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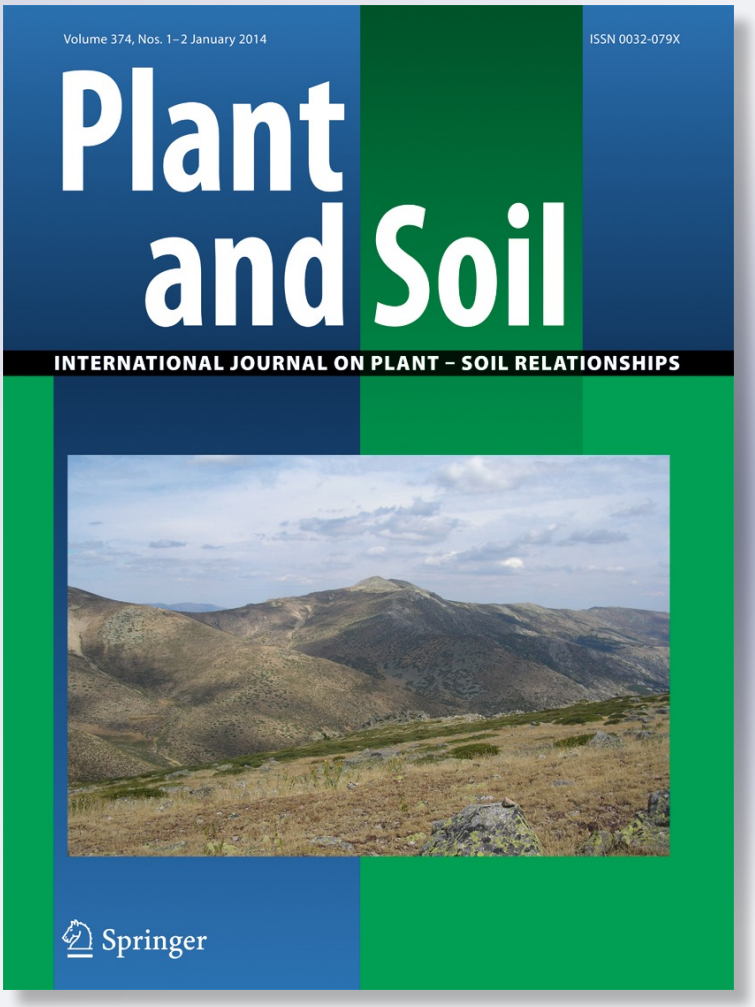

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REGULAR ARTICLE

Serpentine ecotypic differentiation in a polyploid plant complex: shared tolerance to Mg and Ni stress among di- and tetraploid serpentine populations of *Knautia arvensis* (Dipsacaceae)

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Abstract
Background and aims Serpentine soils impose limits on plant growth and survival and thus provide an ideal model for studying plant adaptation under environmental stress. Despite the increasing amount of data on serpentine ecotypic differentiation, no study has assessed the potential role of polyploidy. We tested for links between polyploidy and the response to serpentine stress in *Knautia arvensis*, a diploid-tetraploid, edaphically differentiated complex.
Methods Variation in growth, biomass yield and tissue Mg and Ni accumulation in response to high Mg and Ni concentrations were experimentally tested using hydroponic cultivation of seedlings from eight populations of different ploidy and edaphic origin.
Results Regardless of ploidy level, serpentine populations exhibited higher tolerance to both Mg and Ni stress than their non-serpentine counterparts, suggesting an adaptive character of these traits in *K. arvensis*. The effect of ploidy was rather weak and confined to a slightly better response of serpentine tetraploids to Mg stress and to higher biomass yields in tetraploids from both soil types.
Conclusions The similar response of diploid and tetraploid serpentine populations to edaphic stress corresponded with their previously described genetic proximity. This suggests that serpentine tolerance might have been transmitted during the local autopolyploid origin of serpentine tetraploids.

Keywords Adaptation · Ca/Mg ratio · Metal tolerance · Nickel · Ploidy level · Serpentine

Abbreviations

AFLP	Amplified fragment length polymorphism
Ca	Calcium

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ICP OES	Inductively coupled plasma optical emission spectrometry
Mg	Magnesium
Ni	Nickel

Introduction

Serpentine soils inflict harsh constraints on plant growth because they are characterized by a low Ca/Mg ratio, increased (even toxic) concentrations of heavy metals (especially Ni, Cr, and Co), deficiency of essential macronutrients and a low water-holding capacity (Proctor and Woodell 1975; Kazakou et al. 2008; Harrison and Rajakaruna 2011). Serpentine plants provide excellent systems for the study of adaptive evolution in plants thanks to facts that the physiological response is well described, that major stressing factors are known and amenable to manipulative experiments and that recurrent evolution of adaptive strategies occurs within single species (Brady et al. 2005). Of the limiting factors imposed by serpentine substrates, low Ca/Mg ratio and high Ni content have gained the most attention because they are considered to be the key factors affecting plant growth and survival in serpentine outcrops worldwide (Proctor 1971; Gabbrielli and Pandolfini 1984; Ghasemi and Ghaderian 2009; O' Dell and Rajakaruna 2011). Elevated levels of heavy metals in soils can affect plants through direct toxicity (resulting in stunting and chlorosis), antagonism with other nutrients, and inhibition of root penetration and growth (Antonovics et al. 1971). High Mg content induces Ca deficiency, leading to cell wall disintegration and localized tissue necrosis (O' Dell and Claassen 2006; O' Dell and Rajakaruna 2011).

Plant species differ in their abilities to evolve tolerance against serpentine stress depending on their genetic resources. They can tolerate edaphic challenges either by a constitutive trait present in all members of a species (that grows both on and off serpentine soils) or by an adaptive mechanism present only in tolerant ecotypes (Kazakou et al. 2008). Available case studies provide ambiguous results in this respect since both adaptive and constitutive patterns of tolerance to low Ca/Mg and/or high Ni have been proven experimentally, depending on the model system studied (e.g., Westerbergh 1994; Boyd and Martens 1998; Nyberg Berglund et al. 2004; Ghasemi and Ghaderian 2009). Despite the growing amount of experimental data, evolutionary mechanisms involved in the adaptive process as well as essential prerequisites facilitating colonization of serpentine sites still remain unclear.

Polyploidy, or whole genome duplication, is widely acknowledged as a leading force in plant evolution (Soltis et al. 2009; Otto and Whitton 2000). The presence of several gene copies within a polyploid genome is considered a possible key factor that widens the ecological niche of a polyploid and enhances its expansion to new environments (Ramsey and Schemske 2002; Ramsey et al. 2008; Parisod et al. 2010). On the other hand, it could be the diploid cytotype which survives in extreme environments such as rocky outcrops, xeric habitats or serpentines, where it finds refugia of suitable conditions (e.g., reduced plant competition) in a dramatically changing landscape (Ehrendorfer 1980; Kolář et al. 2012). Despite the widely-known consequences of polyploidy for various ecological and life-history traits, no study has tested specifically for the associations between polyploidy and serpentine tolerance. Correct evolutionary interpretations of ecological patterns, however, require good knowledge of genetic relationships among ecotypes and cytotypes. Inter-cytotype differences in ecological traits may not only be an effect of polyploidization per se and/or subsequent selection but can also simply reflect different evolutionary histories of the cytotypes under investigation (Ramsey and Schemske 1998; Levin 2002). Unfortunately, the genetic background has often been neglected in experimental studies dealing with ecological differences within polyploid complexes (but see e.g., Ramsey 2011; Mráz et al. 2012).

Knautia arvensis (Dipsacaceae) provides an ideal study system for assessing the role of polyploidy in serpentine tolerance. In Central Europe, it comprises two cytotypes (both occurring on and off serpentine) with well-described patterns of their genetic and habitat differentiation (Štěpánek 1997; Kaplan 1998). Interestingly, habitat differentiation largely correspond to the genetic relationships among populations but only partly reflect ploidy level variation (Kolář et al. 2009; Kolář et al. 2012). In Central Europe, the diploid cytotype (2n=2x=20) splits into two major genetic groups: (i) serpentine diploids inhabiting several spatially isolated serpentine outcrops (plus a single lime-rich stand in a subalpine glacial cirque), where it probably survived unfavourable periods of the Holocene (i.e., periods of large forest expansion that restricted heliophilous plants like *Knautia* to small, isolated refugia such as edaphic islands, Ložek 1973), and (ii) non-serpentine diploids inhabiting a wide range of semi-natural habitats in south-eastern parts of C. Europe. Similarly, at

the tetraploid level (2n=4x=40), a serpentine tetraploid lineage is known to be restricted to a single serpentine area whereas non-serpentine tetraploid populations are widespread in semi-natural grasslands throughout most of C. Europe (except for its SE part). Central-European serpentine diploid populations of *K. arvensis* form a genetically distinct lineage within *Knautia* (I. Rešetnik, P. Schönswetter & B. Frajman, unpubl. results) although certain levels of genetic differentiation have been observed among populations, probably as a result of allopatric differentiation among spatially isolated outcrops (Kolář et al. 2012). Importantly, serpentine tetraploid plants are genetically close to their diploid edaphic counterparts, likely as a result of a local autopolyploid origin from diploids inhabiting the same serpentine area. By contrast, non-serpentine diploids represent the genetically most distinct lineage of the whole *Knautia arvensis* group in Central Europe. Finally, non-serpentine tetraploids are genetically closer to serpentine populations (partly also due to hybridization with serpentine tetraploid plants in the area of immediate contact). Their origin is uncertain, however, as they might also encompass gene pools from other, yet uninvestigated populations (Kaplan 1998; Kolář et al. 2009; Kolář et al. 2012).

The available distributional, cytological, and genetic data suggest a key role of serpentines in the evolution of Central European *Knautia arvensis* populations. However, the patterns of evolution of the tolerance and the ways how different cytotypes and edaphic groups respond to serpentine stress remain unknown. Two major contrasting hypotheses regarding the distribution of serpentine tolerance could be postulated: (1) both serpentine and non-serpentine *K. arvensis* populations exhibit similar response to serpentine stress (i.e., serpentine tolerance is a constitutive trait in *K. arvensis*), (2) populations native to the serpentine stands exhibit higher levels of tolerance than their non-serpentine counterparts (i.e., the adaptive explanation applies). If the second was true, the effect of ploidy level itself would still remain to be evaluated as polyploidization and/or subsequent selection might have significant effects on the tolerance traits in the polyploid (e.g., leading to its higher endurance). In this study, we took *K. arvensis* as a model system of serpentine differentiated diploid-polyploid complex and tested for differentiation of its populations in tolerance to manipulated concentrations of magnesium and nickel (i.e., the two key factors affecting plant growth and survival on serpentines). Detailed knowledge on variation patterns of these eco-physiological traits, complemented by the previously described cytological and genetic background, would allow drawing a novel synthetic view on evolutionary pathways of serpentine tolerance in a ploidy variable plant complex.

Materials and methods

Plant material

Ripe achenes were collected in 2009 from approximately ten plants per each of eight natural populations of *Knautia arvensis* in the Czech Republic and Slovakia. The localities were selected in order to achieve a balanced design of the experiment: two diploid serpentine, two diploid non-serpentine, two tetraploid serpentine and two tetraploid non-serpentine populations. For details on the sampled populations, see Table 1. Data on soil characteristics from the rhizosphere of *Knautia* from the original localities were adopted from a previous study (Doubková et al. 2011). The ploidy level of the plants was confirmed using flow cytometry according to the protocol described in Kolář et al. (2009).

Experimental design and hydroponic cultivation

Achenes were germinated on a moist filter paper over a period of 3 weeks. Vital, undamaged seedlings were then carefully fixed onto a floating plastic disc (14 cm in diameter), maintaining uniform gaps between them. Each disc containing eight plants (one per each population) was placed into a 1.5 L light-impermeable experimental container with a standard nutrient solution described in Huss-Danell (1978) with a slight modification ($Co(NO_3)_2$ was used instead of $CoSO_4$ as the cobalt source). A similar solution has been successfully employed in the assessment of Ni and Mg tolerance in other plant systems, e.g., serpentine vs. non-serpentine ecotypes of *Cerastium alpinum* (Nyberg Berglund et al. 2004). The seedlings were grown in the standard nutrient solution for 11 days prior to the start of the experiment. They were then placed into experimental solutions with manipulated concentrations of Mg^{2+} and Ni^{2+} for the next 22 days ($MgSO_4$ and Ni SO_4 were used as sources of Mg and Ni, respectively; the pH was approx. Seven during the whole experiment). The solutions were changed every

Table 1 Details on investigated populations of *Knautia arvensis*

Substrate type	Ploidy level	Population code[a]	Site	Geographic co-ordinates	Altitude (m asl)	Site description	Ca/Mg ratio[b]	Mg ($mg.kg^{-1}$)[b]	Ni ($mg.kg^{-1}$)[b]
Serpentine	2x	S1	Borovsko (E. Bohemia, CZ)	49°40′57.7″N, 15°07′49.7″E	400	Open pine forest	0.7	2478	543
		S2	Staré Ransko (E. Bohemia, CZ)	49°39′04.9″N, 15°48′57.3″E	640	Coniferous forest margins	0.6	3005	155
	4x	S3	Pluhův Bor (W. Bohemia, CZ)	50°03′01.3″N, 12°46′24.3″E	710	Open pine forest	0.6	1657	170
		S4	Křížky (W. Bohemia, CZ)	50°03′54.2″N, 12°45′03.6″E	790	Rock outcrops, semidry grassland	0.8	1760	264
Non-serpentine	2x	NS1	Tvarožná Lhota (S. Moravia, CZ)	48°51′43.6″N, 17°23′23.3″E	290	Mesophilous meadow	18.3	366	7.1
		NS2	Lajdovci (W. Slovakia, SK)	48°28′29.8″N, 17°38′59.2″E	230	Dry meadow	10.1	521	2.4
	4x	NS3	Aš (W. Bohemia, CZ)	50°13′12.9″N, 12°13′19.2″E	670	Abandoned meadow	6.9	59	2.1
		NS4	Chanovice (SW. Bohemia, CZ)	49°24′39.0″N, 13°43′55.5″E	530	Dry meadow	7.5	267	1.9

[a] Population codes correspond with Doubková et al. (2011, 2012)

[b] Data from Doubková et al. (2011); available Mg and Ni concentrations were determined after 1 M ammonium acetate and 0.005 M DTPA extraction, respectively

3 days for a freshly prepared stock over the entire course of the hydroponic cultivation. The cultivations were performed in a controlled environment growth cabinet at the Faculty of Science, University of South Bohemia, Czech Republic with a cycle of 12 h light and 12 h darkness at the constant temperature of 18 °C with a light supply of approx. 450 $\mu mol.m^{-2}\ s^{-1}$ photons (photosynthetically active radiation).

To test the individual and combined effects of Ni and Mg among *K. arvensis* populations on different soil types (factor 'Substrate') and of different ploidy level (factor 'Ploidy'), we used a mixed-effect full-factorial experimental design. Four experimental treatments were applied: the control (Ni-/Mg-), Ni (Ni+/Mg-), Mg (Ni-/Mg+) and Ni + Mg (Ni+/Mg+). Based on a preliminary cultivation experiment, the concentrations of Ni^{2+} were set to 0 μM (control) and 50 μM, and the concentrations of Mg^{2+} were set to 0.55 mM (control) and 5.5 mM (i.e., Ca/Mg ratio of 2 and 0.2, respectively). Each experimental unit (= plastic container filled with one of the four types of the experimental solutions) consisted of eight seedlings, one seedling per population. Each treatment was replicated eight-times, resulting in the total amount of 32 experimental units and 256 seedlings.

Four characteristics were used as proxies of the plant growth response to different treatments: (i) total root growth, (ii) longest root growth and (iii) lateral root formation (proxies for belowground biomass), and (iv) longest leaf growth (proxy of aboveground biomass). Absolute values of the three root characteristics were acquired using the programme RootArch 1.0 (P. Šmilauer, University of South Bohemia, unpublished) from figures recorded at two time points: (i) at the beginning of the experiment, i.e., before setting the seedlings into the experimental solutions with manipulated elemental concentrations (roots were photographed in order not to harm the experimental plants); and (ii) at the end of the experiment (roots were scanned). Standard camera settings and identical distance from the object were kept when acquiring the photographs. The length of the longest leaf was carefully measured with a ruler again both at the beginning and at the end of the experiment. Growth characteristics were then calculated as differences between their initial and final values for each particular individual. Finally, the plants were harvested, the below- and aboveground organs were separated, dried at 60 °C and the dry biomass was weighted.

Determination of Mg and Ni concentrations in plant tissues

Elemental concentrations of Mg and Ni were quantitatively determined from desiccated leaf tissue (elemental contents in roots could not have been evaluated due to small amounts of the material) using inductively coupled plasma optical emission spectrometry (ICP OES, Hansen et al. 2009). We analysed Mg contents in 119 samples (i.e., approx. half of each population/treatment combination); Ni concentration was estimated only in Ni and Ni + Mg treated samples because of the absence of Ni in the control solution (amounting to a total of 64 samples). The samples were decomposed prior to the analysis. Due to the small amounts of our plant samples (8.4 mg of dry biomass on average), we applied the decomposition method using a multi-tube system and the MWS3+ microwave oven (Berghof, Germany). Dried plant tissue (<10 mg) was inserted into digestion tubes and treated with 2 ml nitric acid under the following conditions: 5 min ramp, 10 min hold on 170 °C. 5 min ramp, hold on 200 °C. The tubes were then filled up to the final volume of 10 ml and subjected to the ICP OES analysis. The elemental analysis of nickel and magnesium was carried out with the sequential, radially viewed ICP OES spectrometer INTEGRA XL 2 (GBC, Dandenong Australia) equipped with the microconcentric nebulizer (400 $\mu l.min^{-1}$) and a glass cyclonic spray chamber (both Glass Expansion, Australia). The analytical lines used were 221.6 nm for Ni and 285.2 nm for Mg. The operation conditions of the ICP OES analysis were the following: sample flow rate 1.5 $mL.min^{-1}$, plasma power 1000 W, plasma, auxiliary and nebulizer gas flow rates 10, 0.6, and 0.65 $L.min^{-1}$, respectively, photomultiplier voltage 600 V for nickel and 350 V for magnesium, view height 6.5 mm, three replicated reading on-peak 1 s, fixed point background correction. Calibration standards containing 10 – 5 – 1 – 0.5 – 0.1 $mg.L^{-1}$ of both nickel and magnesium were used for instrument calibration. The external calibration standards were prepared using commercially available stock standard solutions of Mg and Ni, both containing 1 $g.L^{-1}$ (SCP, Baie D'Urfé, Canada). The limits of detection (concentration equal to three times the standard deviation at the point of the background correction) were 5 $\mu g.L^{-1}$ for nickel and 2 $\mu g.L^{-1}$ for magnesium. Certified reference material (bush twigs and leaves GBW 07602 from the China National Analysis Center for Iron and Steel, Beijing) was used to validate the method.

Statistical analyses

Dependent variables (except for root and shoot biomass yield) were log-transformed in order to improve normality and homoscedascity. As we aimed at identification of the overall differences in serpentine tolerance among plants of different ploidy/edaphic origin, we treated the population of origin as a factor with random effect in all statistical analyses. Differences in biomass yield and in growth of above- and belowground biomass under control conditions (i.e., in the standard nutrient solution) among plants of different edaphic origin and ploidy were tested by an ANOVA analysis with the random factor of original population nested in the interaction between Substrate of origin (serpentine, non-serpentine) and Ploidy (diploid, tetraploid). A more complex ANOVA was used for the evaluation of the differences in growth, biomass yields and Mg tissue accumulation in *Knautia* seedlings in response to elevated concentrations of Mg and Ni. The effects of Substrate of origin, Ploidy level, Mg and Ni treatment and all their interactions were tested in a hierarchical ANOVA where the experimental container (nested in Mg and Ni treatment interaction) and population (nested in Substrate of origin and Ploidy interaction) were treated as random factors. Differences in Ni tissue concentrations were analysed only for plants grown in solutions containing nickel (i.e., treatments Ni and Mg + Ni). Differences among genetic groups were not subjected to statistical testing as the main genetic structure has already been represented by the interaction between Ploidy level and Substrate of origin (see the Introduction and Discussion sections). All analyses were calculated in Statistica 8 (StatSoft, Inc. 2007). Note that Statistica uses Satterthwaite's method of denominator synthesis, which finds the linear combinations of sources of random variation that serve as appropriate error terms for testing the significance of the respective effect of interest—for this reason the complete ANOVA tables, the synthesized error MS and synthesized error degrees of freedom are also presented.

Results

Under controlled conditions, plants of serpentine origin exhibited higher growth of the root system than their non-serpentine counterparts ($F_{1,49}$=8.669, p=0.032). By contrast, no significant differences were detected in either leaf growth or final biomass yields. Individuals of distinct ploidy levels also grew differently under

control conditions. Tetraploid plants exhibited higher root growth ($F_{1,49}$=8.501, p=0.033) as well as higher yields of both belowground and aboveground biomass ($F_{1,56}$=21.13, p=0.010; $F_{1,56}$=13.02, p=0.023 for root and shoot biomass, respectively) and a higher root/shoot biomass ratio ($F_{1,56}$=26.99, p=0.001).

Growth and biomass yield under different concentrations of Mg and Ni

The response of the three root growth characteristics was closely correlated and these will therefore be considered together in the further text (r^2=0.82, p<0.01 and r^2=0.83, p<0.01 for the correlation of the longest root growth and lateral root formation, respectively, with total root growth). Elevated concentrations of Mg and Ni significantly reduced the growth of *Knautia* seedlings in both belowground and aboveground organs (Table 2 and Online Resource 1); high Ni content also reduced root and shoot biomass yields (Table 3 and Online Resource 2). In addition, a significant interaction between Mg and Ni was detected, as high Mg content markedly alleviated the negative effects of Ni on growth and biomass yields of belowground organs (Online Resource 3).

Knautia plants of serpentine and non-serpentine origin responded to Mg and Ni stress in a strikingly different way (Tables 2 and 3). Firstly, elevated concentrations of Mg alone strongly reduced both growth and biomass yields of non-serpentine plants, while serpentine plants remained unaffected (this applied to both below- and aboveground organs; see Figs. 1 and 2). Secondly, under high Ni concentrations, elevated Mg amounts alleviated Ni stress more efficiently in serpentine plants, leading to their higher root growth (but not to higher biomass yields) in comparison with non-serpentine plants (Fig. 1 and Online Resource 4; see also Online Resource 5 displaying the response of individual populations). Finally, the effect of Ni alone was also expressed by a slightly higher root growth of serpentine plants under Ni

Table 2 Effect of manipulated Mg and Ni concentrations, ploidy level and substrate of origin on the growth of *Knautia arvensis* plants in hydroponic cultivation

			Total root growth		Longest root growth		Lateral root formation		Longest leaf growth	
Factor/Interaction	Effect	Effect df	MS	F	MS	F	MS	F	MS	F
Experimental container	*Random*	*28*	***0.11***	***2.01****	***0.07***	***1.55****	***0.14***	***1.86****	*0.05*	*0.97*
Population	*Random*	*5*	***0.21***	***3.83****	***0.32***	***7.31*****	*0.11*	*1.53*	***0.14***	***2.60****
Mg	Fixed	1	**0.52**	**4.66***	**0.36**	**5.24***	**0.86**	**6.31***	**0.79**	**15.5*****
Ni	Fixed	1	**8.48**	**76.36*****	**2.24**	**32.94*****	**9.87**	**72.01*****	**0.37**	**7.32***
Ploidy	Fixed	1	0.14	1.20	0.39	2.59	0.04	0.46	0.04	0.44
Substrate	Fixed	1	0.34	2.96	0.01	0.04	**1.27**	**14.38****	0.03	0.38
Mg*Ni	Fixed	1	**3.86**	**34.77*****	**1.28**	**18.81*****	**6.12**	**44.67*****	**0.48**	**9.55****
Ploidy*Mg	Fixed	1	0.01	0.20	0.02	0.41	0.01	0.09	0.00	0.02
Ploidy*Ni	Fixed	1	0.13	2.34	0.13	3.07	0.11	1.50	0.06	1.09
Substrate*Mg	Fixed	1	**2.00**	**36.19*****	**0.64**	**14.75*****	**2.50**	**33.94*****	**0.43**	**8.14****
Substrate*Ni	Fixed	1	**0.50**	**9.06****	**0.18**	**4.05***	0.21	2.86	0.05	0.90
Ploidy*Substrate	Fixed	1	0.21	1.81	0.62	4.16	0.17	1.87	0.06	0.70
Ploidy*Mg*Ni	Fixed	1	0.01	0.18	0.03	0.61	0.09	1.24	0.00	0.00
Substrate*Mg*Ni	Fixed	1	0.13	2.44	0.08	1.80	0.02	0.33	0.15	2.92
Ploidy*Substrate*Mg	Fixed	1	**0.51**	**9.21****	0.10	2.24	**0.75**	**10.18****	0.08	1.52
Ploidy*Substrate*Ni	Fixed	1	0.07	1.36	0.01	0.22	0.00	0.01	0.00	0.03
Ploidy*Substrate*Mg*Ni	Fixed	1	0.02	0.41	0.00	0.01	0.09	1.18	0.01	0.15
Error		207	0.05		0.04		0.07		0.05	

Statistically significant results are in bold, * p<0.05, ** p<0.01, *** p<0.001. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df for each tested term (see Materials and Methods for details) is available in Online Resource 1. Dependent variables were log transformed prior to the analysis.

Table 3 Effect of manipulated Mg and Ni concentrations, ploidy level and substrate of origin on biomass yields of *Knautia arvensis* plants harvested at the end of the hydroponic cultivation

			Belowground biomass		Aboveground biomass		Root/shoot ratio	
Factor/Interaction	Effect	Effect df	MS	F	MS	F	MS	F
Experimental container	*Random*	*28*	*0.000006*	***2.48******	*0.000028*	***2.51*****	*0.032142*	*1.05*
Population	*Random*	*5*	*0.000004*	*1.86*	*0.000014*	*1.24*	*0.042261*	*1.37*
Mg	Fixed	1	0.000015	2.60	0.000002	0.06	0.114927	3.54
Ni	Fixed	1	0.000106	**18.24*** ↓Ni+**	0.000020	0.71	1.109497	**34.20*** ↓Ni+**
Ploidy	Fixed	1	0.000030	**9.59** ↑4x**	0.000057	**4.66* ↑4x**	0.260646	**7.39* ↑4x**
Substrate	Fixed	1	0.000006	1.81	0.000000	0.01	0.129924	3.69
Mg*Ni	Fixed	1	0.000046	**7.97****	0.000011	0.40	1.195312	**36.85*****
Ploidy*Mg	Fixed	1	0.000005	2.32	0.000001	0.07	0.113254	3.67
Ploidy*Ni	Fixed	1	0.000005	2.06	0.000006	0.56	0.001787	0.05
Substrate*Mg	Fixed	1	0.000057	**24.28****	0.000328	**29.40*****	0.174101	**5.64***
Substrate*Ni	Fixed	1	0.000000	0.20	0.000001	0.11	0.005466	0.17
Ploidy*Substrate	Fixed	1	0.000002	0.61	0.000010	0.78	0.015727	0.44
Ploidy*Mg*Ni	Fixed	1	0.000005	2.06	0.000000	0.00	0.003240	0.10
Substrate*Mg*Ni	Fixed	1	0.000000	0.08	0.000015	1.31	0.031809	1.03
Ploidy*Substrate*Mg	Fixed	1	0.000015	**6.45***	0.000061	**5.43***	0.008287	0.26
Ploidy*Substrate*Ni	Fixed	1	0.000000	0.01	0.000001	0.06	0.001190	0.03
Ploidy*Substrate*Mg*Ni	Fixed	1	0.000000	0.05	0.000001	0.10	0.000000	0.00
Error		207	0.000002		0.000011		0.030833	

Statistically significant results are in bold, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. For factors with a significant effect, arrows indicate the direction of change. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df (see Materials and Methods for details) is available in Online Resource 2. Dependent variables were not transformed

stress (on the contrary, its effect on final biomass yields was not significant; Table 3 and Online Resource 2).

A significant effect of ploidy level on root growth and biomass was manifested by a complex interaction with edaphic origin and Mg treatment (Tables 2 and 3). Generally, the difference between plants of different edaphic origin was more pronounced within the tetraploid cytotype. Under elevated concentrations of Mg, serpentine tetraploids exhibited higher and non-serpentine tetraploids lower root growth than their particular edaphic diploid counterparts (Fig. 1). In the case of biomass yields, the differences among cytotypes were more pronounced in the control (higher yields in non-serpentine vs. serpentine tetraploids but no marked difference among diploids); under high Mg stress, tetraploid populations of both edaphic types yielded relatively more root and shoot biomass than their diploid edaphic counterparts (Online Resource 6). In addition, ploidy level alone had a significant effect on biomass production since tetraploids generally accumulated significantly more below- and aboveground biomass than diploids and also allocated relatively more biomass to the roots (Table 3).

Mg and Ni accumulation in aboveground tissues

Elevated concentrations of Mg and Ni in the experimental solution significantly increased accumulation of both elements in *Knautia* aboveground biomass (Table 4 and Online Resource 7; see also Online Resource 8 for details on the values). Mg concentration in leaf tissues was significantly affected by the interaction between ploidy level and its concentration in the solution (tetraploids accumulated slightly more Mg than diploids in the Mg rich solution; for details see Online Resource 9) but not with the substrate of origin (serpentine vs. non-serpentine). On the contrary, the accumulation of Ni in leaf tissues was significantly affected by the serpentine vs. non-serpentine origin in the interaction with the Mg treatment. Serpentine plants reduced their Ni accumulation when Mg concentrations in the solution were

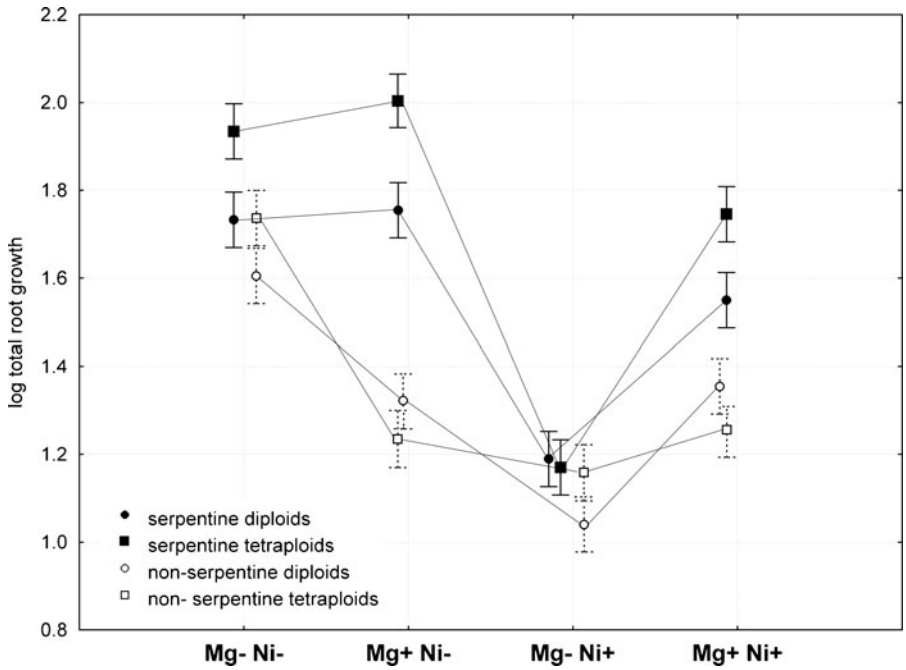

Fig. 1 Different response in total root growth of serpentine and non-serpentine *Knautia arvensis* plants (diploid vs. tetraploid) to the low (−) vs. high (+) concentrations of Mg and the absence (−) vs. presence (+) of Ni in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

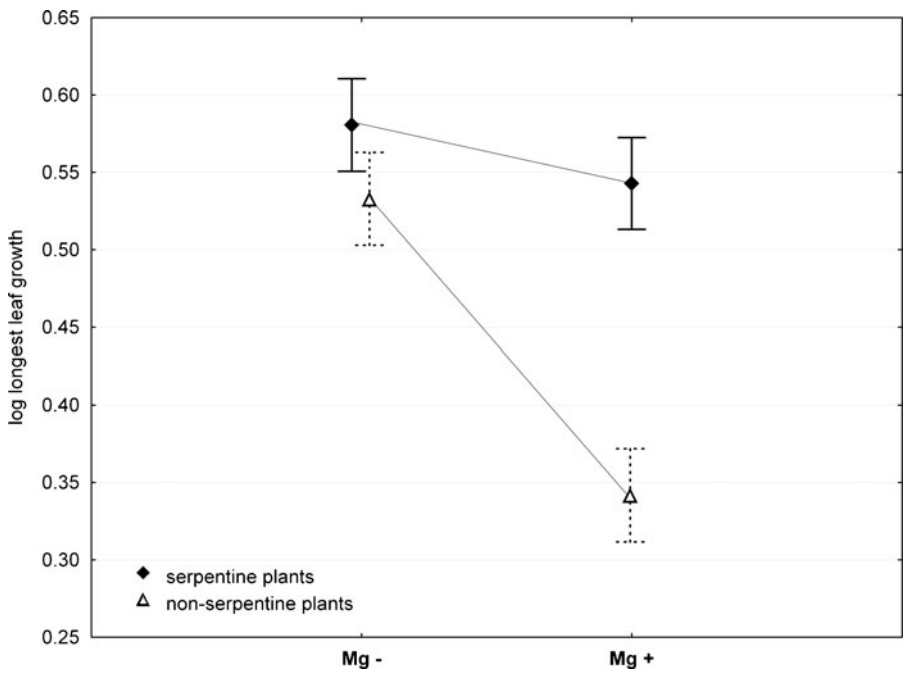

Fig. 2 Different response in the longest leaf growth of serpentine and non-serpentine *Knautia arvensis* plants to the low (−) vs. high (+) concentrations of Mg in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

Table 4 Effect of manipulated Mg and Ni concentrations in an experimental solution, ploidy level and substrate of origin on the concentrations of Mg and Ni in *Knautia arvensis* aboveground biomass. Differences in Ni accumulation were tested only for plants grown in Ni-enriched solutions

		Mg concentration in leaf tissue			Ni concentration in leaf tissue		
Factor/Interaction	Effect	Effect df	MS	F	df	MS	F
Experimental container	*Random*	*12*	*0.05*	*1.60*	*6*	*0.09*	*0.74*
Population	*Random*	*4*	*0.01*	*0.24*	*4*	*0.13*	*1.09*
Mg	Fixed	1	**21**	**629.18*** **	**1**	**1.67**	**19.32****
Ni	Fixed	1	0.08	2.31		–	–
Ploidy	Fixed	1	0	0.08	1	0.01	0.06
Substrate	Fixed	1	0.01	0.17	1	0.82	6.45
Mg*Ni	Fixed	1	0	0.04		–	–
Ploidy*Mg	Fixed	1	**0.2**	**5.96***	1	0.41	3.57
Ploidy*Ni	Fixed	1	0.02	0.49		–	–
Substrate*Mg	Fixed	1	0.05	1.47	**1**	**2.09**	**17.84*****
Substrate*Ni	Fixed	1	**0.22**	**6.45***		–	–
Ploidy*Substrate	Fixed	1	0.03	0.9	1	0.03	0.26
Ploidy*Mg*Ni	Fixed	1	0.01	0.42		–	–
Substrate*Mg*Ni	Fixed	1	0.02	0.64		–	–
Ploidy*Substrate*Mg	Fixed	1	0.02	0.67	1	0.02	0.16
Ploidy*Substrate*Ni	Fixed	1	0.05	1.46		–	–
Ploidy*Substrate*Mg*Ni	Fixed	1	0.02	0.65		–	–
Error		87	0.03		46	0.12	

Statistically significant results are in bold, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Population identity and experimental container were treated as random factors; the full ANOVA table including Synthetic error MS and df (see Materials and Methods for details) is available in Online Resource 7. Dependent variables were log transformed prior to the analysis

elevated whereas non-serpentine plants exhibited approximately the same levels of Ni in their tissues in both external Mg concentrations (Fig. 3, see also Online Resource 10 for the response of individual populations).

Discussion

Ecotypic response of *K. arvensis* to Mg and Ni stress

Plant adaptation to the stressful conditions of serpentine soils may occur either only in populations experiencing the stress or it can be widespread across all populations of a species. Both diploid and tetraploid cytotype of *Knautia arvensis* occur on and off serpentine, the available distributional data, however, did not allow any conclusion which of the above described scenarios applies to *Knautia*. In the hydroponic cultivation, serpentine and non-serpentine *Knautia arvensis* responded to the low Ca/Mg ratio and high Ni concentration in markedly different ways, suggesting that serpentine tolerance is an adaptive rather than a constitutive trait within this species. Firstly, serpentine plants, unlike their non-serpentine counterparts, did not reduce their growth under elevated concentrations of Mg (Figs. 1 and 2), which were present not only in the solution but also in plant tissues (Online Resource 8, see also Doubková et al. 2012). Tolerance to elevated Mg and/or high Mg requirement has been found to be an almost ubiquitous trait of serpentine-adapted plants that represents an integral part of serpentine tolerance (see Brady et al. 2005; Kazakou et al. 2008 for review). Secondly, Ni toxicity seems to be less harmful to serpentine than to non-serpentine plants when both Mg and Ni are present in elevated concentrations, i.e., in conditions close to those in natural serpentine stands (Fig. 1). A similar effect of certain elements such as Mg and Ca on reduction of Ni toxicity has been experimentally

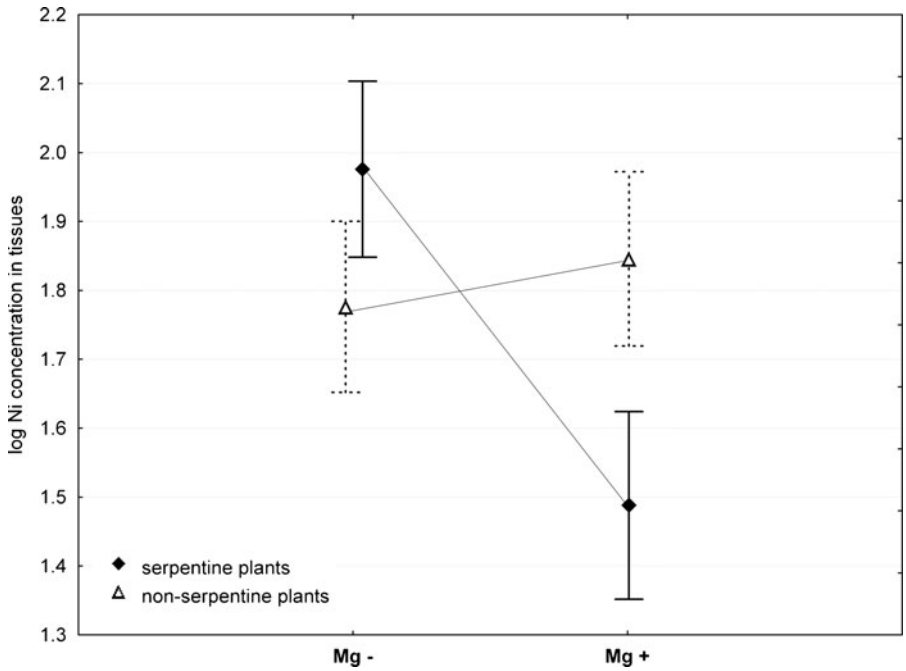

Fig. 3 Different response in the accumulation of Ni in aboveground tissues of serpentine and non-serpentine *Knautia arvensis* plants to the low (−) vs. high (+) concentrations of Mg in experimental solutions. Symbols and vertical bars denote the mean and standard error of the mean, respectively

demonstrated in several other plant species (e.g., *Avena sativa*, Proctor and McGowan 1976; *Alyssum bertolonii*, Gabbrielli and Pandolfini 1984; *Zea mays*, Robertson 1985). The physiological basis of this effect still remains rather unclear, but it possibly reflects a direct interference among both elements during their uptake by roots (Chen et al. 2009). Finally, serpentine plants also exhibited a significant decrease in Ni accumulation in their leaf tissues under elevated Mg, while non-serpentine plants accumulated approximately the same (high) amounts of Ni irrespective of external Mg concentrations (Fig. 3). Regulation of heavy metal uptake (Ni in particular) is often stressed as an important factor in serpentine tolerance, but the opposite—i.e., tolerance to excessive Ni accumulation—has in many cases been taken as evidence of serpentine tolerance (e.g., Ni hyperaccumulator plants such as *Thlaspi goesingense*, Reeves and Bakwer 1984; *T. caerulescens*, Boyd and Martens 1998; *Alyssum bertolonii*, Galardi et al. 2007). Nevertheless, restricted Ni uptake and translocation was also documented in several serpentine-tolerant plant species (e.g., Vergnano et al. 1982; Gabbrielli et al. 1990). To sum up, the consistently better response of the serpentine populations to Mg and Ni stress indicate that the tolerance is an adaptive trait characteristic for serpentine *Knautia arvensis* populations rather than a pre-adaptation (Brady et al. 2005) shared by all members of the complex.

Knautia serpentine populations seem to have evolved a complex mechanism of tolerance against serpentine chemical stress that is based on tolerance to high Mg accumulation and a restriction of Ni uptake. Such a combined response to both Mg and Ni stress has been revealed in several other case studies which examined the effects of both elements (e.g., Gabbrielli and Pandolfini 1984; Nyberg Berglund et al. 2004; Asemaneh et al. 2007). Interestingly, in *Cerastium alpinum,* serpentine populations exhibited considerable variation in the direction of Mg-Ni tolerance, as some populations exhibited a positive effect of Mg on Ni toxicity, while the opposite applied to other populations (well reflecting soil properties at sites of original populations, Nyberg Berglund et al. 2004). By contrast, we have detected a largely congruent pattern of growth response to both Mg and Ni among *Knautia* serpentine populations (see Online Resources 5 and 10), what also corresponds to the rather constant concentrations of both elements in the original soils (Table 1 and Doubková et al. 2011). Our data thus do not indicate any strong local adaptation to Mg and Ni stress within the serpentine *Knautia* ecotype.

Serpentine soils provide a complex set of chemical and physical factors influencing plant life, collectively

summarized under the term 'serpentine syndrome' (Jenny 1980; Brady et al. 2005). However, chemical stress caused by extremely low Ca/Mg ratios and high Ni concentrations is generally perceived as the principal trigger promoting serpentine ecotypic differentiation and adaptation (Brady et al. 2005; Kazakou et al. 2008). Moreover, other important components of the serpentine syndrome such as low levels of macronutrients and drought are probably less important in *Knautia* because its native serpentine soils are rich in nitrogen and organic carbon (in amounts similar to non-serpentine soils, Doubková et al. 2011). Within serpentine areas, *Knautia* plants avoid dry zones with obvious water limitation. Instead, they occupy the forest floor and various depressions where they co-occur with other mesophilous plant species (pers. obs.). Thus, for simplicity, we use the general term 'serpentine tolerance' to describe the different response of serpentine and non-serpentine *Knautia* ecotypes. We are nevertheless aware that other, untested physical factors and/or biotic interactions may still contribute to the tolerance of *Knautia* to the serpentine syndrome. Indeed, the ecotypic differentiation of a whole plant-fungus assemblage in relation to phosphorus uptake has recently been documented among serpentine vs. non-serpentine *Knautia arvensis* populations (the same as those used in our study, Doubková et al. 2012). The specific combination of (i) a serpentine-native arbuscular mycorrhizal fungus strain and (ii) *Knautia* plants represented the most efficient system of phosphorus uptake in serpentine soils. Because the pattern of response was congruent with our results, we can consider mycorrhizal association as another factor contributing to serpentine tolerance in *Knautia*.

Evolutionary background of *K. arvensis* serpentine tolerance

Serpentine ecotypic differentiation ranks among the best documented examples of plant adaptive differentiation, and it has been recorded in various areas around the world (Kruckeberg 1967; Proctor 1971; Ghasemi and Ghaderian 2009). Yet it has never been directly examined in relation to polyploidy. In contrast to classical views on polyploidy associated changes in important plant life-history traits (Levin 2002), polyploidy alone seems to play a rather minor role in the observed differentiation of serpentine compared to non-serpentine *Knautia* ecotypes. A simple effect of ploidy level on tolerance to Mg or Ni stress was not apparent in any of the examined traits in *K. arvensis*. It should nevertheless be noted that our experimental approach was targeted at a single (yet critical) developmental stage (seedlings), so we cannot exclude that some inter-cytotype differences might become pronounced in later stages of the plants' life cycle and/or during reproduction. The effect of polyploidy in *Knautia* seedlings, however, appeared when the serpentine vs. non-serpentine origin was also taken into account. Specifically, serpentine tetraploids exhibited higher growth of their root system under Mg stress than their diploid edaphic counterparts (whereas non-serpentine tetraploids performed even worse than diploids of the same edaphic origin, see Fig. 1). The better growth response to Mg stress as well as overall higher biomass yields detected in serpentine tetraploids of *Knautia* could have contributed to their establishment and spread in the serpentine locality (where they currently prevail over their diploid ancestors, M. Hanzl and F. Kolář, unpubl. results). Still, due to the parapatric distribution of the populations included in our study, we cannot exclude the possibility that the observed inter-cytotype differences might, at least partly, reflect local adaptation of individual populations.

Instead of the effect of polyploidy per se, we argue that the observed pattern of serpentine tolerance (shared tolerance among serpentine di- and tetraploids) more likely reflects the genetic relationships among the populations. As has been evidenced by genome size, AFLP and chloroplast DNA markers evaluated in our previous studies (Kolář et al. 2009; Kolář et al. 2012), *Knautia arvensis* in Central Europe has a distinct genetic structure which only loosely corresponds to ploidy levels but strongly reflects the patterns of edaphic differentiation. While non-serpentine diploids are genetically very distinct and the position of non-serpentine tetraploids is ambiguous, serpentine populations of both ploidy levels are genetically close to each other and probably followed a common evolutionary trajectory (restriction of diploids to edaphic refugia followed by local polyploidization; Štěpánek 1989; Kaplan 1998; Kolář et al. 2012). The shared adaptations to serpentine chemical stress among serpentine diploids and tetraploids, revealed by the present study, correspond with the autopolyploid origin of the serpentine tetraploids within the edaphic refugium (that was detected during our previous investigations, Kolář et al. 2012). We assume that the genes enhancing serpentine tolerance have been probably directly transmitted during the polyploidization process. Alternative explanations such as acquisition of the tolerance through subsequent 2x–4x gene flow can be ruled out based on the existence of strong interploidal reproductive barriers in *Knautia*

(Ehrendorfer 1962; Kolář et al. 2009). In addition, the tetraploids probably originated directly at the edaphic island (Kolář et al. 2012) and thus they should have been serpentine tolerant already in the initial phases of their establishment. Recent serpentine literature documents several evolutionary scenarios describing the acquisition and spread of serpentine tolerance such as gradual selection, catastrophic selection, hybridization and cross-tolerance to other stresses (see Brady et al. 2005; O' Dell and Rajakaruna 2011 for review). Polyploidization, however, represents a novel pathway through which serpentine tolerance could be transmitted during the origin of a new serpentine-tolerant evolutionary unit.

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