

Polyploidy affects responses to Nickel in Ni-hyperaccumulating plants: Evidence from the model species *Odontarrhena bertolonii* (Brassicaceae)

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ABSTRACT

Compared with their diploid progenitors, polyploid plants often display enhanced vigor and adaptive ability to extreme conditions. Previous studies investigated polyploid fitness under various forms of environmental stress, but whether the whole-genome duplication can affect growth, accumulation ability and tolerance levels to trace metals in metal-accumulating species remains obscure. We investigated this topic using an experimental approach and *Odontarrhena bertolonii* as model system. This obligate serpentine endemic includes diploid and tetraploid accessions with allopatric distribution in Tuscany and Liguria. Three diploid ($2n = 2x = 16$) and three natural autotetraploid ($2n = 4x = 32$) accessions were compared for growth, leaf traits, photosynthetic parameters and metal accumulation after cultivation on natural serpentine soil and in Ni-containing hydroponic solutions. In soil cultivation, shoot Ni concentration was similar in diploid and tetraploid plants, but the metal content per plant was significantly higher in polyploids because of their higher biomass production. The latter was mainly due to the production of larger leaves, coupled to a lower specific leaf area, which pointed to a better adaptation of the tetraploids to serpentine soil. In hydroponics, all the accessions showed a hormetic-like Ni effect, with stimulation of growth and photosynthesis in the low-dose zone. The data fitting to the hormetic model displayed significant differences in some parameters, with the tetraploid plants showing higher maximal length of roots and shoots and higher requirement of both external and internal Ni concentrations for optimal growth, in respect to diploid plants. On the other hand, Ni tolerance to both external and internal metal concentrations was similar in the plants of the two ploidy levels. Our results suggest that whole-genome duplication can affect the potential for growth and Ni-hyperaccumulation in obligate Ni-accumulator plants like *O. bertolonii*. This points to the opportunity of selecting polyploid instead of diploid accessions for environmental restoration and metal cropping practices, when the candidate species includes both chromosome races.

1. Introduction

Since its discovery at the beginning of the last century, polyploidy, the whole-genome duplication process, has been recognized as a major driving force of plant evolution. Although estimates about its incidence among flowering plants are still uncertain (Ramsey and Schemske, 1998; Doyle and Sherman-Broyles, 2017), it is likely that 15–30% of all angiosperm species is of polyploid origin (Wood et al., 2009; Mayrose et al., 2011; Soltis et al., 2016). A wealth of cytological and genetic data supports that auto-polyploids are likely prevalent on allo-polyploids (Barker et al., 2016). Both allo- and auto-polyploids usually display increased vigor, higher adaptability, and competitive advantage over

their diploid progenitors, in different functions and environmental contexts. Evidence from many studies supports that these plants are able to occupy new ecological niches (Stebbins, 1950; Blaine Marchant et al., 2016) thanks to their higher tolerance to various forms of abiotic stress (McIntyre, 2012; Allario et al., 2013;). Hence polyploidization is widely acknowledged as an active ongoing force that promotes plant speciation (Comai, 2005; Alix et al., 2017), and it is predicted that this will be an increasingly important mechanism in the present age of global climate change (Levin, 2019).

Among the most effective forms of abiotic stress that plants can experience is the presence of trace metals in toxic concentrations in their growth substrate. Most terrestrial plants display severe toxicity

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symptoms to very low concentrations of metals, such as Cr, Co, Zn, Pb, Cu, Ni and others. A remarkable exception, however, is given by the metal-tolerant species, which are provided by peculiar physiological mechanisms that allow them to exclude the metal from the root system or, alternatively, to take up and translocate some of them to the shoot tissues where they are usually sequestered and accumulated into the cell vacuole (Wójcik et al., 2017). Among the naturally occurring trace elements, Ni is the most widely accumulated one in the plant kingdom. Conventionally, Ni accumulation takes place when concentration in the plant tissue exceeds $1000 \mu\text{g metal g}^{-1}$ of shoot dry weight (Brooks et al., 1977; Reeves, 1992; van der Ent et al., 2013). At present, this ability is known to occur in 530 angiosperm taxa belonging to relatively few families and phylogenetic lineages, which originated through adaptive speciation on Ni-rich metalliferous soils derived from serpentine rocks across the main five Earth's continents (Reeves et al., 2018). Serpentine soils are metal-rich substrates with phytotoxic concentration of trace elements, such as Ni, Cr and Co, low levels of macronutrients (N, P, K and Ca) and an unfavorable Ca:Mg ratio (Gonnelli and Renella, 2012). Along with some physical characteristics, such as a low water retention, these chemical properties make serpentine soils habitable only by specialized and distinctive communities, often including rare and endemic taxa (Kruckeberg, Kruckeberg, 2004). Among the serpentinophytes, the so-called Ni-hyperaccumulating plants represent the most specialized ones (Brooks, 1987).

Metal hyperaccumulation in plants was discovered around the mid of the 20th century by Minguzzi and Vergnano (1948), who first analyzed the ashes of *Odontarrhena bertolonii* (Desv.) Jord. & Fourr. (Brassicaceae) from serpentine outcrops in central Italy. Ni-concentration in the leaf tissue of this obligate endemic species was found to range between 4000 and 20,000 $\mu\text{g g}^{-1}$ d.w., showing its capacity to take up and translocate the metal from the roots to the shoots, where it is accumulated (Galardi et al., 2007b, 2007a; Bettarini et al., 2021). After this remarkable discovery, some 80 species in the genus *Odontarrhena* and others of Brassicaceae in tribes Alysseae, Coluteocarpeae and Schizopetaleae from serpentine soils in Western Eurasia and North America were found to be strong Ni-hyperaccumulators (Brooks and Radford, 1978; Reeves et al., 1983, 2018).

Present-day evidence shows that polyploidy has played a prominent role in the evolutionary processes of these metal-accumulating lineages, especially the Alysseae, where it is estimated that ca. 25% of the taxa are of polyploid origin and 19% involve both diploid and polyploid cytotypes (Španiel et al., 2015; Alybase, <https://www.alysseae.sav.sk/>). *Odontarrhena* is the most diverse genus of Ni-accumulators in Western Eurasia and tetraploidy based on $x = 8$ is typical of several obligate or facultative serpentine taxa, such as the Italian *O. argentea* (All.) Ledeb. (Cecchi et al., 2013), the Balkan endemics *O. chalcidica* and *O. smolikana* (Cecchi et al., 2018) and the Aegean endemic *O. cyprica* (Nyár.) Španiel, Al-Shehbaz, D.A.German & Marhold (Vogt and Aparicio, 1999). A relatively restricted group of Ni-accumulating species in this genus includes both diploid and tetraploid cytotypes, and these provide a unique opportunity to investigate the relationship between ploidy level and responses to the metal in terms of growth, tolerance and accumulation capacity. Despite the numerous studies about the fitness of polyploids under various forms of abiotic stress (recently reviewed by Tossi et al., 2022), whether conspecific diploids and polyploids behave differently when experimentally exposed to increasing metal concentration remains obscure. So far, the polyploid fitness hypothesis in response to stress due to elevated concentrations of trace metals in the growth substrate has not been experimentally tested. To this purpose, it is necessary to compare infraspecific cytotypes growing in similar site conditions, especially soil chemical and physical characteristics, to exclude the effects of species-specific constraints or preadaptive traits that may influence the responses to metals. Our previous studies revealed that *O. bertolonii* includes phenotypically distinct diploid and tetraploid accessions, which likely originated via union of unreduced gametes (Cecchi et al., 2013; Selvi and Vivona, 2022). These two cytotypes show

an allopatric distribution in Tuscany and Liguria (central-northern Italy) but are found on serpentine soils with very similar chemical and physical characteristics, including comparable levels of Ni, and other trace metals such as Cr, Co and Mg, thus allowing to exclude heritable adaptation to different metal levels. In addition, our previous observations (Selvi and Vivona, 2022) showed that seed germination occurs significantly faster and at higher percentages in the polyploid cytotypes, and that germination capacity is better conserved through time than in diploid accessions. Overall, these findings supported a higher fitness in the polyploids at the initial life stages and prompted further investigation about the responses of the two cytotypes to elevated Ni concentrations. As reported by Feng et al. (2019), a better understanding of the effects of chromosome doubling on metal responses in accumulating species may also have relevant implications, especially in the selection of accessions to be used in practices such as phytoremediation and agromining (Kidd et al., 2018; van der Ent et al., 2021).

Hence, we performed the first ad hoc designed experiment to test the effect of polyploidy on the responses to serpentine soil and to elevated Ni concentrations in Ni-hyperaccumulators, using *O. bertolonii* as model species. According to the above-reported polyploid fitness hypothesis, tetraploid accessions are expected to have enhanced capacity to grow under elevated Ni concentration coupled with the ability to accumulate it to higher intracellular concentrations in respect to diploid accessions.

2. Materials and Methods

2.1. Plant material and soil sample collection

Plant material and seeds were collected from native populations in July 2020, in six distant serpentine sites of Tuscany and Liguria. The three more western accessions are diploid ($2n = 2x = 16$), including the one from the "locus classicus" (type locality) in eastern Liguria, while the three from the eastern sector of the species range (Arezzo province) are tetraploid ($2n = 4x = 32$). Plants of the latter are auto-tetraploids performing diploid-like chromosome pairings at meiosis, where bivalents are selected randomly from four homologs, and are thus able to produce subsequent generations. Information about the population localities is given in Table 1, while details of chromosomal features of the accessions can be found in Selvi and Vivona (2022).

At each site, shoots from five adult plants were arbitrarily collected, together with over 1000 mature seeds from 10 to 15 fruiting plants at a distance of at least 10 m from each other. In addition, ten bulk soil samples (ca. 20 g) were arbitrarily collected at ca. 5–15 cm depth in the same sampling area. These samples were then pooled together to obtain a single bulk soil sample of ca. 200 g.

2.2. Nickel concentrations in soil and field-collected samples

Bulk soil samples were dried at room temperature, sieved with a 2 mm mesh stainless steel sieve, and then oven-dried at 50 °C for 7 days (Pignattelli et al., 2012). From each sample, five subsamples (0.5 g d.w.) were digested using 10 mL of 69% HNO_3 in a microwave digestion system (Mars 6, CEM) as in Bettarini et al. (2019). Nickel concentrations in the digests were determined by atomic absorption spectroscopy (PinAAcle 500, PerkinElmer, Waltham, Massachusetts, USA).

Dried samples of plant shoot tissues were carefully washed three times with deionized water, dried at 50 °C for 48 h (Selvi et al., 2017), and then the dried materials (0.1 g d.w.) were analyzed in the same way as described for soils. Each plant sample analysis was replicated three times.

2.3. Pot cultivation

To compare Ni-accumulation capacity in the six *O. bertolonii* accessions, plants were cultivated in pots filled both with natural serpentine soil (Tuscany, Grosseto province), with total Ni = $1869 \pm 66 \mu\text{g g}^{-1}$ d.

Table 1

List of seed collection sites of *O. bertolonii*, with accession code, coordinates, altitude and chromosome number (after Selvi and Vivona, 2022). Nickel concentrations ($\mu\text{g g}^{-1}$ d.w., mean of 5 values \pm standard deviation) in the native soil (HNO_3 -extracted, total concentration) and in shoots of field collected plants.

Accession	Code	Lat, Long	Altitude (m)	Chromosome number	[Ni] soil	[Ni] shoot
Tuscany, Impruneta, Sassi Neri	Im	43°40'37", 11°16'07"	270 – 280	2 n = 16	1089 \pm 83	3350 \pm 822
Tuscany, Galceti, Mt. Ferrato	Ga	43°54'43", 11°05'05"	120 – 140	2 n = 16	1591 \pm 94	6848 \pm 1725
Liguria, Falcinello	Fa	44°08'36", 9°57'10"	90 – 120	2 n = 16	2128 \pm 175	4935 \pm 1257
Tuscany, Pieve S. Stefano, Mt. Petroso	Pi	43°38'57", 12°03'22"	450 – 480	2 n = 32	1412 \pm 71	5399 \pm 1302
Tuscany, Anghiari, Mt. Rognosi	An	43°34'32", 12°00'57"	440 – 470	2 n = 32	1534 \pm 87	6806 \pm 1665
Tuscany, Pieve S. Stefano, Formole	Pif	43°38'38", 12°03'06"	420 – 430	2 n = 32	1332 \pm 75	6977 \pm 798

w., mean of 5 samples \pm SD) and with garden soil (hereafter indicated as control soil, commercial peat-clayed soil: Geotec Fioriture Tonerde), with metal concentration below the detection limit. After seed sowing, pots with one plant each (12 pots/plants per treatment) were placed in a growth chamber (24/16 °C day/night; light intensity 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$, 16-h (day) photoperiod; relative humidity 60–65%). Plants were watered regularly two times a week and 12 individuals for population were sampled after 6 weeks to measure shoot height, fresh and dry weight. Leaf traits were analyzed as described in Colzi et al. (2022) following standard trait measurement protocols (Pérez-Harguindeguy et al., 2013). For each treatment, four fully expanded leaves from each of the 12 individuals were collected. Projected Leaf Area (LA), Specific Leaf Area (SLA = LA/Leaf Dry Weight) and Leaf Dry Matter Content (LDMC = LDW/Leaf Fresh Weight) were determined. Subsequently, plants were rinsed with milliQ-water and roots were carefully washed with 10 mM $\text{Pb}(\text{NO}_3)_2$ at 4 °C for 30 min to desorb metals adhering to the root cell wall, as in Bazihizina et al. (2015). Samples were analyzed for Ni concentration as described above for soils. The Ni content per plant shoot was calculated by multiplying the shoot dry biomass by the shoot Ni concentration.

2.4. Chlorophyll fluorescence and content

Chlorophyll content ($\text{Chl}_{\text{index}}$) was measured immediately before sample harvesting using a Chl content meter (CCM-300, Opti-Science, Hudson, USA) and expressed as total Chl per m^2 of leaf material (mg m^{-2} ; Gitelson et al., 1999). Chlorophyll fluorescence was measured on 15-min dark-adapted leaf samples using a portable fluorimeter (Plant Efficiency Analyzer - Handy PEA, Hansatech Instruments Ltd). Samples were flashed for 1 s with a saturated ($1800 \mu\text{mol m}^{-2}\text{s}^{-1}$) red light pulse (650 nm) from a LED into the fluorimeter sensor. Leaf health was evaluated using the potential quantum efficiency of photosystem II, i.e., the indicator of photosynthetic efficiency F_v/F_m (F_v indicates the difference between the maximal F_m and the minimal F_0 fluorescence), and by the performance index (P_{index}), the general indicator of the photosystem I and II efficiency.

2.5. Nickel tolerance and accumulation in hydroponics

Seeds were sown in peat soil and 6-week-old seedlings were then transferred to hydroponic cultures in 1-L polyethylene pots (one plant per pot) containing a modified half-strength Hoagland's solution (Hoagland and Arnon, 1950) in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulphonic acid, adjusted to pH 5.5 with KOH. Background Ni concentration in the growing medium was about 0.1 μM and this was considered as the control condition, as in Bettarini et al. (2020). Nutrient solutions were changed weekly, and plants were grown in the same growth chamber described above. After 3 weeks, plants of homogeneous size were selected and treated as in Bettarini et al. (2021): root and shoot length of each plant was measured and subsequently plants were exposed for 7 days to a series of NiSO_4 concentrations (0, 50, 100, 250, 500, 1000, 2000, 3000 μM , 12 plants per treatment), in a background solution of the same composition as the pre-culture solution. The applied Ni concentrations were previously proved to be adequate for serpentine plants of *Odontarrhena*

(Bettarini et al., 2020, 2021) and were in the range of toxicity for plants not adapted to metal excess (5 μM , Marschner, 1995). After seven days of growth, the photosynthetic parameters were measured as described above. Root and shoot length of all plants was measured again to assess the increment (calculated subtracting length values at the beginning of the treatment to values at the end of the treatment). Plants were rinsed with milliQ-water and roots were carefully desorbed with 10 mM $\text{Pb}(\text{NO}_3)_2$ as described above. Shoots and roots were dried at 70 °C for 24 h and Ni accumulation was determined as previously stated.

2.6. Data analyses

The experimental data points were fitted to the Brain-Cousens model for the analysis of the growth response to Ni treatment, as in Bettarini et al., (2020, 2021). The increment in root and shoot length was measured as response variable for all tested accessions, while the Ni concentration of the growth medium was considered as the predictor variable. The Brain-Cousens model allows to validate the presence of significant hormetic effect and to estimate the following parameters: the external maximum stimulation dose (MSD_{ext}), the maximum mean response (MAX, necessary for a reliable calculation of the percentage of the hormetic effect as $[100 * (\text{MAX-length increment in control condition}) / \text{length increment in control condition}]$, here named hormetic percentage = HP) and the half-maximal effective external concentration (EC50_{ext}). The Brain-Cousens model was used to estimate also the internal maximum stimulation dose (MSD_{int}) and the half-maximal effective internal concentration (EC50_{int}) basing on data of Ni concentrations accumulated in the root symplast, instead of the Ni concentrations present in the culture medium. This last parameter (EC50_{int}) was used as an index of the accumulation capacity as in Galardi et al. (2007a), Adamidis et al. (2014), Colzi et al. (2014) and Bettarini et al. (2021). The drc package (Ritz et al., 2015) as implemented in R Studio version R 3.4.3 (R Core Team 2017) was used to fit the curve of concentration-response data.

The significance of differences between the means was analyzed by one-way and two-way ANOVA followed by HSD-Tukey test for post-hoc comparisons using GraphPad Prism 7 (GraphPad Software, San Diego, CA), after checking data normality distribution (assessed with the Shapiro-Wilk test).

Linear regression analyses were performed to investigate the relationships between the parameters obtained by data fitting.

3. Results

3.1. Plant growth and Ni-accumulation on serpentine soil

After 6 weeks of cultivation on serpentine and control soil, all examined accessions of *O. bertolonii* showed no visible toxicity symptoms such as foliar chlorosis or necrosis. However, shoot biomass values (fresh and dry weight) and plant height were significantly lower in plants grown on serpentine soil (Fig. 1). Regarding shoot height, the interaction population \times treatment was not significant (Table S1a) and no differences occurred between diploid and tetraploid accessions on the two soil types (Table S1b). Concerning shoot fresh and dry weight, the interaction population \times treatment was significant when pooling all

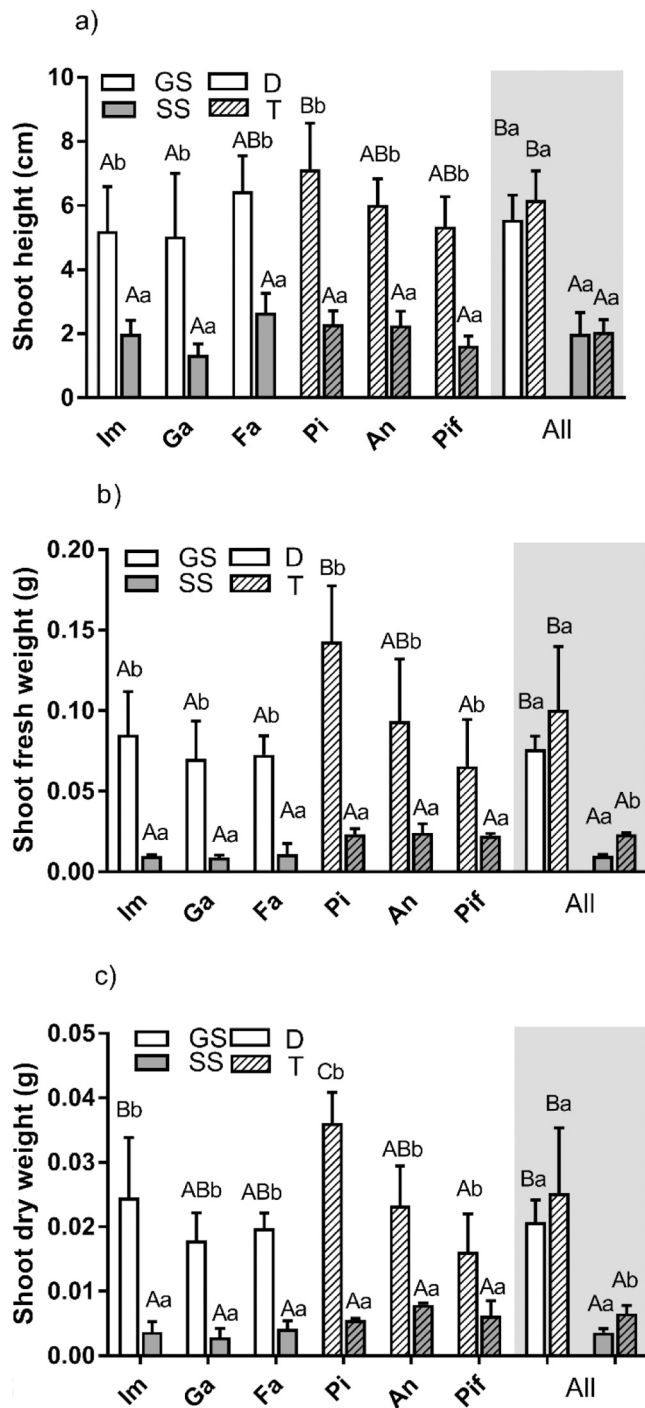


Fig. 1. Shoot length (a) fresh (b) and dry (c) weight at harvest of the three diploid (D) and three tetraploid (T) *O. bertolonii* accessions grown for six weeks in pots with garden soil (GS) and with the same natural serpentine soil (SS). The bars in the grey box group the mean values of all diploid and tetraploid accessions. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates \pm standard deviation.

accessions together but not for the two ploidy groups separately (Table S2 and S3). The mean biomass values of plants grown on serpentine soil were significantly lower in diploid than in tetraploid plants (Fig. 1).

Concerning leaf traits, LA was significantly lower in serpentine plants, and there was a significant interaction accession \times treatment (Fig. 2; Table S4a); tetraploid plants displayed a higher LA on both soil

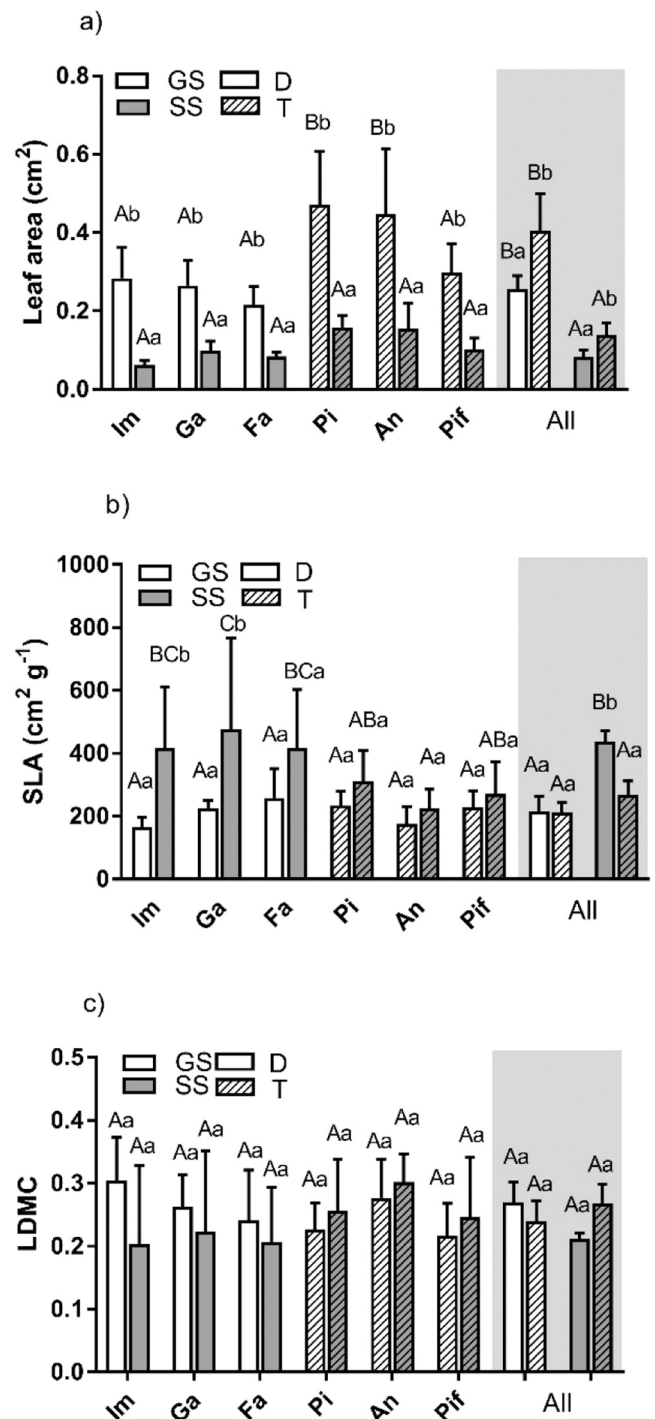


Fig. 2. Leaf traits at harvest of the three diploid (D) and three tetraploid (T) *O. bertolonii* accessions grown for six weeks in pots with garden soil (GS) and with the same natural serpentine soil (SS). The bars in the grey box group the mean values of all diploid and tetraploid accessions. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates \pm standard deviation. a) Leaf area; b) Specific leaf area (SLA); c) Leaf dry matter content (LDMC).

types, compared to diploids, but with no significant interaction ploidy \times treatment (Table S4b). Conversely, the traits SLA and LDMC were not significantly different between serpentine and control plants (Fig. 2; Table S5a and S6a), despite higher mean values in a few serpentine accessions. In plants grown on serpentine soil, however, the effect of ploidy level on SLA resulted significant, as the mean values of this trait

in tetraploids were lower than in diploids (Table S5b).

Serpentine and control plants did not differ in the F_v/F_m values, whereas the P_{index} values and Chl content were lower in the serpentine accessions, significantly for some of these (Fig. 3). No interactions treatment \times accessions were detected, nor significant effects of ploidy level on these variables (Tables S7, S8 and S9).

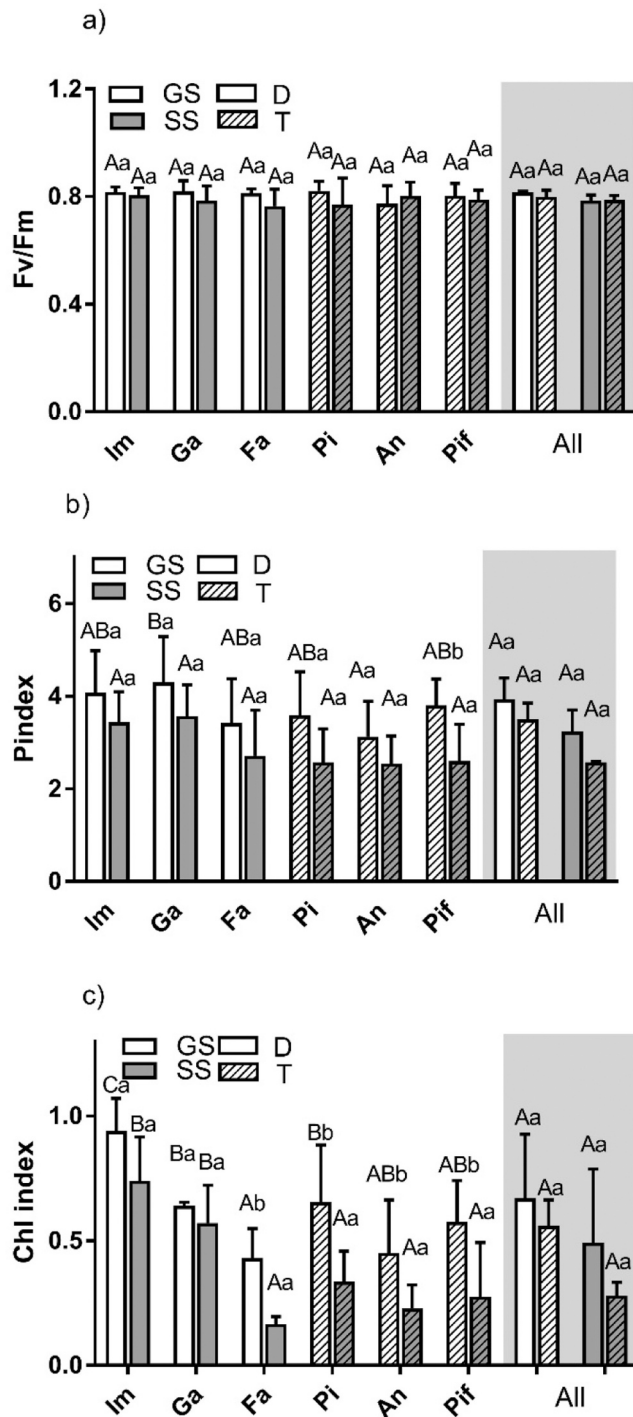


Fig. 3. Parameters of photosynthetic efficiency at harvest of the three diploid (D) and three tetraploid (T) *O. bertolonii* accessions grown for six weeks in pots with garden soil (GS) and with the same natural serpentine soil (SS). The bars in the grey box group the mean values of all diploid and tetraploid accessions. Letters indicate significant differences among the means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates \pm standard deviation. a) F_v/F_m ratio; b) P index; c) Chl index.

Nickel concentration in roots was negligible in plants from control soil, but significantly higher in those from serpentine soil (Fig. 4). The diploid accessions showed significantly higher Ni root concentrations, indicating a ploidy level effect (Table S10). Nickel levels in roots were much lower than in shoots. Shoot Ni-concentration in control plants were lower than in plants from serpentine soil, that always exceeded the $1000 \mu\text{g Ni g}^{-1}$ d.w. threshold. Differences in shoot Ni concentration between accessions were significant, but apparently not related to ploidy level (Table S11).

Concerning the shoot Ni content per plant (Fig. 5), the tetraploid accessions always showed significantly higher values than diploid ones on both soil types, whereas the interaction treatment \times ploidy level was not significant (Table S12).

3.2. Plant growth in hydroponics

Root and shoot length variations of the *O. bertolonii* accessions exposed to increasing NiSO_4 concentrations are shown as increment after 7 treatment days (Fig. 6). In the low-dose zone, plants showed generally a significantly higher growth increment in respect to control conditions, which was variable depending on metal doses and accessions. Tetraploid accessions Pi, An and Pif displayed significant

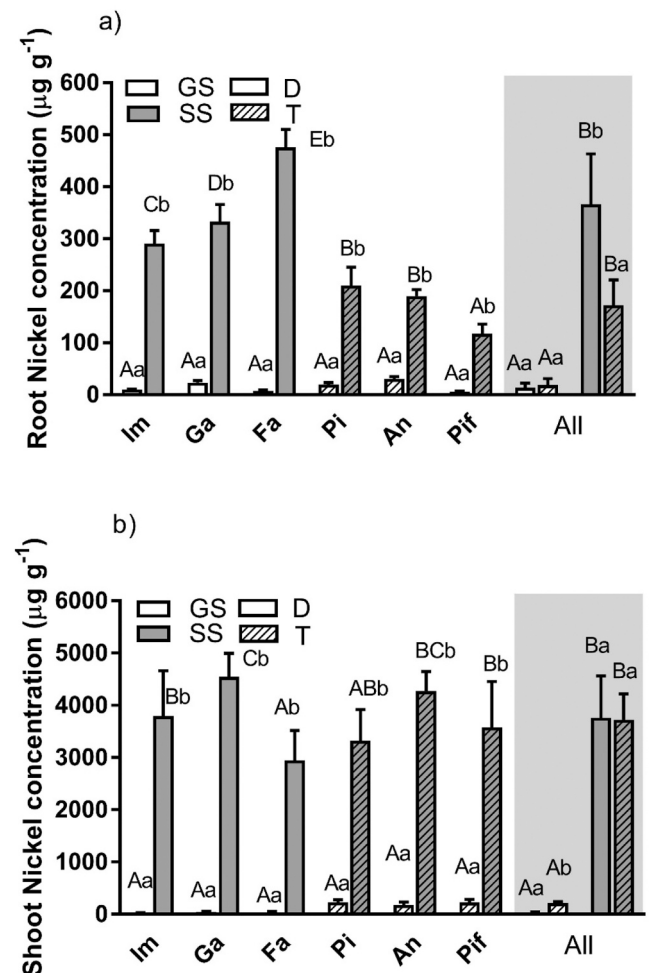


Fig. 4. Root (a) and shoot (b) Ni-concentration at harvest of the three diploid (D) and three tetraploid (T) *O. bertolonii* accessions grown for six weeks in pots with garden soil (GS) and the same natural serpentine soil (SS). The bars in the grey box group the mean values of all diploid and tetraploid accessions. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates \pm standard deviation.

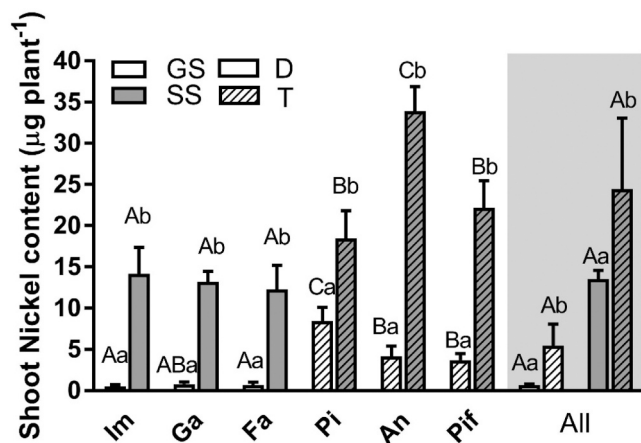


Fig. 5. Shoot Ni content ($\mu\text{g Ni per plant}^{-1}$) at harvest of the three diploid (D) and three tetraploid (T) *O. bertolonii* accessions grown for six weeks in pots with garden soil (GS) and the same natural serpentine soil (SS). The bars in the grey box group the mean values of all diploid and tetraploid accessions. Letters indicate significant differences among means (Tukey test), capital case among accessions and lower case within accessions. Values are means of 12 replicates \pm standard deviation.

increments up to the treatment 500 $\mu\text{M NiSO}_4$ for roots and 150 $\mu\text{M NiSO}_4$ for shoots, with Pif shoots continuing to grow up to concentrations of 500 $\mu\text{M NiSO}_4$. At the highest metal levels, there was a general reduction in plant length increment, which occurred at 2000 $\mu\text{M NiSO}_4$ for roots and 3000 $\mu\text{M NiSO}_4$ for shoots. Two-way ANOVA showed significant interpopulation variation in root and shoot elongation in response to Ni treatments (Table S13) and a significant interaction accession \times treatment.

The Brain-Cousens hormetic model provided a significant fitting; the lack-of-fit test resulted in p-values in the range of 0.061–0.301 for roots (Table 2) and 0.063–0.727 for shoots (Table 3), thus validating the model for all the examined accessions. Plants showed different values for the parameters calculated by the data fitting. Significant variation in $\text{EC}_{50\text{ext}}$ was found among the accessions (Table S14a and S15a for roots and shoots, respectively). Compared with diploid plants, tetraploid accessions showed higher mean values of MAX in roots and of MAX and MSD_{ext} in shoots.

The relationships between the estimated values of MSD_{ext} and HP and between MAX and HP were significant for shoots (Table S17) but not for roots (Table S16).

The photosynthetic parameters of the *O. bertolonii* accessions exposed to increasing NiSO_4 concentrations are reported in Table 4. The F_v/F_m values were not affected by the Ni-treatment, whereas some differences were observed among accessions, except for the control condition and the 150 $\mu\text{M NiSO}_4$ treatment. Two-way ANOVA supported significant differences among accessions and for the interaction population \times treatment (Table S18a). In all accessions, the P_{index} increased in

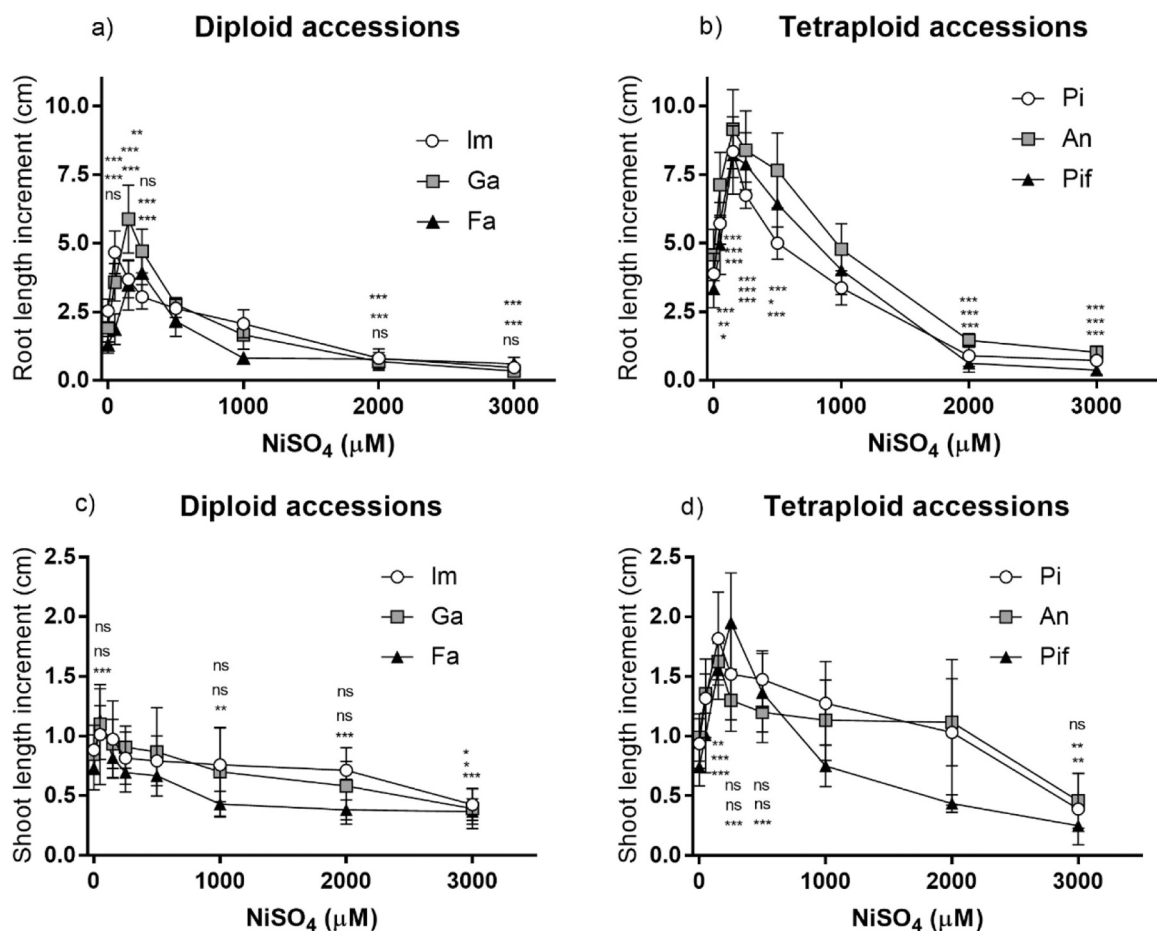


Fig. 6. Increment in root and shoot length (cm) in diploid and tetraploid *O. bertolonii* accessions treated with increasing NiSO_4 concentrations. a) root length of diploid accessions; b) root length of tetraploid accessions; c) shoot length of diploid accessions; d) shoot length of tetraploid accessions. Asterisks indicate the significant differences in root and shoot length within each accessions according to the Tukey's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (the sequence of the indications reflects the one of the legend: Im, Ga, Fa and Pi, An, Pif respectively). Values are means of 12 replicates \pm standard deviation.

Table 2

Parameters of the root dose-response curves: p-values of the lack-of-fit test and growth parameters calculated with the Brain-Cousens model for six *O. bertolonii* accessions grown at eight NiSO₄ concentrations. Letters indicate significant differences among accessions according to the Tukey's test ($p < 0.05$). Asterisks indicate significant differences between the mean values of the diploid and tetraploid accessions (* $p < 0.05$, ** $p < 0.01$).

Population	Lack-of-fit test	MSD _{ext} (μM)	MAX (cm)	HP (%)	EC50 _{ext} (μM)	MSD _{int} (μg g ⁻¹ d.w.)	EC50 _{int} (μg g ⁻¹ d.w.)
Diploids							
Im	0.061	56	4.6	82	1574 ± 94ab	390	2340 ± 187a
Ga	0.301	154	5.5	189	1497 ± 93a	834	2559 ± 195c
Fa	0.088	213	3.7	185	1546 ± 107a	788	2379 ± 168c
Mean ± sd		141 ± 79	4.6 ± 0.9	152 ± 61	1539 ± 39	670 ± 244	2426 ± 117
Tetraploids							
Pi	0.065	161	7.8	101	1471 ± 102a	899	2175 ± 129b
An	0.081	233	9.2	102	1681 ± 96bc	1391	3759 ± 169e
Pif	0.070	248	8.4	152	1699 ± 105c	1252	3243 ± 159d
Mean ± sd		214 ± 46	8.5 ± 0.7 **	118 ± 29	1617 ± 127	1180 ± 253 *	3059 ± 808

Table 3

Parameters of the shoot dose-response curves: p-values of the lack-of-fit test and growth parameters are calculated with the Brain-Cousens model for six *O. bertolonii* accessions grown in presence of eight NiSO₄ concentrations. Letters indicate significant differences among accessions according to Tukey's test ($p < 0.05$). Asterisks indicate significant differences between the mean values of diploid and tetraploid accessions (* $p < 0.05$, ** $p < 0.01$).

Population	Lack-of-fit test	MSD _{ext} (μM)	MAX (cm)	HP (%)	EC50 _{ext} (μM)	MSD _{int} (μg g ⁻¹ d.w.)	EC50 _{int} (μg g ⁻¹ d.w.)
Diploids							
Im	0.380	74	1.0	13	2917 ± 208 f	154	3595 ± 235c
Ga	0.727	61	1.1	30	2425 ± 203a	385	2885 ± 91b
Fa	0.577	88	1.4	91	2544 ± 250bc	342	2570 ± 317a
Mean ± sd		74 ± 13	1.2 ± 0.2	45 ± 41	2629 ± 257	294 ± 123	3017 ± 525
Tetraploids							
Pi	0.096	218	1.7	81	2979 ± 167d	1090	3095 ± 351b
An	0.063	132	1.5	52	3378 ± 198e	890	4220 ± 230d
Pif	0.328	255	1.8	142	2268 ± 212b	1215	2799 ± 252ab
Mean ± sd		202 ± 63 *	1.7 ± 0.2 *	92 ± 46	2875 ± 562	1065 ± 164 **	3371 ± 749

Table 4

Parameters of photosynthetic efficiency of the six *O. bertolonii* accessions treated with increasing concentrations of NiSO₄. Letters indicate significant differences among the means according to the Tukey's test, capital case among accessions and lower case within accessions. Values are means of 12 replicates ± standard deviation.

Treatment NiSO ₄ (μM)	Diploids			Tetraploids		
	Im	Ga	Fa	Pi	An	Pif
F_v/F_m						
Control	0.77 ± 0.06 aA	0.79 ± 0.03 aA	0.78 ± 0.05 aA	0.77 ± 0.09 aA	0.81 ± 0.04 aA	0.72 ± 0.05 aA
50	0.77 ± 0.04aAB	0.79 ± 0.06aAB	0.83 ± 0.02aB	0.80 ± 0.03 aAB	0.82 ± 0.03 aA	0.73 ± 0.09 aA
150	0.79 ± 0.04 aA	0.83 ± 0.02 aA	0.82 ± 0.03 aA	0.76 ± 0.06 aA	0.79 ± 0.09 aA	0.76 ± 0.06 aA
250	0.74 ± 0.04 aA	0.84 ± 0.01aB	0.79 ± 0.05aAB	0.78 ± 0.06aAB	0.79 ± 0.03aAB	0.77 ± 0.08 aAB
500	0.76 ± 0.05aB	0.83 ± 0.02aB	0.82 ± 0.01aB	0.76 ± 0.07aB	0.79 ± 0.05aB	0.66 ± 0.08 aA
1000	0.73 ± 0.04aAB	0.81 ± 0.02aB	0.79 ± 0.03aB	0.78 ± 0.05aB	0.79 ± 0.03aB	0.68 ± 0.07 aA
2000	0.74 ± 0.07aB	0.82 ± 0.03aB	0.75 ± 0.03aB	0.80 ± 0.05aB	0.77 ± 0.09aB	0.64 ± 0.05 aA
3000	0.74 ± 0.04aAB	0.81 ± 0.05aB	0.81 ± 0.02aB	0.73 ± 0.14aAB	0.77 ± 0.04aB	0.68 ± 0.04abcA
P_{index}						
Control	2.8 ± 0.9abA	4.1 ± 0.6abBC	4.0 ± 0.9abBC	4.0 ± 0.8abcBC	4.9 ± 0.7deC	3.3 ± 0.7abAB
50	2.8 ± 0.4abA	4.2 ± 0.9abB	6.1 ± 0.9cB	4.4 ± 0.7cB	4.6 ± 0.4cdeB	4.2 ± 0.7bcB
150	3.8 ± 0.73ba	4.9 ± 0.9bcdAB	5.5 ± 0.9cdB	4.1 ± 0.4bcAB	5.4 ± 0.8eB	4.8 ± 0.9cAB
250	2.3 ± 0.8 aA	5.7 ± 0.9dC	4.8 ± 0.4bcBC	4.2 ± 0.9cB	4.4 ± 0.9cdeB	4.4 ± 0.7bcB
500	2.2 ± 0.5 aA	5.4 ± 0.9cdE	4.8 ± 0.6bcDE	3.5 ± 0.6abcBC	4.2 ± 0.7bcdCD	2.6 ± 0.6aAB
1000	1.8 ± 0.3 aA	4.6 ± 0.7abcdC	3.3 ± 0.8aB	3.5 ± 0.6abcBC	3.1 ± 0.7abB	2.8 ± 0.4aB
2000	1.8 ± 0.5 aA	4.3 ± 0.5abcC	3.2 ± 0.6aBC	3.0 ± 0.6abB	3.5 ± 0.6abcBC	2.4 ± 0.5aAB
3000	1.8 ± 0.7 aA	3.7 ± 0.8aC	3.1 ± 0.4aBC	2.9 ± 0.5aABC	2.6 ± 0.7aABC	2.4 ± 0.4aAB
Chl (mg m⁻²)						
Control	558 ± 90aAB	765 ± 32aC	671 ± 96abcBC	501 ± 84 aA	622 ± 66aB	569 ± 57abcAB
50	597 ± 62aAB	751 ± 91aC	749 ± 80cC	533 ± 65abA	640 ± 90abABC	673 ± 43cdBC
150	624 ± 68aAB	738 ± 44aB	723 ± 84bcB	546 ± 81abcA	634 ± 64aAB	699 ± 71dAB
250	560 ± 93 aA	789 ± 57aC	642 ± 60abcAB	601 ± 73abcA	754 ± 88bBC	741 ± 60dBC
500	535 ± 61 aA	737 ± 82aB	605 ± 53 aA	631 ± 78bAB	626 ± 67aAB	651 ± 59bcdAB
1000	543 ± 81 aA	749 ± 89aB	606 ± 51aB	657 ± 86cAB	577 ± 53 aA	529 ± 96 aA
2000	508 ± 66 aA	783 ± 89aB	618 ± 69aB	602 ± 41abcA	550 ± 82 aA	553 ± 85aB
3000	520 ± 52 aA	760 ± 73aB	589 ± 61 aA	586 ± 59abcA	562 ± 77 aA	564 ± 47abcA

the low-dose zone and decreased to values lower than those in control conditions at the highest NiSO₄ concentrations, with significant differences depending on metal doses and accessions. Differences among the accessions were significant (Table S18b), though variable, with the Im

population always showing the lowest P_{index} values. The interaction population × treatment was significant. The Chl content showed the same P_{index} trend, with significant changes in all the accessions except for Im and Ga. Significant differences among the accessions and a

significant interaction accession \times treatment were detected (Table S18c).

3.3. Ni-accumulation in hydroponics

In all accessions, Ni concentration in roots and shoots increased with increasing external Ni concentration. Shoots showed values higher than roots in almost all treatments (Fig. 7). However, accessions differed significantly in root and shoot Ni accumulation. Two-way ANOVA indicated significant variation among the accessions and a significant effect accession \times treatment for both roots and shoots (Table S19).

Accumulation data were entered in the Brain-Cousens model to calculate hormetic and tolerance parameters based also on internal Ni concentration (MSD_{int} and $EC50_{int}$, Tables 2 and 3). Values of $EC50_{int}$ were different among accessions, but not related to ploidy level. Instead, the mean values of MSD_{int} in roots and shoots were higher in the tetraploid accessions. The parameters $EC50_{ext}$ and $EC50_{int}$ were positively related in roots (Table S16). Positive relations in roots and shoots were also observed for the two pairs of variables $MAX - MSD_{int}$ and $MSD_{ext} - MSD_{int}$, as well as in shoots for $EC50_{ext} - EC50_{int}$ (Table S17).

4. Discussion

4.1. Plant growth and Ni accumulation on serpentine soil

All the examined accessions exhibited an increase in plant biomass production when cultivated on artificial control soil, likely due to a higher nutrient availability as compared with the natural serpentine soil, which is typically infertile (Brooks, 1987; Kazakou et al., 2008).

However, the stressful conditions of serpentine soil caused different responses in diploid vs. tetraploid plants, as the latter were more able to grow and produce biomass. This finding is in line with the higher vigor of native tetraploid plants of *O. bertolonii*, shown by their larger size and more robust habit, the thicker and more branched woody stems, coupled with the increased size and mass of the seeds, and their faster rate and higher percentages of germination, in respect to diploids (Selvi and Vivona, 2022). A similar result was obtained by Feng et al. (2019) who found that colchicine-induced tetraploid plants of the Cd-hyperaccumulator *Sedum alfredii* Hance (Crassulaceae) produced higher biomass than diploid ones due to the so-called “gigas” effect (Stebbins, 1950). The latter is associated with the increased number of gene copies, which usually induces enhanced growth performances under various conditions (Bretagnolle and Thompson, 1995; Corneille et al., 2019) and tolerance to various forms of environmental stress (McIntyre, 2012; Allario et al., 2013; Godfree et al., 2017; Van De Peer et al., 2017; Stevens et al., 2020; Tossi et al., 2022).

The general reduction of leaf area in the *O. bertolonii* plants grown on serpentine substrate was in line with the decreased biomass production mentioned above. Similar results were obtained by Sambatti and Rice (2007) with Californian sunflowers and interpreted as an adaptive mechanism to reduce transpiration on the usually open and dry serpentine soil (Ivalú Cacho and Strauss, 2014; Coppi et al., 2022). The diploid *O. bertolonii* plants on serpentine soil showed a reduction in their leaf area and thickness, thus explaining their increased SLA. The consequence of this was a stronger decrease in leaf dry mass production, which further contributed to the lower biomass of the diploid plants. Plants often respond to low soil fertility by developing leaves with a high

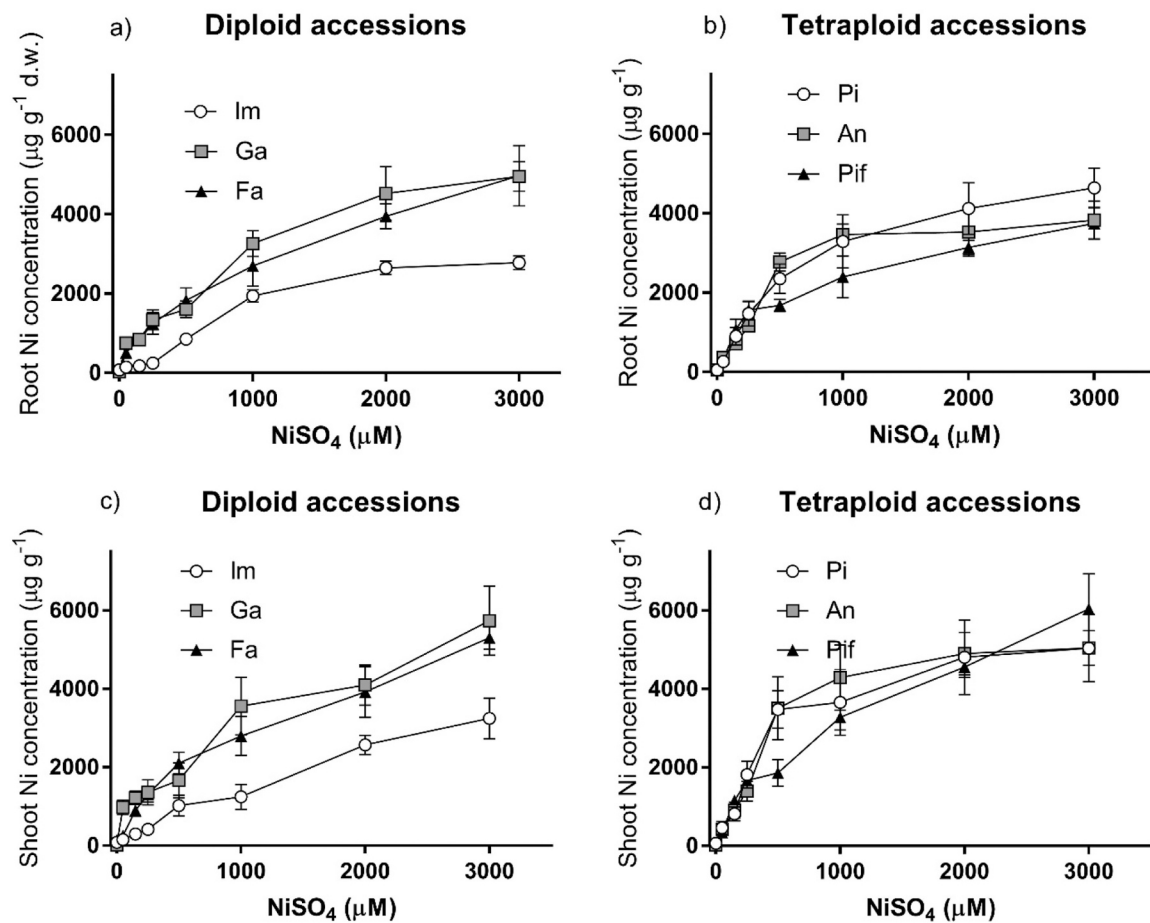


Fig. 7. Nickel accumulation ($\mu\text{g g}^{-1}$ d.w.) in roots and shoots of the diploid and tetraploid *O. bertolonii* accessions treated with eight NiSO_4 concentrations for seven days. a) root Ni concentration in diploid accessions; b) root Ni concentration tetraploid accessions; c) shoot root Ni concentration diploid accessions; d) shoot root Ni concentration tetraploid accessions. Values are means of 12 replicates \pm standard deviation.

mass per area as a resource-conservative strategy (Reich et al., 1997; Garnier et al., 2016), and low SLA values are considered to enhance serpentine tolerance (Fernandez-Goñig et al., 2012; Lazzaro et al., 2021). Hence, the lower SLA of the tetraploid plants from serpentine substrate is likely associated with their higher ability to tolerate the drought and infertility of this soil in respect to the diploids. In our experiment, however, there were no significant differences in LDMC, whose alterations are generally linked to stress-induced changes in leaf tissue density and, therefore, in the leaf morphological development and mesophyll/structural component ratio (Van Arendonk and Poorter, 1994; Niinemets and Kull, 2003).

Regarding the photosynthetic parameters, the reduced growth of *O. bertolonii* cultivated on serpentine soil was not due to an impaired photosynthetic efficiency, unlike in non-serpentine plants that display a strong decrease in F_v/F_m and P_{index} when grown on serpentine soils (Karatassiou et al., 2021). The photosynthetic efficiency was preserved despite the lower chlorophyll content in samples from the serpentine soil in comparison to those from control soil, probably caused by the imbalance in nutrients characteristic of serpentine (Brooks, 1987).

As typical for Ni-hyperaccumulators, shoot Ni concentration was invariably higher than root concentration and significantly above the threshold of $1000 \mu\text{g g}^{-1}$ d.w. of this metal (van der Ent et al., 2013; Deng et al., 2018;), with no significant differences between diploids and tetraploids. The only difference between the two chromosome races was the higher root Ni concentration showed by the diploid plants, that however did not result in any variation in shoot concentration. Because of the higher dry biomass of the tetraploids on serpentine soil (ca. 180% of the dry biomass of the diploids), the total amount of Ni in the above-ground parts of a single plant resulted, on average, significantly larger than in the diploids. Enhanced plant growth and biomass production are basic features for Ni-agromining applications (Kidd et al., 2018), thus suggesting tetraploid accessions as best candidates for the recovery of trace metals from serpentine soils. For the same reason, plants of *S. alfredii* with duplicated genome were indicated as a feasible alternative to diploid plants to promote the efficiency of phytoremediation (Feng et al., 2019). When these applications are based on metal accumulating species with infraspecific variation in ploidy level, the polyploid accessions are likely to adapt to new conditions and perform better than diploids (Prasad and De Oliveira Freitas, 2003; Paape et al., 2020; Tossi et al., 2022). In fact, only autotetraploid accessions of the facultative metallophyte *Arabidopsis arenosa* (L.) Lawal. were found on Cd and Zn contaminated sites in central Europe, most of them showing metal hyperaccumulation features, whereas diploid ones populated non-metalliferous substrates and showed low shoot metal concentrations (Gierón et al., 2021). In *O. bertolonii*, moreover, the degree of variation in shoot Ni content among tetraploid accessions was larger than among diploid ones, suggesting that further experimental screening within polyploids would be desirable to select the most efficient extractors.

4.2. Plant growth and Ni accumulation in hydroponics

The increase in root and shoot growth shown by all *O. bertolonii* accessions in the Ni low-dose zone of the hydroponics experiment was in line with recent results in other *Odontarrhena* taxa from the Balkan serpentine outcrops (Bettarini et al., 2021). This growth stimulatory effect of Ni in hyperaccumulators of this genus was partly attributed to an indirect ameliorative action of the metal on the plant photosynthetic machinery (Scartazza et al., 2022). The same mechanisms are likely to play a role also in the case of *O. bertolonii*, due the increase in P_{index} and Chl content in the metal low-dose zone. According to Bettarini et al. (2021), however, the Ni-mediated stimulation on growth and photosynthesis could also be related to a hyperaccumulation-mediated Ni cytosolic depletion in the low-dose zone. Actually, this feature was found in all the serpentine taxa of *Odontarrhena* analysed so far (Bettarini et al., 2021).

All the dose-response curves significantly fitted the Brain-Cousens model (Brain and Cousens, 1989), and the magnitude of the stimulating effect (HP) was always well above the 10% hormetic threshold proposed by Calabrese and Blain (2009). The hormetic parameters showed significant differences both among the accessions and between the two chromosomal races. In both roots and shoots, the higher maximum length (MAX) was reached by the tetraploid accessions and was coupled with a higher Ni concentration in the substrate required for the maximum stimulatory effect (MSD_{ext}), though not significantly for roots. Compared with diploids, tetraploid accessions thus needed higher Ni concentrations in the growth substrate for an optimal growth, as also supported by the positive correlation of shoot MAX with both MSD_{ext} and HP found when pooling all accessions together. This response is likely not associated with pre-adaptation of polyploids to higher Ni levels in their native serpentine soils in eastern Tuscany, as these levels were similar to those in the native sites of the diploids in the central and western outcrops of the region (Table 1).

In hydroponics, the range of Ni concentrations at which half of the reduction of plant growth was observed in all accessions (around $2000 \mu\text{M}$) was remarkably high in comparison to those of normal plants (below $5 \mu\text{M}$, Marschner, 1995), thus corroborating the known hyper-tolerance of Ni-hyperaccumulators (Manara et al., 2020). The $EC50_{ext}$ values showed significant variation among accessions, without any apparent effect of ploidy level, suggesting that the higher external metal requirement of the tetraploid plants was not coupled with a higher tolerance. Therefore, the latter could not be advocated to explain the larger size of the tetraploid plants. More probably, the enhanced vigour of the tetraploid accessions in nature and in the pot experiment is because the Ni stimulatory effect occurs in a low-dose zone comparable with the Ni concentrations in the circulating solutions of serpentine soils (Johnston and Proctor, 1981).

Concerning Ni accumulation, metal shoot concentration in all accessions was always higher than root concentration, as typical of hyperaccumulating plants (Deng et al., 2018). Remarkably, the tetraploid accessions showed a higher mean MSD_{int} than the diploid ones. To reach their maximal growth, therefore, the tetraploids were not only likely to need higher external Ni concentrations, but also higher ones inside their tissues, as confirmed by the positive relationship between the parameters MSD_{ext} and MSD_{int} in roots and shoots when pooling all the accessions together. This effect was likely due to more efficient hyperaccumulation mechanisms inducing higher Ni compartmentation and, therefore, cytosolic depletion in the tetraploid accessions. The presence of doubled gene copies controlling the vacuolar uptake, such as the IREG transporters indicated as overexpressed in some Ni hyperaccumulators (van der Pas and Ingle, 2019), could lead to a severe reduction of cytosolic Ni below the deficiency threshold, when the metal is at low concentration in the environment.

Our experimental findings suggest *O. bertolonii* to be a suitable model for further studies about the effects of (auto)polyploidy and genome duplication on the molecular and ecophysiological mechanisms of Ni-accumulation and tolerance in metallophytes. In line with Gierón et al. (2021), who found a link between Cd and Zn-hyperaccumulation and autotetraploidy in the facultative metallophyte *Arabidopsis arenosa*, we propose that infraspecific ploidy variation in these species can offer opportunities for novel discoveries that are not possible with taxa possessing “a compact diploid genome” ($2n = 16$), which have been formerly proposed as the optimal genetic models for the investigation of metal (Ni and Zn) accumulation in the Brassicaceae family (Peer et al., 2003, 2006).

When grown on the same serpentine soil, however, the higher values of MSD_{int} of the tetraploids, that is their putative higher Ni-tissue concentration for optimal growth, did not result in differences in shoot metal concentration with respect to diploids. Probably, the Ni concentration in the substrate, along with the longer exposure time, was able to generate a tissue metal concentration far above the MSD_{int} found in hydroponics, thus hiding any difference between the groups of plants.

As reported above for the external Ni concentrations, the tolerance parameter based on the internal Ni concentration ($EC_{50_{int}}$) showed variation among accessions but not apparently related with ploidy level. Since $EC_{50_{int}}$ can be considered a proxy of accumulation capacity (Galardi et al., 2007a), the diploids and tetraploids were likely not different in their mean level of hyperaccumulation. Therefore, the differences between the two chromosomal races appeared only at the low-dose zone for the internal concentration as well. When pooling all the accessions together, the significant relation between $EC_{50_{ext}}$ and $EC_{50_{int}}$ corroborated the positive relation between Ni tolerance and accumulation already observed in this and other species of *Odontarrhena* (Galardi et al., 2007a; Bettarini et al., 2021).

5. Conclusions

This study provides the first experimental insights into the effects of whole-genome duplication on the responses to Ni in a hyperaccumulating model plant with both diploid and tetraploid accessions. Despite a similar shoot Ni concentration, tetraploid plants contained a significantly higher amount of Ni per plant, due to a larger production of biomass. In hydroponics, a higher Ni requirement for optimal growth and a stronger stimulatory effect of low Ni-concentrations occurred in the tetraploids, whereas Ni tolerance to both external and internal element concentrations was similar to diploids. Thus, the selection of suitable ‘metal crops’ for environmental restoration practices and field biotechnological applications should be in favour of polyploid accessions, rather than diploid, when the candidate species includes both cytotypes.

CRedit authorship contribution statement

Iliaria Colzi: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Cristina Gonnelli:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Isabella Bettarini:** Methodology, Formal analysis, Data curation. **Federico Selvi:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2023.105403](https://doi.org/10.1016/j.envexpbot.2023.105403).

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