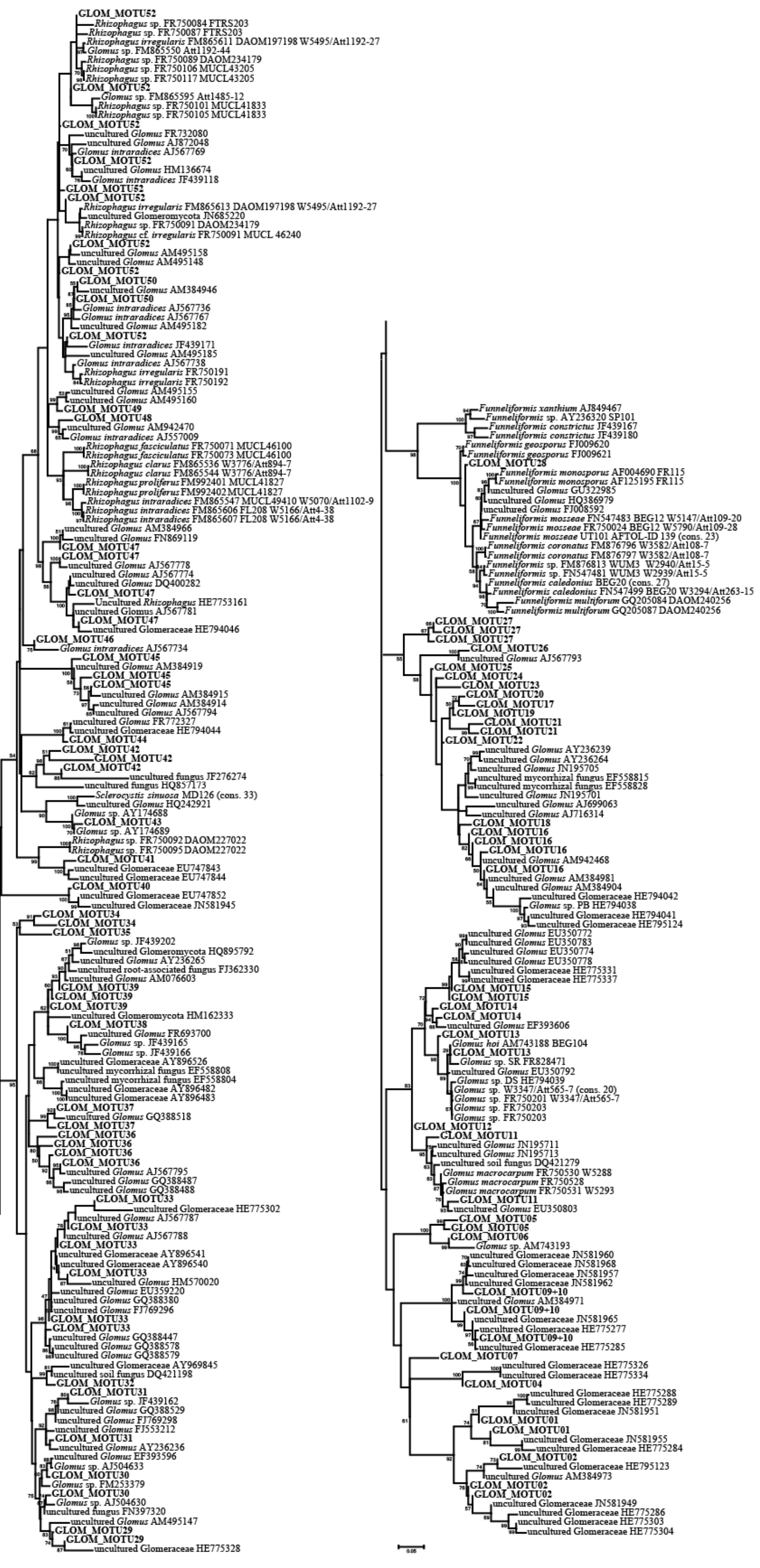
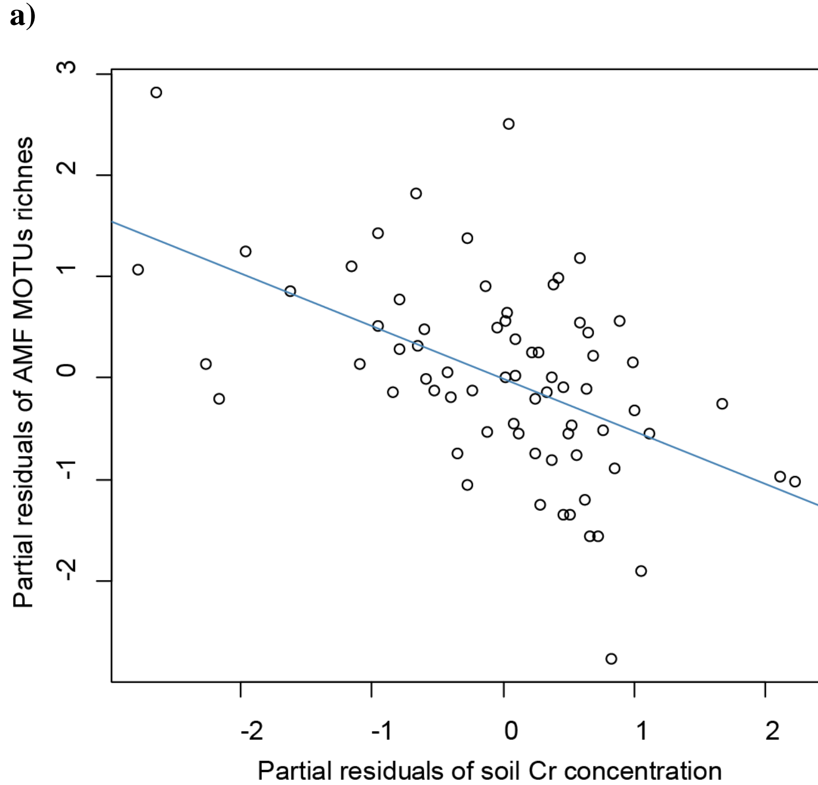
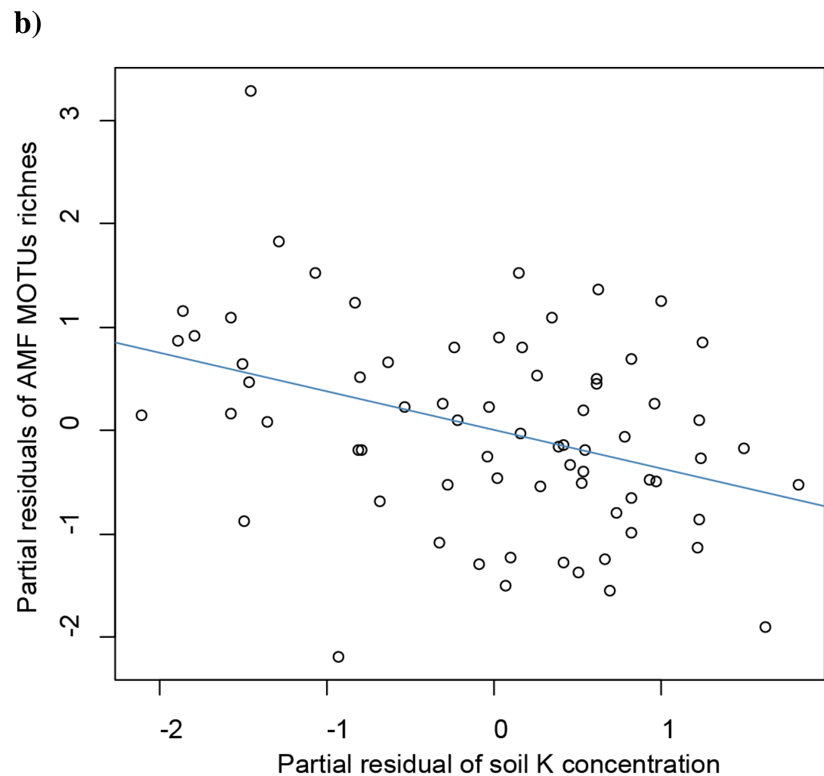


Fig. 3 A plot of the residuals of AMF MOTUs richness against soil (a) Cr concentration (R^2 = 0.259; $p < 0.001$), (b) K concentration (R^2 = 0.128; p = 0.001), and (c) pH value (R^2 = 0.295; $p < 0.001$). Significant environmental factors were identified according to the AIC method. The line represents the best fitting linear regression model.

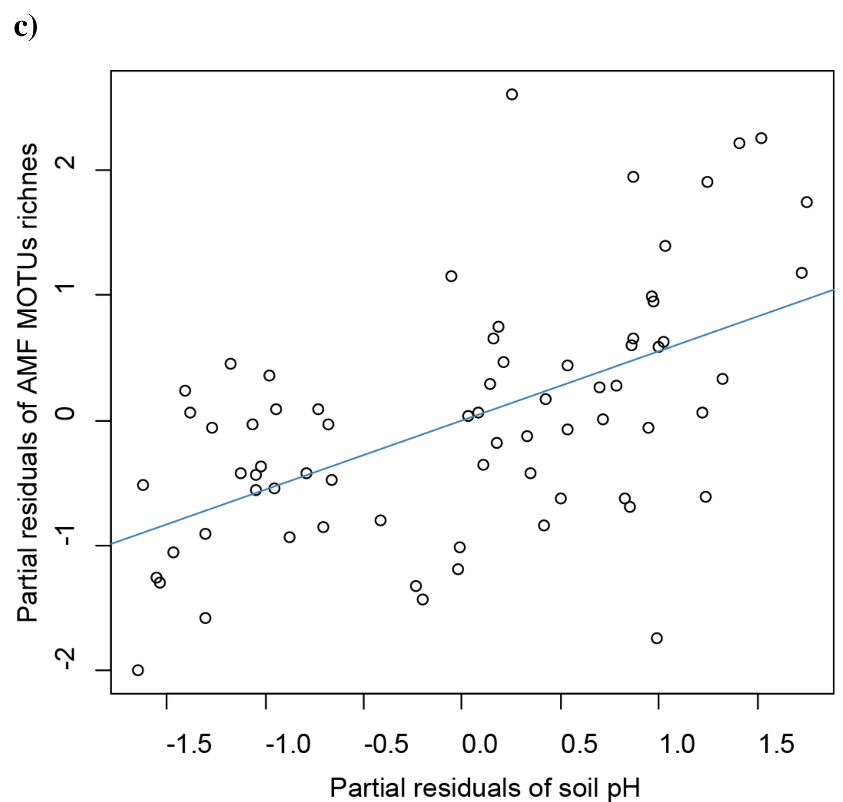
a)



b)



c)



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Supplementary material

Fig. S1 Schematic map of the sampled sites of *Knautia arvensis* in the Slavkovský les Mts.

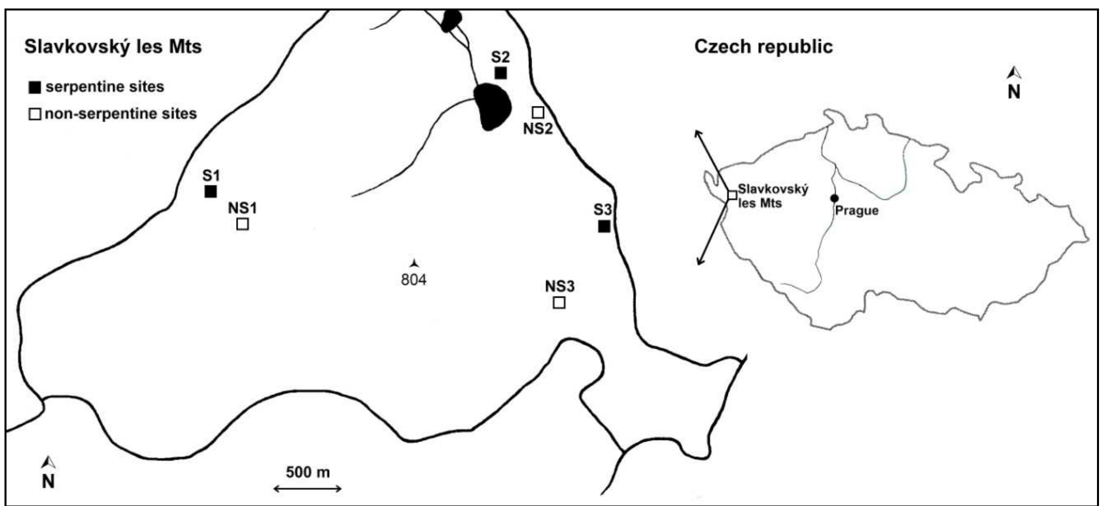


Fig. S2 Correlation between observed AMF MOTUs richness and residuals of linear model of MOTUs richness and sequence numbers per sample

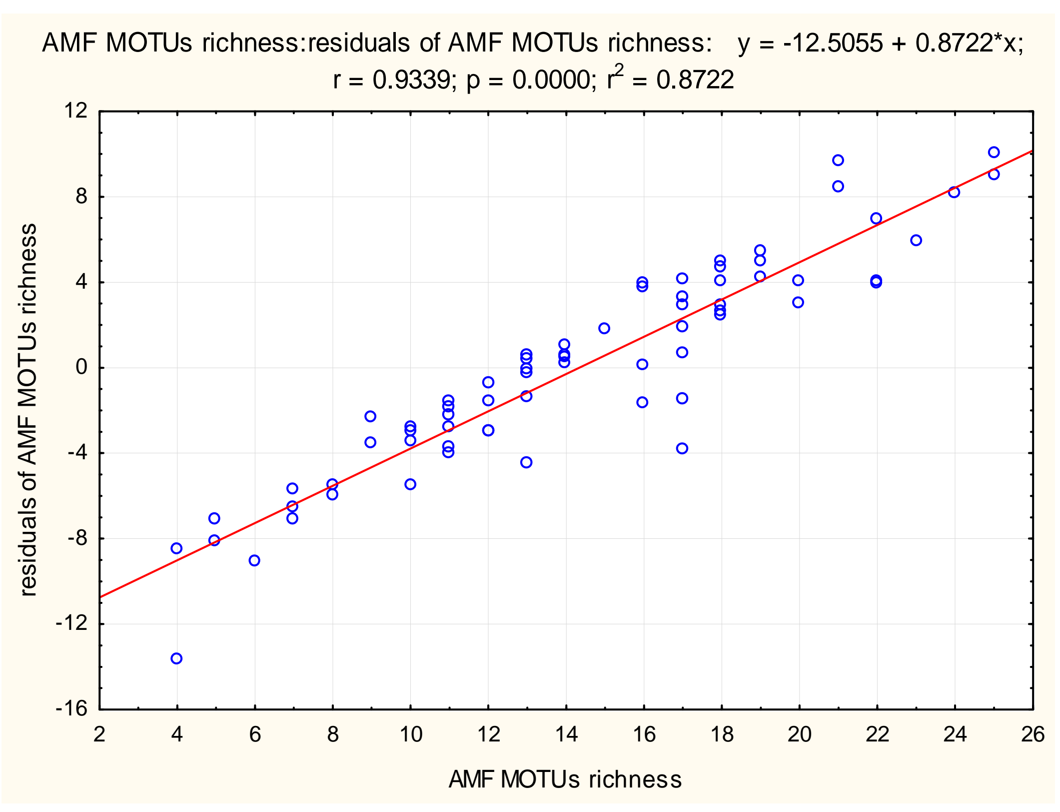
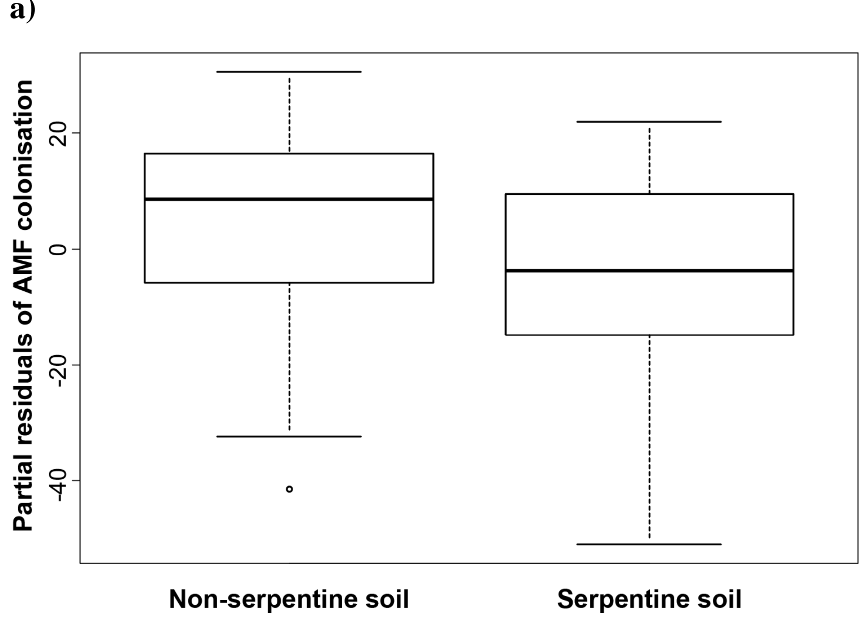


Fig. S3 The significant effects of (a) soil type (R^2 = 0.068; p = 0.02) and (b) soil Fe concentration (R^2 = 0.094; p = 0.007) on residuals of AMF colonisation model according to the AIC method.

a)



b)

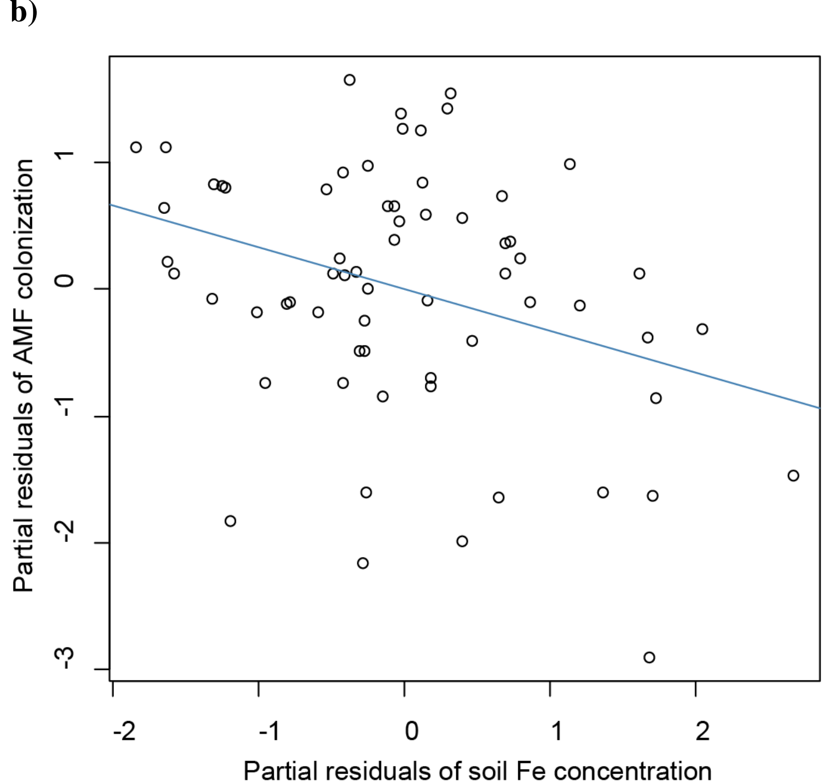
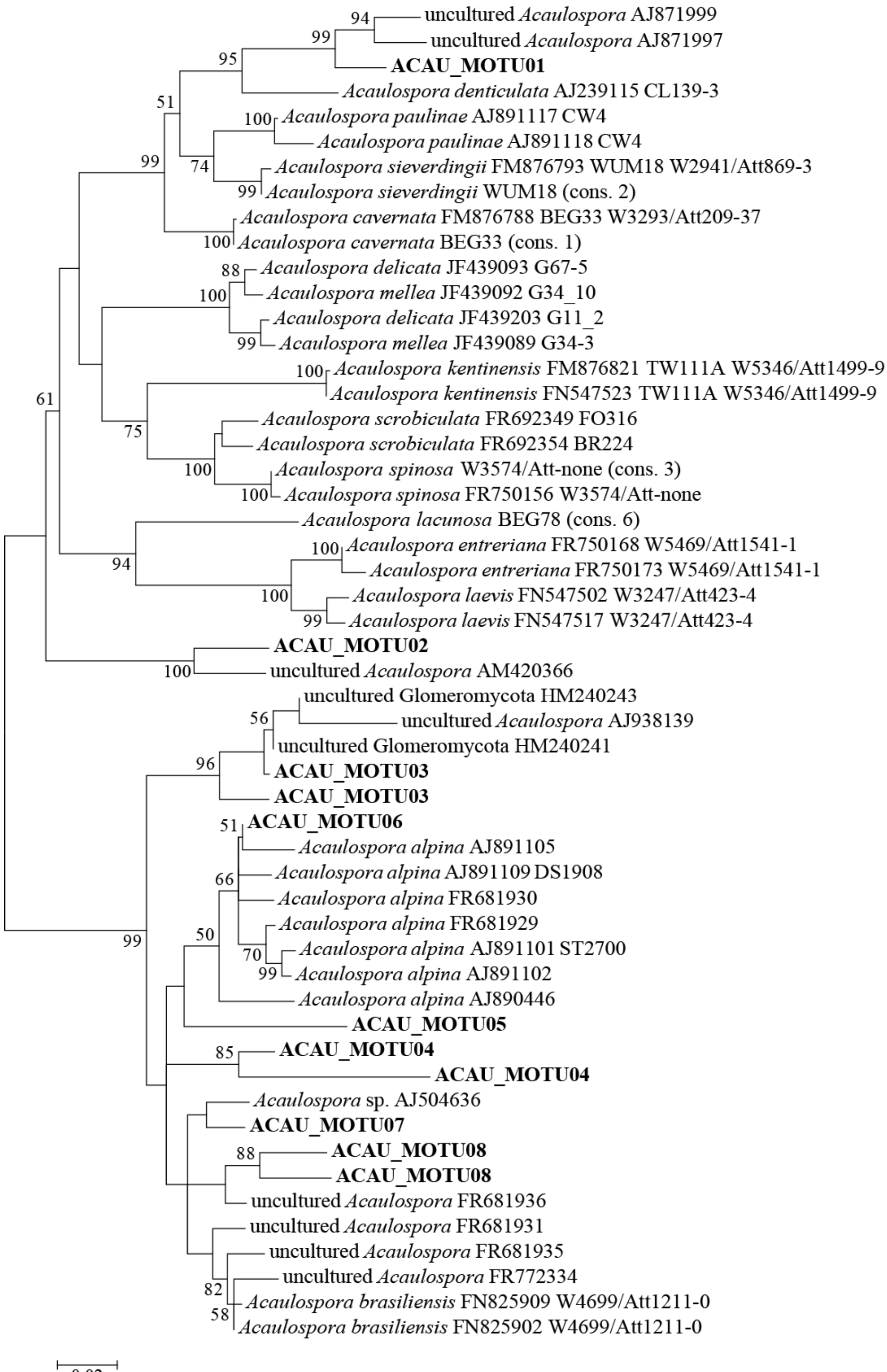
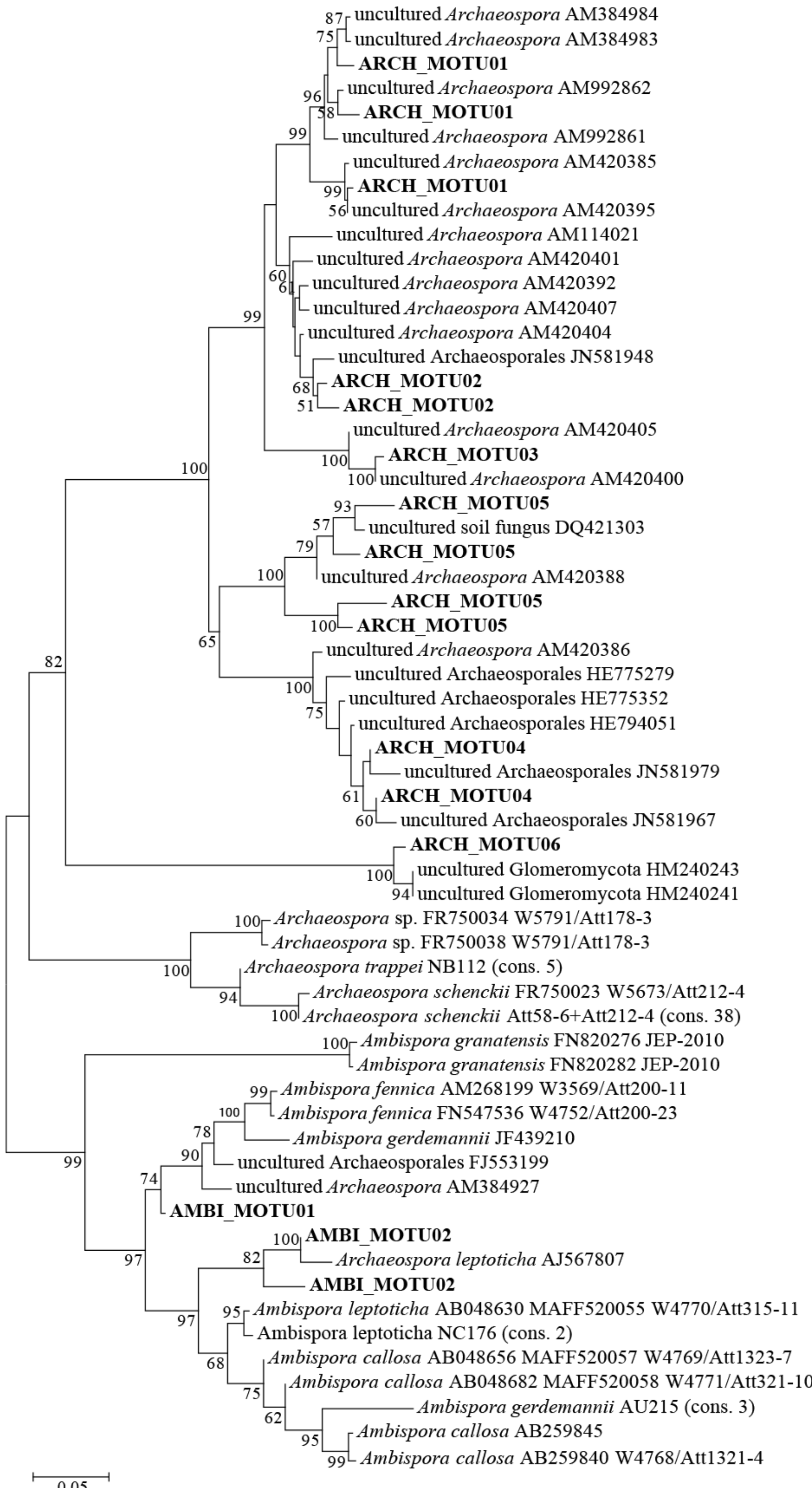


Fig. S4 Phylogenetic trees of the (a) Acaulosporaceae, (b) Archaeosporales, (c) Claroideoglomeraceae, (d) Diversisporaceae, and (e) Gigasporaceae based on a neighbour-joining analysis of partial SSU, ITS and partial LSU regions of rDNA. The numbers above or below branches denote neighbour-joining bootstrap values from 1,000 replications. Consensus sequences obtained in this study are highlighted in bold.

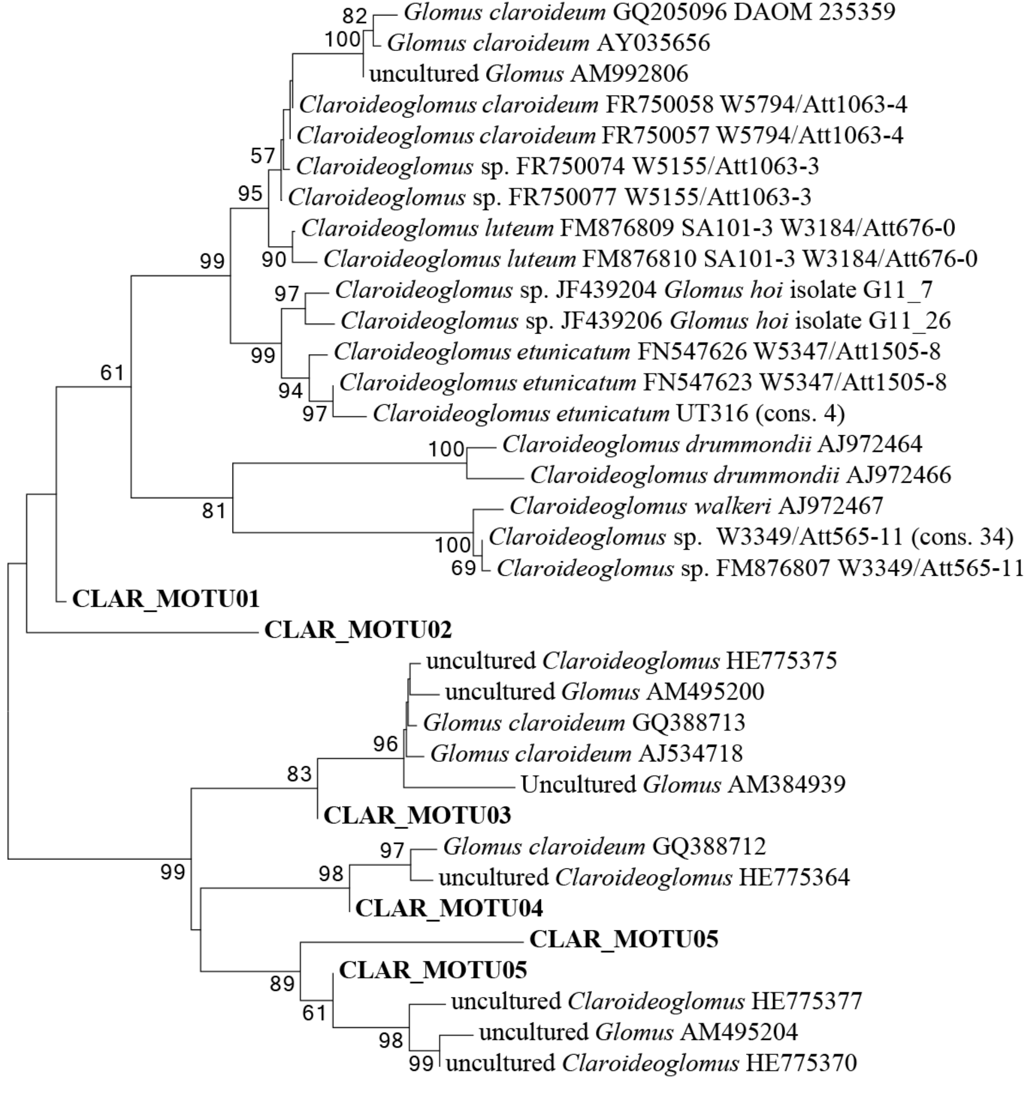
a) Acaulosporaceae



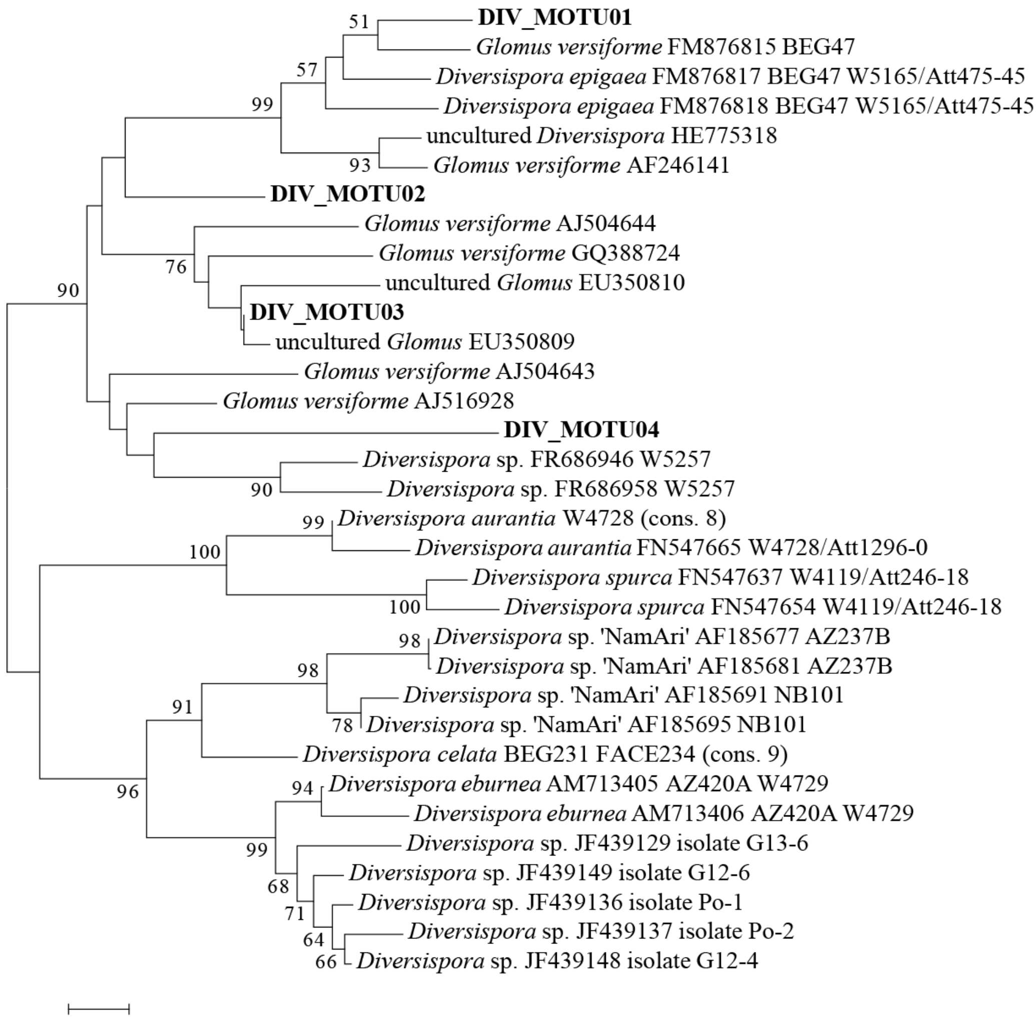
b) Archaeosporales



c) Claroideoglomeraceae



d) Diversisporaceae



e) Gigasporaceae

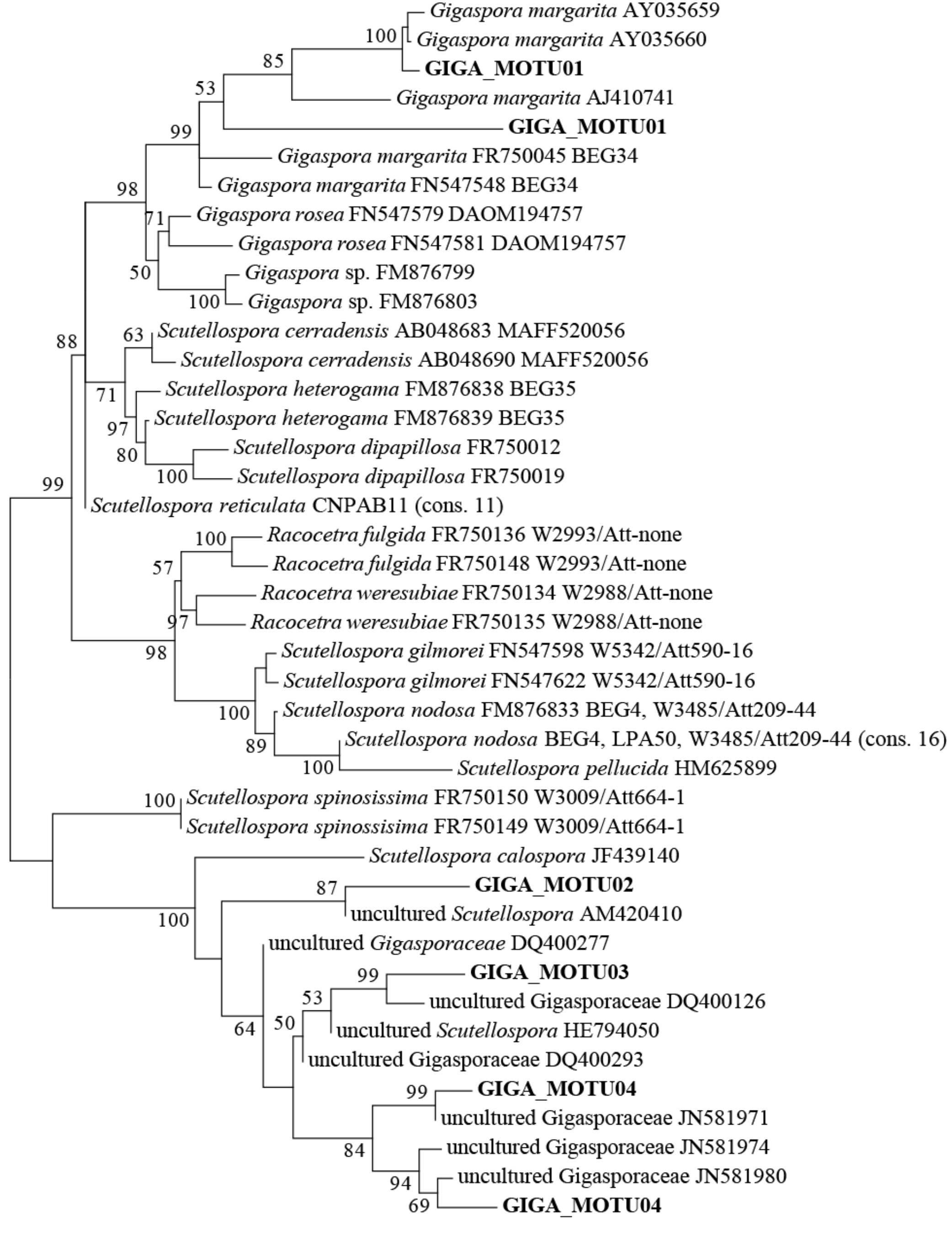


Fig. S5 Species accumulation curves calculated for each sampled plant at the individual sampling sites

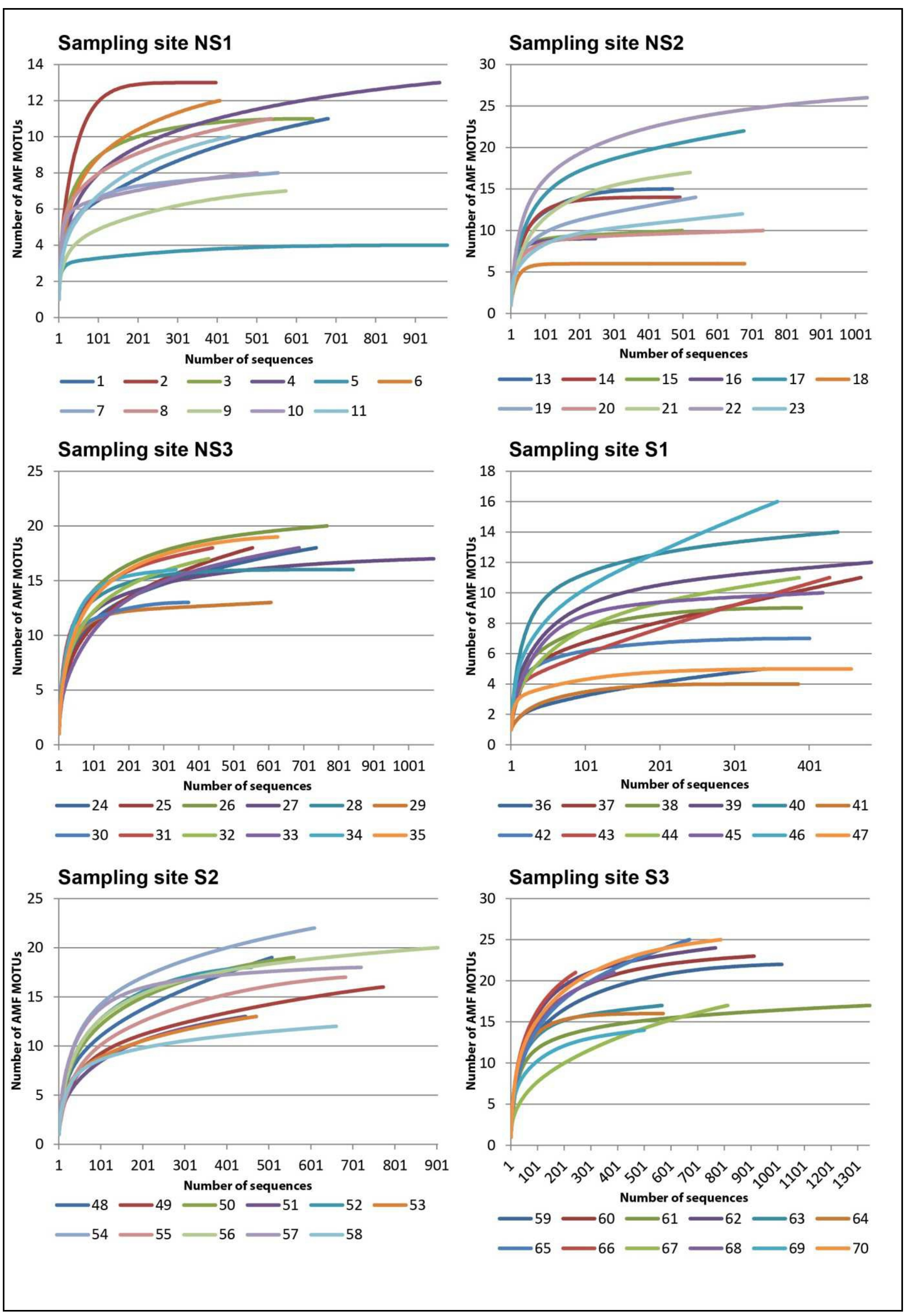


Table S1. Chemical characteristics of *K. arvensis* rhizosphere soil. The data represent means of 11 or 12 replicates (SE values given in brackets).

Soil type	Site	pH_{H2O}	CEC ($mmol_c kg^{-1}$)	N [a]	C_{org} [a]	C_{carb} [a]	P [b]	Ca [c]	Mg [c]	K [c]	Fe [d]	Mn [d]	Ni [d]	Cr [d]	Co [d]	Ca:Mg
				(%)			(mg kg^{-1})									
Non-serpentine	NS1	4.98 (0.05)	68.6 (3.8)	0.38 (0.02)	3.69 (0.11)	1.23 (0.10)	17.3 (1.2)	946 (54)	149 (13)	316 (29)	144 (10)	27.9 (1.2)	0.65 (0.06)	23.36 (12.28)	0.50 (0.04)	6.62 (0.32)
	NS2	4.81 (0.06)	57.0 (1.5)	0.23 (0.01)	3.20 (0.19)	0.68 (0.11)	21.3 (2.8)	407 (20)	403 (8)	90 (7)	320 (22)	18.4 (4.8)	6.55 (0.22)	0.13 (0.01)	1.41 (0.23)	1.01 (0.05)
	NS3	5.62 (0.05)	110.5 (9.7)	0.29 (0.02)	3.01 (0.21)	1.06 (0.14)	20.5 (0.9)	1443 (108)	274 (44)	593 (64)	80 (10)	47.6 (2.4)	1.72 (0.10)	46.63 (14.53)	0.22 (0.03)	6.23 (0.60)
Serpentine	S1	5.64 (0.15)	302.4 (33.9)	1.00 (0.10)	11.63 (1.36)	2.93 (1.08)	58.4 (7.9)	832 (145)	3038 (354)	339 (41)	399 (32)	55.8 (7.1)	48.77 (7.76)	0.89 (0.07)	3.16 (0.42)	0.28 (0.04)
	S2	5.42 (0.07)	220.3 (22.5)	0.61 (0.06)	7.06 (1.13)	0.69 (0.13)	38.9 (5.5)	734 (147)	2123 (197)	304 (46)	373 (16)	30.9 (3.4)	63.94 (12.76)	8.53 (8.41)	2.07 (0.27)	0.33 (0.04)
	S3	5.59 (0.04)	122.9 (6.9)	0.29 (0.01)	3.35 (0.21)	0.75 (0.13)	14.0 (1.0)	525 (56)	1152 (62)	56 (3)	211 (14)	67.6 (4.7)	43.16 (1.81)	8.00 (7.75)	2.12 (0.25)	0.46 (0.04)

[a] combustion method (CHN Carlo Erba NC2500 analyser, Italy)

[b] 0.5M sodium bicarbonate-extractable (Unicam UV4-100, UK)

[c] 1M ammonium acetate-extractable, pH 7.0 (ContrAA 700, Germany)

[d] 0.005M DTPA-0.1M triethanolamine-0.01M $CaCl_2$-extractable (ContrAA 700, Germany)

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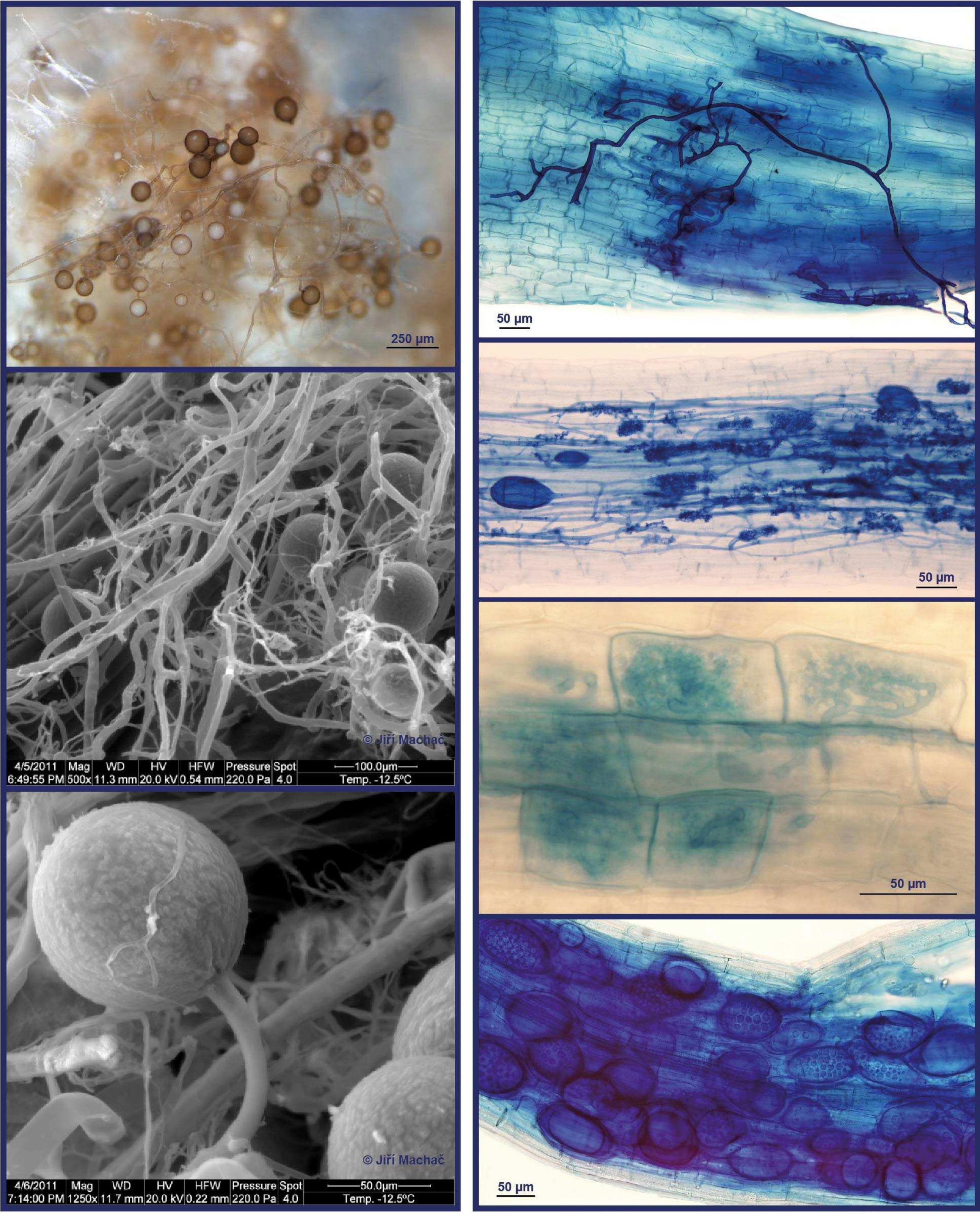
7. Supplementary

I. Serpentine sites and habitus of the model plant species *Knautia arvensis* (for more information on the sites, see section 2.3. and MS1)

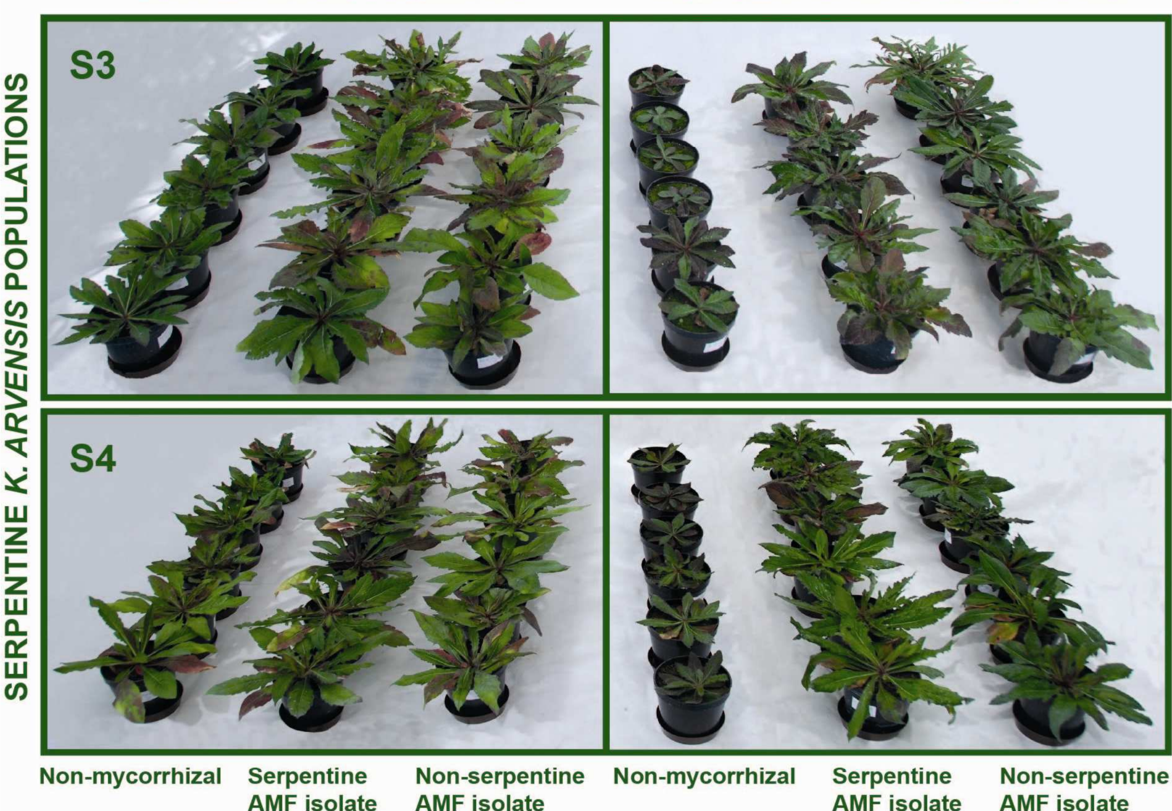
II. *Glomus* sp. SR, isolate of arbuscular mycorrhizal fungus originating from the serpentine *Knautia arvensis* population S2 (for more details, see MS2)

Extraradical fungal hyphae with spores

Mycorrhizal colonisation in roots of *K. arvensis* plants



III. The reciprocal transplant experiment: serpentine vs. non-serpentine origin of substrate, AMF and host plant (for more details, see MS2); only selected populations are presented.



IV. **Effect of water regime (% field capacity, FC) and AMF inoculation on serpentine *Knautia arvensis* plants (for more details, see MS3); only selected inoculation treatments are presented.**

NON-MYCORRHIZAL TREATMENT | **SERPENTINE AMF ISOLATE**

55 % FC

45 % FC

35 % FC

25 % FC