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ORIGINAL ARTICLE

Structural, physiological and genetic diversification of *Silene vulgaris* ecotypes from heavy metal-contaminated areas and their synchronous in vitro cultivation

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Abstract

Main conclusion **Results provide significant comparison of leaf anatomy, pigment content, antioxidant response and phenolic profile between individuals from miscellaneous populations and describe unified cultivation protocols for further research on stress biology.**

The plant communities growing on heavy metal-polluted areas have attracted considerable attention due to their unique ability to tolerate enormous amounts of toxic ions. Three ecotypes of *Silene vulgaris* representing calamine (CAL), serpentine (SER) and non-metallicolous (NM) populations were evaluated to reveal specific adaptation traits to harsh environment. CAL leaves presented a distinct anatomical pattern compared to leaves of SER and NM plants, pointing to their xeromorphic adaptation. These differences were accompanied by divergent accumulation and composition of photosynthetic pigments as well as antioxidant enzyme activity. In CAL ecotype, the mechanism of reactive oxygen species scavenging is based on the joint action of superoxide dismutase and catalase, but in SER ecotype on superoxide dismutase and guaiacol-type peroxidase. On the contrary, the concentration of phenylpropanoids and flavonols in the ecotypes was unchanged, implying the existence of similar pathways of their synthesis/degradation functioning in CAL and SER populations. The tested specimens showed genetic variation (*atpA*/*Msp*I marker). Based on diversification of *S. vulgaris* populations, we focused on the elaboration of similar in vitro conditions for synchronous cultivation of various ecotypes. The most balanced shoot culture growth was obtained on MS medium containing 0.1 mg l^{-1} NAA and 0.25 mg l^{-1} BA, while the most abundant callogenesis was observed on MS medium enriched with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} BA. For the first time, unified in vitro protocols were described for metallophytes providing the opportunity to conduct basic and applied research on stress biology and tolerance mechanisms under freely controlled conditions.

Keywords Anatomy · Antioxidants · Facultative metallophyte · Photosynthetic pigments · Restriction fragments length polymorphism · Tissue culture

Abbreviations

CAL	Calamine ecotype
HMs	Heavy metals
NM	Non-metallicolous ecotype
SER	Serpentine ecotype

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Introduction

Many centuries of metalliferous ore exploitation have contributed to the creation of waste heaps, mining pits or quarries that have a profound negative impact on the surrounding environment. As habitats for plants, post-industrial terrains constitute a combination of unfavorable conditions that are often inimical to successful vegetation establishment. The substratum analysis has shown that waste material is characterized by a low concentration of organic matter, unfavorable pH and nutrient deficit and, at the same time, extremely high concentration of heavy metals (HMs; Koszelnik-Leszek 2007; Ciarkowska et al. 2017). Additionally, plants on waste heaps are exposed to drought, high insolation and strong wings. These environmental factors govern the process of spontaneous succession and significantly reduce the pool of plant species that are able to colonize chemically degraded areas.

Plant species or specialized ecotypes have adapted to excess amounts of HMs and exhibit a greater ability to survive in contaminated habitats than species from unpolluted sites (Mohtadi et al. 2012; Muszyńska et al. 2018a). One of them is *Silene vulgaris* which in Poland can be often found on meadows, fields and in forests (Koszelnik-Leszek and Bielecki 2013). The exceptional adaptation abilities of this species has led to the occurrence of diversified ecotypes that can thrive even in extremely harsh environments such as waste heaps created as a result of calamine or serpentine exploitation (Wierzbicka and Panufnik 1998; Koszelnik-Leszek 2017). On HMs-contaminated areas, plant tolerance to potentially toxic elements may be achieved by extracellular strategies that include the modification of soil pH, ion complexation with root exudates or symbiosis with microorganisms (Maestri et al. 2010). Another mechanism to cope with metal toxicity is metal exclusion from the shoots. Thereby, the majority of species appearing on metal-enriched areas behave as "excluders" that retain most of the HMs in their roots and reduce the transport of ions to the shoots. This strategy has also been observed in populations of *S. vulgaris* growing on metal-contaminated soils (Koszelnik-Leszek 2007; Ciarkowska and Hanus-Fajerska 2008; Mohtadi et al. 2012). Some other species, called "accumulators", exhibit a contrasting behavior and accumulate significantly higher concentrations of toxic ions in shoots than in roots. Among them, a relatively small number of species, called hyperaccumulators, can accumulate HMs at extraordinarily high concentrations in their aboveground tissues rather than in roots (Mohtadi et al. 2012). The presence of metal ions in plant tissues suggests the existence of defense mechanisms that allow avoiding their harmful effects. Studies on the adaptation and acclimatization to HMs have been also carried out in the *Silene* genus. For *S. vulgaris*, the complexation of toxic ions with organic acids (Harmens et al. 1994) or free amino acids (Nadgórska-Socha et al. 2009) has been demonstrated. Many experiments have indicated the important role of phytochelatins and glutathione in the response of *S. vulgaris* to HMs (Nadgórska-Socha et al. 2009; Sobrino-Plata et al. 2013; Koszelnik-Leszek 2017). Compared to the abundant knowledge on tolerance strategy in *S. vulgaris*, relatively little is known about the antioxidant machinery preventing oxidative stress which occurred as a consequence of toxic ion penetration into the protoplast.

The ability of certain plants to tolerate, detoxify and store high HMs concentrations in their tissues is of a great importance for development of biological methods of soil cleanup. Nevertheless, the current possibility to exploit plant potential in environmental remediation is slightly restricted by limited understanding of plant metabolic pathways and adaptation mechanisms. Plant cell tissue and organ cultures offer a range of experimental advantages in research on biotic and abiotic stress physiology or genetic and biochemical basis of tolerance (Al Khateeb and Al-Qwasemeh 2014; Muszyńska et al. 2017). In vitro techniques provide fully controlled conditions, particularly with regard to medium composition and thus eliminate many interactions that could disturb the straightforward effect of the studied factors. The growth in aseptic environment enables distinguishing the plant responses and their capabilities to contaminant detoxification from the actions of associated microbes normally present in the rhizosphere or within plant tissues (Lebeau et al. 2008). The opportunity to standardize in vitro conditions and the relative homogeneity of cultured explants helps to enhance the repeatability of results in comparison with environmental studies and to shorten the time of cultivation (Doran 2009). Besides basic research, in vitro methods allow to efficiently propagate valuable plant material excluding the variability between individual specimens that can be directly used on contaminated areas (Ciarkowska and Hanus-Fajerska 2008; Muszyńska et al. 2017). The optimization of micropropagation protocols is also necessary for genetic manipulation and selection of plants tolerant to various abiotic and biotic stresses (El-Minisy et al. 2016; Muszyńska and Hanus-Fajerska 2017). The elaboration of aseptic culture conditions seems to be a crucial stage of successful experiments to predict plant responses to environmental contaminants as well as to improve phytoremediation technologies.

In the current research, we have compared three contrasting *Silene vulgaris* ecotypes at various levels of organism organization to reveal specific features of metal-tolerant and reference specimens growing in natural conditions. The first aim of this study was to evaluate the anatomic, physiologic and genetic diversification of *S. vulgaris* specimens

taken from calamine, serpentine and non-metallicolous populations. Taking into account the possibilities created by in vitro techniques to study the adaptation mechanisms and improvement of environmental technologies, we decided to work out protocols to cultivate the tested ecotypes in a synchronic manner under aseptic conditions. Thus, in the second experimental step, the protocols of tissue and organ cultures that enable standardizing in vitro methods for further basic and applied research in the domain of stress reactions in dicotyledonous plants were elaborated.

Materials and methods

Plant material

Three ecotypes of *Silene vulgaris* that spontaneously appear in various ecological niches in Poland were investigated in this study. The control, non-metallicolous ecotype originated from natural non-contaminated stand in Zielonka near Warsaw (described further as NM). The metallicolous ecotypes originated from calamine (described further as CAL) and serpentine (described further as SER) waste heaps. CAL ecotype colonizes post-flotation tailing created as a result of lead and zinc ore mining and processing, near Bolesław city in Olkusz Ore-Bearing Region, southern Poland (50°17′N, 19°30′E). In the substratum on which tested CAL specimens occurred, the concentration of total Zn, Pb and Cd forms reached about 10,690 mg, 8060 mg and 85 mg kg^{-1}, respectively (Ciarkowska et al. 2017). SER ecotype grows on a post-mining dump connected with exploitation of serpentine rocks, near a small town Wiry, localized not far from western slopes of the Ślęża Massif (50°50′N, 16°38′E). In the place of SER plant sampling, the average concentration of total forms of Ni was 1300 mg kg^{-1}, while Cr was 461 mg kg^{-1} (Koszelnik-Leszek 2007). On both sites vegetation cover is rather poor and dispersed (occurring on about 35% of area). However, *S. vulgaris* more and more frequently appears on calamine waste heap or even dominates and creates more or less even patches on serpentine one.

Plant characterization in their natural habitats

Leaf blade morphology and anatomy

Biometric measurements, such as leaf blade length and width in the middle of its length, were evaluated for 25 leaf blades of each type (five randomly chosen leaves from each plant). For anatomical observation, ten fragments (approximately 5 × 5 mm) of fully expanded leaves taken from plants growing in natural conditions were fixed for 3 h according to Karnovsky (1965) and rinsed four times in cacodylate buffer. The samples were dehydrated in an ethanol series, substituted by propylene oxide and finally embedded in glycid ether 100 epoxy resin (Serva, Heidelberg, Germany). Polymerization was performed at 60 °C for 24 h. Semithin sections (3 µm thick) were prepared with a Jung RM 2065 microtome and stained with methylene blue and azure B prior to examination under a light microscope (Olympus-Provis). Several leaf anatomical features such as total thickness, number and thickness of palisade and spongy cell layers, size of parenchyma cells and stomata number per 300 µm of adaxial and abaxial surface were measured with cellSens Standard program (Olympus-Provis). For each ecotype, the measurements of whole leaf thickness and particular tissue were carried out on ten leaf fragments, and the size of palisade and spongy parenchyma cells was taken from 100 cells of these ten chosen leaves.

Determination of photosynthetic pigment content

Whole aboveground parts of plants for physiological and genetic analyses were collected from natural habitats, immediately placed on dry ice in a styrofoam container and transported to the laboratory where leaf samples were ground in liquid nitrogen and frozen at − 80 °C.

Spectrophotometric determination of photosynthetic pigments was performed according to Lichtenthaler (1987). Leaf samples (0.2 g) were homogenized with 5.0 ml of 80% acetone in ice-cold conditions and centrifuged (4 °C, 15 min, 4800*g*). The absorbance of chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and total carotenoids (car) was measured at 470, 646 and 663 nm, respectively. The Wellburn's equations (Wellburn 1994) were used to calculate the pigment content. Total chlorophylls (chl $a+b$), the chlorophyll *a*/*b* ratio (chl *a*/*b*) and the ratio of total chlorophylls to carotenoids (chl $a+b$/car) were also calculated.

Radical scavenging activity

Stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to test the radical scavenging activity of *S. vulgaris* leaves (Pekkarinen et al. 1999). The changes in absorbance of DPPH· solution, following reduction of DPPH, were measured at 517 nm at the moment of methanolic extract addition and after 30 min. The antioxidant activity of extracts was expressed in % of reduced DPPH· radical by a unit of plant extract.

Assessment of secondary metabolites profile

Leaf samples (0.2 g) were homogenized with 10 ml of 80% (v/v) methanol and centrifuged (4 °C, 15 min, 4800*g*). The Folin–Ciocalteu assay (Swain and Hillis 1959) was used to estimate polyphenol content. The absorbance of the samples was measured at 740 nm with BioSpectrometer kinetic

(Eppendorf, Hamburg, Germany). Gallic acid was used as a standard. Additionally, the concentration of total secondary metabolites with double bonds in their structure, phenylpropanoids, flavonols and anthocyanins was determined according to Fukumoto and Mazza (2000). This method allows to detect compounds showing maximum absorbance at 280, 320, 360 and 520 nm, respectively. The methanolic supernatant was mixed with 0.1% (v/v) HCl (in 96% ethanol) and 2% (v/v) HCl (in water), and after 15 min the absorbance was measured. The content of phenolic compounds was expressed in mg of the respective standard equivalents per 100 g of fresh weight (FW).

Measurements of antioxidant enzyme activity

Enzyme extracts were prepared by grinding 200 mg shoot samples in a mortar with quartz sand and an ice-cold extraction buffer containing: 50 mM potassium phosphate buffer (pH 7.0), 2 mM 2-mercaptoethanol, 0.5% (v/v) Triton X-100, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Next, samples were incubated on ice bath for 20 min and centrifuged (4 °C, 20 min, 16,000*g*); the obtained extracts were used to determine the enzyme activities.

Superoxide dismutase (EC 1.15.1.1, SOD) activity was assayed according to Kostyuk and Potapovich (1989). An activity reagent was prepared by mixing equal volumes of 67 mM K/Na phosphate buffer (pH 7.8) and 25 mM EDTA. The pH value of this reagent was adjusted to 10.0 by tetramethylethylenediamine (TEMED). Next, 1 ml of activity reagent was added to 0.1 ml of extract (first diluted with Milli-Q water, 1:00). The reaction was started by the addition of 0.1 ml of 2.5 µm quercetin in DMSO and the absorbance at 406 nm was recorded immediately and again after 20 min. SOD activity was expressed in arbitrary units (the amount of SOD that inhibits superoxide-driven oxidation of quercetin by 50%) per gram of FW.

The activity of the other three antioxidant enzymes—catalase (EC 1.11.1.6, CAT), guaiacol-type peroxidase (EC 1.11.1.7, GOPX) and glutathione peroxidase (EC 1.11.1.9, GPX)—was measured as described previously by Muszyńska et al. (2018a) with minor modifications relying on the appropriate selection of the volume of enzyme extract used for the measurements.

Restriction fragments length polymorphism (RFLP) analysis

Bulked sample analysis, which is widely used in plant population biology (Liu et al. 2018), was also applied in this study. Leaves of 25 individuals from each population were sampled and bulked for one sample and the genomic DNA was then purified from bulked samples. The PCR–RFLP procedure followed has been described previously by Welch et al. (2006). Total genomic DNA was extracted from the leaves of NM, CAL and SER plants using Plant and Fungi DNA Purification Kit (EURx, Gdansk, Poland). The amounts of isolated DNA were measured spectrophotometrically with BioSpectrometer kinetic (Eppendorf, Hamburg, Germany) equipped with µCuvette G1.0 and its purity and integrity were checked on a 1.2% (w/v) agarose gel stained with SimplySafe (EURx). *S. vulgaris*-specific primers for mitochondrial genes *atpA* (GenBank:DQ422872) and *cox1* (GenBank:DQ422877) were adopted from Welch et al. (2006) and primer oligonucleotide sequences are shown in Suppl. Table S1. PCR amplifications were conducted in 25-µl reaction volumes that contained 6 ng of double-stranded (ds)DNA, 10 mM of each gene-specific primer pairs, 0.2 mM dNTPs, 1 × reaction buffer, 1.25 U DreamTaq DNA polymerase (Fermentas/Thermo Scientific, Waltham, MA, USA) and sterile water. The PCR program started with initial hot-start activation at 95 °C for 5 min and next consisted of 35 cycles of 95 °C for 30 s, 60 °C for 30 s, then 72 °C for 60 s and final elongation at 72 °C for 20 min. Negative controls were run without dsDNA templates. Amplified fragments were electrophoresed on a 1.2% (w/v) agarose gels in 1 × TBE running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.3), visualized by SimplySafe and photographed using Molecular Imager Gel Doc™XR + Imaging System (BioRad, München, Germany). Next for RFLP profiling, 5 µl of PCR products were digested with restriction endonucleases (EURx) according to the manufacturer's instruction and visualized on 2.5% (w/v) agarose gels as described above. *atpA* amplicons were digested in separate reactions with *Alu*I and *Msp*I, while the digestion with *Dde*I and *Msp*I for *cox1* was performed. All experiments were conducted in three biological replicates including three independent genomic DNA extractions.

Laboratory study under in vitro conditions

The culture initiation

To start in vitro experiments, seeds collected from specimens growing in natural conditions as described above were used. The time of effective seed surface decontamination was established experimentally. The seed samples were immersed in 70% (v/v) ethanol for 1 min and decontaminated with 0.05% mercuric chloride for 3, 4, 5 or 10 min. After three washes with sterile distilled water, the seeds of the respective ecotype were placed in Petri dishes covered with MS medium (Murashige and Skoog 1962) devoid of plant growth regulators (PGR). After elaboration of the optimal time for surface decontamination, the influence of lighting on seed germination was tested. 30 seeds of each ecotype were placed on a Petri dish with MS medium without PGR.

Three plates, each with ten seeds of the respective ecotype, were kept either under white fluorescent light or in darkness. Germination test was carried out in a growth chamber at 24 °C day/20 °C night. The number of germinated seeds was evaluated 10 days after sowing when it did not change significantly.

Shoot multiplication protocol

Shoots of aseptically obtained seedlings were used as primary explants to establish a proliferating shoot culture. The seedling shoots bearing an apical meristem were placed onto MS basal medium salts and vitamins supplemented with sucrose (20 g l^{-1}). The composition of PGR and others additives was chosen on the basis of previously elaborated protocols for specific *S. vulgaris* ecotypes or other taxonomically related species from Caryophyllaceae (Hanus-Fajerska 2011; Muszyńska and Hanus-Fajerska 2017; Muszyńska et al. 2018b). The assumption of this experimental stage was to obtain a comparable growth of all studied *S. vulgaris* ecotypes. Therefore, the following media were tested:

1. MS + 0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BA (described further as M1);
2. MS + 0.1 mg l^{-1} NAA + 0.25 mg l^{-1} BA (described further as M2);
3. MS + 0.05 mg l^{-1} NAA + 0.5 mg l^{-1} BA (described further as M3);
4. MS + 0.2 mg l^{-1} IAA + 1.0 mg l^{-1} 2iP + 0.65 g l^{-1} calcium gluconate (described further as M4).

In the course of micropropagation experiments, vessels of 200 ml capacity filled with 50 ml of respective media were used, and five shoot explants were put in a single vessel onto the freshly prepared medium. In total, 50 explants in every single treatment were evaluated (ten flasks per treatment). The subcultures were done every 4 weeks, and after 12 weeks the obtained shoots and spontaneously regenerated roots (if there were any) were counted and measured. The micropropagation coefficient (MC) was calculated using the following formula:

$$MC = \text{number of induced adventitious shoots} / \text{total number of explants.}$$

The rooting phase

The in vitro obtained shoots of about 20 mm were used to investigate the rooting efficiency. Five explants per 200 ml vessel were explanted on the respective rooting medium consisting of the same ingredients that provided the best shoot growth (described above as M2), but the concentration of macro- and micronutrients was reduced to 1/2 (described further as 1/2 MSR) or 1/3 (described further as 1/3 MSR). After 4 weeks, the adventitious roots were counted and accurately measured.

The organ culture during proliferation and rooting stage was maintained in a growth chamber at 24 °C day/20 °C night, under a 16 h photoperiod. The irradiance at the shoot/plantlet level was equal to 80 µmol $m^{-2}s^{-1}$.

Microcutting acclimatization

The rooted plantlets were transplanted to plastic pots (90 mm in diameter), filled with a mixture of perlite, horticulture soil and calamine substratum for CAL microplants or serpentine substratum for SER microplants (1:1:3, by vol.), whereas for NM microplants both calamine and serpentine substrata were applied. As a control substrate, a mixture of perlite and horticultural soil (1:1, v/v) was used. The chemical properties of calamine and serpentine substratum have been previously described in detail by Ciarkowska et al. (2017) and Koszelnik-Leszek (2007), respectively. For each treatment ten microplants of each ecotype were planted. During the first 2 weeks, plants were protected with transparent containers to provide optimum humidity and were kept in a conditioned room at a temperature of 18–20 °C. The percentage of survived specimens was calculated after 4 weeks of ex vitro cultivation.

Callus culture initiation and further proliferation

The cotyledons, hypocotyls and fully expanded leaves of aseptically obtained seedlings were used for callus induction and proliferation. Both adaxial and abaxial fragments of leaf explants were put on B5 (Gamborg et al. 1968) or MS medium supplemented with PGR according to the protocol described by Jack et al. (2005) and Hanus-Fajerska (2011):

1. B5 + 1.2 mg l^{-1} 2.4-D + 0.2 mg l^{-1} BA (described further as C1);
2. MS + 1.2 mg l^{-1} 2.4-D + 0.2 mg l^{-1} BA (described further as C2);
3. MS + 0.05 mg l^{-1} NAA + 0.5 mg l^{-1} BA (described further as C3);
4. MS + 0.5 mg l^{-1} NAA + 5.0 mg l^{-1} BA (described further as C4).

The media were solidified with 0.9% Bacto agar, and their pH was adjusted to 5.8 before autoclaving (121 °C for 20 min). For each combination, five explants were placed in each of ten Petri dishes. Explants were cultured at 24 ± 1 °C both in light (5 dishes) and in darkness (5 dishes). After 4 weeks of culture initiation, the percent of explants forming callus tissue was evaluated. The obtained callus tissue was passaged every 4 weeks on analogous fresh media to stabilize culture and proliferation. After 12 weeks, callus

morphology and their visual increase were assessed. Anatomical features were evaluated in four callus mass of each ecotype (approximately, 5 × 5 mm in size) prepared in the same way as described above for leaf samples.

Statistical analysis

All results from both field and laboratory studies were subjected to one-way ANOVA analysis (STATISTICA Software) with the exception of seed germination ability for which two-way ANOVA was applied (factors: ecotype and light condition). To determine differences between ecotypes at $\alpha = 0.05$, a post hoc Fisher's test was performed. The experimental setup under in vitro conditions was repeated three times. Microcuttings were randomly assigned to the treatments. The data obtained for shoot and callus cultures were separately verified for each medium.

Results

The comparison of different *Silene vulgaris* ecotypes

Morphological and anatomical leaf features

Silene vulgaris leaves were different between ecotypes. The smallest and the most narrowed leaves were characteristic for CAL specimens, while the size and shape of SER and NM ones were similar to each other (Table 1). The microscopic measurements revealed a significant variation in the analyzed traits (Table 1). The metallicolous populations had thicker leaves than non-metallicolous ones, which ranged from 76 to 99 µm for CAL and SER, respectively, versus 67 µm for NM specimens (in the maximum distance). The increase in leaf thickness resulted from the volume of parenchyma cells, rather than the number of palisade and spongy layers that did not vary between ecotypes. In NM leaves the thickness of the palisade layer was similar to the spongy one, while in metallicolous ecotypes a thicker layer of spongy parenchyma was noticed (Fig. 1a–d). The most typical arrangement of leaf anatomy was observed in CAL leaves, in which the average length of palisade and spongy cells amounted to 16.3 and 10.4 µm, respectively (Fig. 1a). In the mesophyll of CAL leaves, many crystals were observed (Fig. 1b). In SER (Fig. 1c) and NM leaves (Fig. 1d), the size of both types of parenchyma cells was not so clearly differentiated. In NM ecotype, the number of stomata was almost two times higher in the abaxial leaf surface than in the adaxial one. On the contrary, in CAL leaves the stomata number per 300 µm of epidermis did not change on both surfaces (4.6), while in SER leaves the value was quite similar, independent of leaf side, and ranged from 8.3 to 9.3.

Physiological analysis: photosynthetic pigments, radical scavenging activity and non-enzymatic and enzymatic antioxidants

To determine if *S. vulgaris* ecotypes differ in the composition of photosynthetic pigments and in the antioxidant apparatus efficiency, spectrophotometric analyses were performed. It was found that both chl *a* and *b* amounts varied significantly in all tested ecotypes, namely the highest contents were observed in NM, lower in CAL and the lowest in SER specimens (Table 2). Such tendency was also well reflected in total chlorophyll content (chl $a+b$). The chl a/b ratio was considerably decreased in CAL ecotype in comparison to the control NM. In case of carotenoids, the highest content was noticed in NM leaves, while the lowest was in SER leaves in which carotenoids presented an about 3.5-fold lower concentration than in the reference NM ecotype. The calculated ratio of total chlorophylls to carotenoids considering them as physiological indicator of plant fitness reached significantly higher values in NM specimens than in CAL and SER ones. In both metallicolous ecotypes, this value

Table 1 Leaf anatomical traits determined on *Silene vulgaris* specimens from non-metallicolous (NM), calamine (CAL) and serpentine (SER) populations

Traits	NM ecotype	CAL ecotype	SER ecotype
Leaf blade length (mm)	38.67 a* ± 3.23	20.42 b ± 2.42	36.65 ± 5.79
Leaf blade width (mm)	9.85 a ± 1.26	6.04 b ± 1.22	9.48 a ± 1.13
Total leaf thickness (µm)	67.11 c ± 5.68	76.79 b ± 3.32	97.52 a ± 5.03
Number of palisade cell layers	2	1–2	2
Thickness of palisade parenchyma (µm)	20.61 c ± 2.35	22.01 b ± 2.03	25.57 a ± 2.56
Length of palisade cells (µm)	13.60 b ± 1.61	16.29 a ± 1.89	12.95 b ± 1.99
Number of spongy cell layers	2	2	2–3
Thickness of spongy parenchyma (µm)	19.27 c ± 1.37	27.76 b ± 3.44	36.14 a ± 1.96
Length of spongy cells (µm)	9.29 b ± 0.81	10.44 b ± 1.59	12.95 a ± 0.86
Stomata number per 300 µm of adaxial surface	8.33 a ± 0.47	4.66 c ± 0.47	6.33 b ± 0.58
Stomata number per 300 µm of abaxial surface	11.33 a ± 0.57	4.66 c ± 0.47	9.33 b ± 0.94

*Means indicated by the same letter do not significantly differ at $\alpha = 0.05$ according to Fisher's test

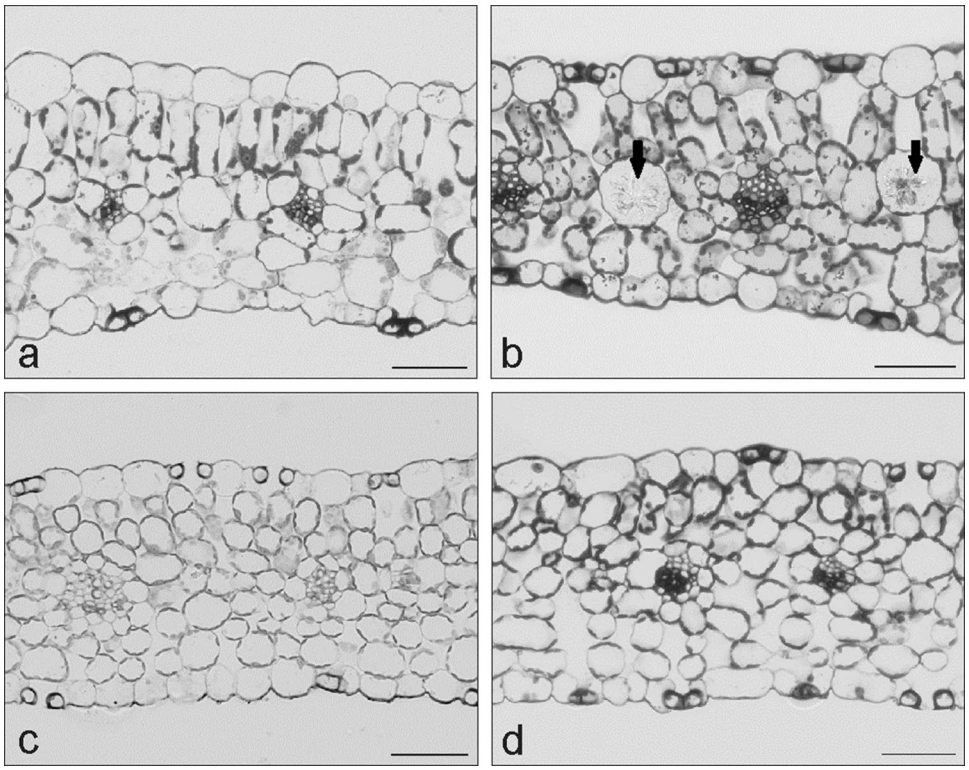

Fig. 1 Anatomical structure of leaves collected from plants representing various ecological niches. **a** Transverse section of calamine leaves with typical arrangement of palisade and spongy parenchyma. **b** Crystals (arrows) in parenchyma cells of calamine leaves. **c** Transverse section of leaves taken from serpentine specimens. **d** Transverse section through leaves of non-metallicolous specimens. Bar = 50 µm

Table 2 Photosynthetic pigment content and its ratios in *Silene vulgaris* leaves depending on ecotype

Parameter	NM ecotype	CAL ecotype	SER ecotype
Chlorophyll *a* (mg g^{-1} FW)	1.31 a* ± 0.10	0.66 b ± 0.02	0.33 c ± 0.02
Chlorophyll *b* (mg g^{-1} FW)	0.39 a ± 0.04	0.24 b ± 0.01	0.12 c ± 0.01
Chlorophyll *a*+*b* (mg g^{-1} FW)	1.71 a ± 0.13	0.90 b ± 0.03	0.45 c ± 0.02
Chlorophyll *a*/*b*	3.31 a ± 0.07	2.79 b ± 0.17	2.99 ab ± 0.21
Carotenoids (mg g^{-1} FW)	0.28 a ± 0.03	0.19 b ± 0.01	0.08 c ± 0.01
Chlorophyll *a*+*b*/carotenoids	6.06 a ± 0.38	4.72 b ± 0.09	5.10 b ± 0.37

Means indicated by the same letter do not significantly differ at $\alpha = 0.05$ according to Fisher's test

NM non-metallicolous ecotype, *CAL* calamine ecotype, *SER* serpentine ecotype

*Values are means of three replicates ± SD

was similar and amounted to 4.7–5.1, while in the NM one it was higher, about 15–20% (Table 2).

The antioxidant capacity of *S. vulgaris* leaves was significantly elevated in CAL ecotype, in which the highest efficiency of radical scavenging occurred (Table 3). Leaf extracts of NM and SER ecotypes showed similar activity to reduce DPPH (2,2-diphenyl-1-picrylhydrazyl) radical at the level of 9.5%.

Leaves of non-metallicolous ecotype accumulated significantly higher amounts of total secondary metabolites as well as particular phenol groups than metallicolous ecotypes (Table 3). In CAL and SER specimens, the content of examined compounds was similar and did not differ statistically; however, higher values were obtained for CAL leaves. The exception was polyphenols, the levels of which varied significantly between ecotypes and reached 126 mg for NM ecotype, 76 mg for CAL and 57 mg for SER ectotypes per 100 g of FW. The highest content of anthocyanins (about 136 mg per 100 g FW) was accumulated in NM leaves, while a decrease amounting to two to five times was noticed in CAL and SER leaves, respectively.

The activity of glutathione peroxidase (GPX) and superoxide dismutase (SOD) was comparable in both metallicolous ecotypes and significantly lower in the case of GPX or higher in the case of SOD compared to NM specimens (Table 4). The measurements of catalase (CAT) and guaiacol-type peroxidase (GOPX) activity revealed a diversification of metallicolous ecotypes. In CAL leaves, CAT activity reached the highest value (306 µmol min^{-1} g^{-1}), while GOPX activity was the lowest (187 µmol min^{-1} g^{-1}) in comparison to the other

Table 3 Radical scavenging activity (RSA, in %) and secondary metabolites profile (mg 100 g^{-1} FW) in leaves of non-metallicolous (NM), calamine (CAL) and serpentine (SER) *Silene vulgaris* specimens

Parameter	NM ecotype	CAL ecotype	SER ecotype
RSA (%)	9.26 b* ± 0.94	11.91 a ± 0.53	9.47 b ± 0.51
Total secondary metabolites	1569.35 a ± 24.30	1193.78 b ± 81.21	1085.01 b ± 52.45
Phenylpropanoids	495.85 a ± 13.31	410.71 b ± 27.69	383.38 b ± 16.63
Flavonols	689.47 a ± 5.95	601.39 b ± 30.98	558.67 b ± 28.43
Polyphenols	126.09 a ± 8.07	76.03 b ± 4.93	57.28 c ± 5.86
Anthocyanins	136.08 a ± 14.06	58.93 b ± 4.06	25.16 c ± 2.63

Means indicated by the same letter do not significantly differ at $\alpha = 0.05$ according to Fisher's test

*Values are means of three replicates ± SD

Table 4 Activity of antioxidant enzymes in leaves of non-metallicolous (NM), calamine (CAL) and serpentine (SER) *Silene vulgaris* specimens growing in natural conditions

Antioxidant enzyme	NM ecotype	CAL ecotype	SER ecotype
Glutathione peroxidase (μmol min^{-1} g^{-1})	214.83 a* ± 9.89	165.05 b ± 14.17	163.14 b ± 24.94
Superoxide dismutase (U g^{-1})	56.39 b ± 11.58	178.88 a ± 24.43	179.32 a ± 11.72
Catalase (μmol min^{-1} g^{-1})	278.03 ab ± 18.38	306.66 a ± 13.58	247.96 b ± 31.35
Guaiacol peroxidase (μmol min^{-1} g^{-1})	466.79 b ± 33.01	187.97 c ± 28.19	841.17 a ± 33.23

Means indicated by the same letter do not significantly differ at $\alpha = 0.05$ according to Fisher's test

*Values are means of three replicates ± SD

ecotypes. In SER leaves, these enzyme activities exhibited the opposite relationship to that in CAL leaves, and thus their activity was about 1.2-fold lower and almost 4.5-fold higher for CAT and GOPX, respectively.

Genetic profiling

To find out possible differences at the genetic level between the tested *S. vulgaris* ecotypes, PCR–RFLP was conducted. Using the *S. vulgaris*-specific primers for ATP synthase subunit alpha (*atpA*) gene, an amplified product of about 750 bp was obtained, while the primers for cytochrome oxidase subunit 1 (*cox1*) gene amplified a product of about 1400 bp length. These results were representative for all examined ecotypes. We showed that after digestion of amplicon *atpA* with *Msp*I, the polymorphism was disclosed (Fig. 2). The restriction banding pattern differentiated NM ecotype from CAL and SER ecotypes. No polymorphism of restriction fragments was observed for the restriction endonuclease *Alu*I and for digestion of *cox1* amplified fragment with *Msp*I and *Dde*I (Fig. 2).

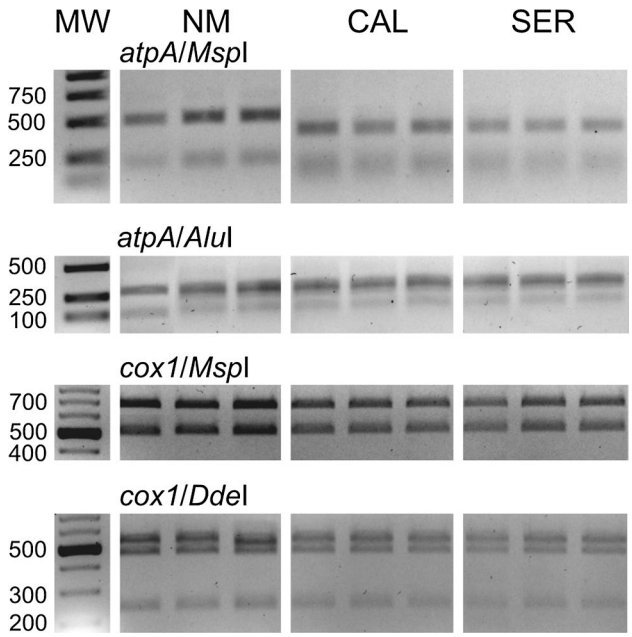

Fig. 2 Analysis of restriction fragments length polymorphism (RFLP) variation of non-metallicolous (NM), calamine (CAL) and serpentine (SER) *Silene vulgaris* ecotypes

The synchronous cultivation of *Silene vulgaris* ecotypes under tissue culture

Considering the diversity of the tested ecotypes, we aimed at elaboration of culture conditions that would be identical for all tested ecotypes. Such standardization of a unified in vitro approach was undertaken for further basic and applied research on *S. vulgaris.*

Culture initiation

To start in vitro cultures, different sterilizing regimes of *S. vulgaris* seeds were tested. The most effective surface decontamination of seed samples representing the studied ecotypes was achieved using 0.05% solution of $HgCl_2$ for 4 min. To optimize the condition for germination, seeds were

kept either in white light or in darkness. It was found that both ecotype and light treatment significantly affected germination ability; however, the combinations of these two factors did not influence this process. The seeds of NM ecotype germinated in about 90%, independent of light/no light condition (Fig. 3). The germination ability of metallicolous ecotypes was significantly lower than that of non-metallicolous one, and ranged from 58 to 53% for CAL and from 53 to 33% for SER. The greatest number of properly shaped CAL and SER seedlings that could easily develop to aseptic plantlets was obtained in light. Under this condition, the germination of CAL seeds was irregular and differed strongly between repetitions.

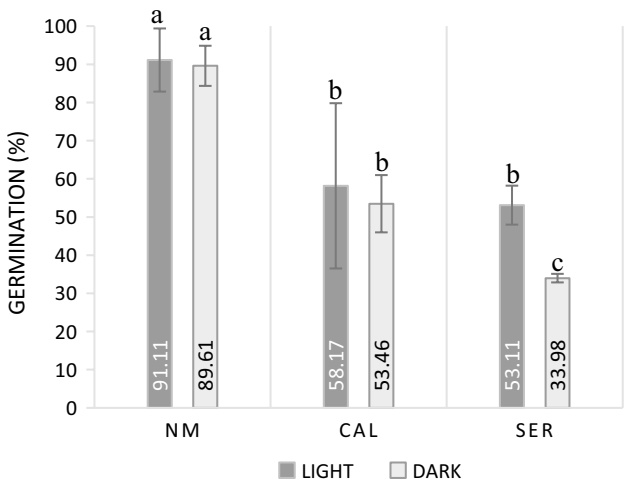

Fig. 3 Germination ability of non-metallicolous (NM), calamine (CAL) and serpentine (SER) *Silene vulgaris* seeds under different light conditions. Different letters indicate statistical significance of means ($n=30$ for each ecotype) according to two-way ANOVA and post hoc Fisher's test at $\alpha=0.05$

Shoot proliferation

Aseptically obtained seedlings were used to initiate shoot cultures. The morphogenetic response of these cultures was variable depending on the particular genotype and the treatment medium (Fig. 4; Table 5). On M1 medium consisting of 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA, shoots of CAL ecotype proliferated the most intensively (MC = 5.6); however, they were thick, relatively short (about 27 mm long), and sometimes vitreous. On the contrary, the highest shoot length of 54 mm and the highest parameters of examined rooting characteristics were noted on NM culture. The adventitious roots developed from callus that occurred abundantly in the shoot bases of all ecotypes, but with the highest intensity on SER shoots. Undesirable in such a case, an intensive callus development occurred in shoot bases during their cultivation. To regenerate multiple shoots without any intervening callus phase, MS medium with a concentration of auxin (medium M2) or cytokinin (medium M3) reduced by half was tried. Micropropagation coefficient calculated after 12 weeks of cultures on M2 medium reached comparable values of 2.2–2.3 for all tested ecotypes (Fig. 4). Microshoots that were formed by axillary branching reached similar length of about 45 mm and the minimal callus proliferation during the whole time of cultivation was observed (Table 5). Although such a combination of plant growth regulators sometimes stimulated spontaneous rhizogenesis, their efficiency was unsatisfactory for metalliferous ecotypes in which the percentage of rooted seedlings varied from 37 to 45% for CAL and

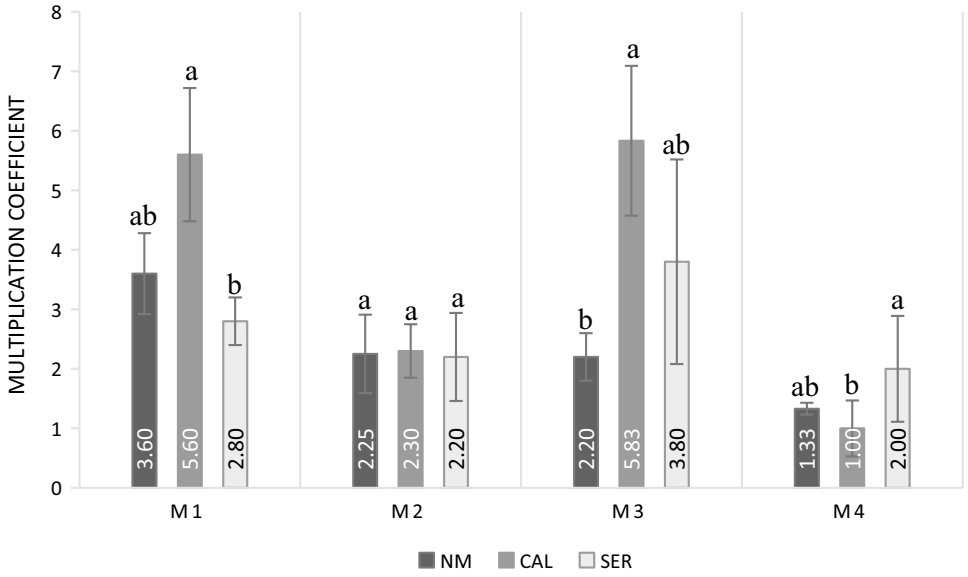

Fig. 4 Micropropagation coefficient calculated as the total number of regenerated shoots per primary explants for non-metallicolous (NM), calamine (CAL) and serpentine (SER) ecotypes of *Silene vulgaris* cultivated on different media (M1–M4). Different letters indicate statistical significance of means ($n=50$ within each medium) according to one-way ANOVA and post hoc Fisher's test at $\alpha=0.05$

Table 5 Effectiveness of *Silene vulgaris* micropropagation after 12 weeks on different media modifications

Medium code	Ecotype	Shoot length (mm)	Rooted shoots (%)	No. of roots/microplant	Root length (mm)
M1	NM	53.78 a* ± 10.55	20.00 a	0.80 a ± 1.12	25.60 a ± 8.80
	CAL	27.25 b ± 9.13	10.00 b	2.40 a ± 1.46	25.00 a ± 10.15
	SER	35.62 ab ± 6.46	0.00	0.00	0.00
M2	NM	42.41 a ± 9.64	100.00 a	3.50 a ± 0.86	15.20 a ± 4.59
	CAL	45.46 a ± 5.48	37.50 c	1.50 b ± 0.99	14.71 a ± 6.77
	SER	43.38 a ± 6.08	45.00 b	2.00 b ± 1.29	20.33 a ± 2.35
M3	NM	25.45 b ± 3.82	20.00	1.40 ± 0.99	15.00 ± 2.44
	CAL	34.25 a ± 11.68	0.00	0.00	0.00
	SER	18.30 c ± 6.11	0.00	0.00	0.00
M4	NM	38.82 a ± 12.40	60.00 b	1.75 b ± 0.43	24.25 a ± 6.41
	CAL	15.55 c ± 7.68	33.00 c	0.86 c ± 0.78	19.33 a ± 8.19
	SER	24.81 b ± 6.44	75.00 a	5.60 a ± 2.15	26.40 a ± 11.12

Means indicated by the same letter within the columns do not significantly differ at $\alpha = 0.05$ according to Fisher's test

NM non-metallicolous ecotype, *CAL* calamine ecotype, *SER* serpentine ecotype

*Values are means of three replicates ± SD

SER, respectively. The values of examined rooting characteristics, such as average root number regenerated from one explant or root length, did not reach sufficient levels. Considering all tested combinations of plant growth regulators, the highest inhibition of rhizogenesis was noted on medium M3. In this treatment, only 20% of NM seedlings developed roots, the number of which did not exceed 1.4 roots/explant, and their length was about 15 mm (Table 5). An average length of shoots obtained on M3 medium was diversified and amounted to 18 mm for SER, 25 mm for NM and 34 mm for CAL. Shoots cultured in the presence of 0.05 mg l^{-1} NAA and 0.5 mg l^{-1} BA (M3) were also characterized by differential multiplication efficiency, and thus the values of MC varied from 2.2 to 5.8 (Fig. 4). Regarding the M4 medium, an application of 0.2 mg l^{-1} IAA, 1.0 mg l^{-1} 2iP and calcium gluconate, added to the medium due to the presence of excess amount of Ca ions on calamine substratum, resulted in strong inhibition of *S. vulgaris* growth and multiplication efficiency (Fig. 4; Table 5). The lowest values of all analyzed parameters were ascertained in CAL culture in which additional anthocyanin's coloration of shoots was observed.

Rooting and acclimatization

Since the main objective of our study was to obtain balanced growth and development of all *S. vulgaris* ecotypes, the medium described as M2 containing a combination of 0.1 mg l^{-1} NAA and 0.25 mg l^{-1} BA was arbitrarily chosen as the optimal variant (Fig. 5a, b). In the elaborated protocol of clonal propagation, a separate rooting stage was necessary. The regeneration of roots and their number from one explant proved to be variable depending on the particular medium treatment. Nevertheless, a better characteristic of rhizogenesis was observed on 1/3 MSR in comparison to 1/2 MSR medium. On 1/2 MSR, the number of rooted shoots ranged from 60% for SER, through 75% for CAL to 100% for NM ecotype, while on 1/3 MSR medium it amounted to 100%. Better root regeneration was also manifested by twofold increase of average root number per explant on 1/3 MSR than on 1/2 MSR. The length of roots regenerated on 1/3 MSR was similar in each ecotype and varied from 18 to 19 mm. Thus, MS medium with micro- and macronutrient reduced to one-third and enriched with 0.1 mg l^{-1} NAA and 0.25 mg l^{-1} BA is proposed to initiate root regeneration of the tested *S. vulgaris* ecotypes.

During the acclimatization step, NM shoots lost turgor much faster than those of CAL or SER when they were not covered with transparent containers and thus required longer protection time. Despite it, the survival rate of NM, CAL and SER microplants reached 100% on the control substratum (Fig. 5c). Similarly, no mortality of microplants on calamine or serpentine substratum was noticed; however, the rate of *S. vulgaris* growth was slightly delayed in this treatment (Fig. 5d).

Callus tissue initiation and characterization

Although various explant sources were used to initiate callus development, only the fully developed leaves from

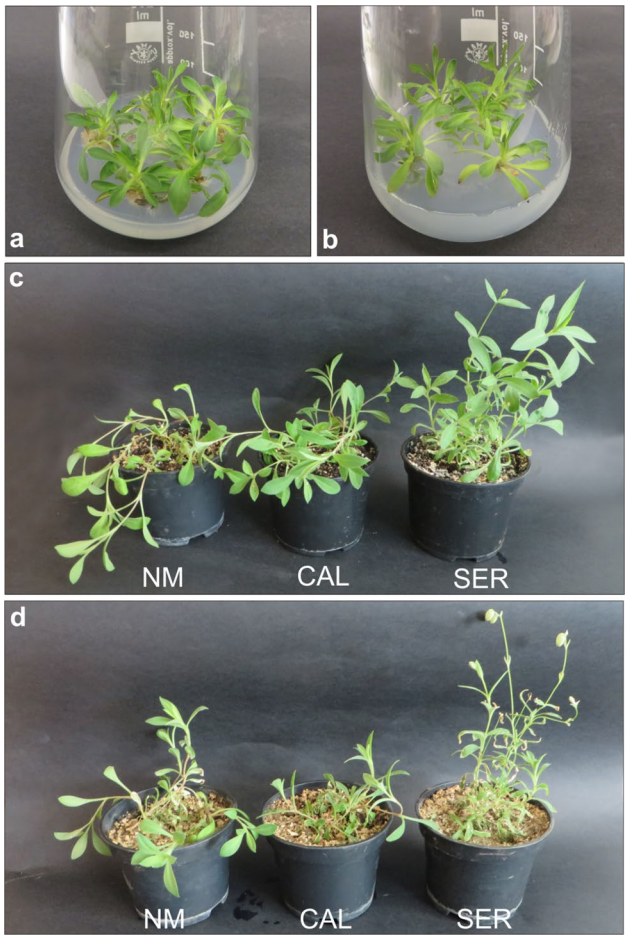

Fig. 5 **a**, **b** Synchronous propagation of non-metallicolous (**a**) and serpentine (**b**) *Silene vulgaris* shoots on MS medium enriched with 0.1 mg l^{-1} NAA and 0.25 mg l^{-1} BA. **c**, **d** Specimens of non-metallicolous (NM), calamine (CAL) and serpentine (SER) ecotypes transferred to ex vitro conditions after 8 weeks of cultivation on horticulture (**c**) and calamine (**d**) substratum

aseptically obtained shoots reacted on culture conditions. Thus, they were used in further experimental steps. On medium described as C1 and C2 consisting of 0.2 mg l^{-1} 2.4-D and 0.2 mg l^{-1} BA, no callus formation was observed independently on light condition. On C3 medium enriched with 0.05 mg l^{-1} NAA and 0.5 mg l^{-1} BA, the abaxial fragments of leaf explants taken from all ecotypes exhibited callus induction, but its growth rate was slow and the calli became necrotic within 4 weeks of cultivation both in light and in darkness. Similarly, adaxially oriented explants did not respond on C3 medium. The most abundant callus formation was observed on C4 medium with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} BA; however, this process was affected by the explant position. When abaxial leaf surfaces were put on the medium, callus tissue appeared independent of light condition in 100% of explants in the case of CAL and SER ecotypes. In turn, leaves of CAL and SER ecotypes placed in adaxial orientation produced more callus tissue in the dark than in light, in which calli proliferation was strongly inhibited. Considering NM ecotype, callus was induced in all explants regardless of the orientation and light condition, yet after 3 weeks the development was arrested and the cells died. After 8 weeks of cultivation, the callus color was bright green when explants were cultivated in light. Callus from CAL explant formed compact nodules (Fig. 6a), whereas the structure of the SER callus was more granular (Fig. 6b). Different browning of clumps in the place of contact with the medium was observed very often in case of CAL callus (Fig. 6a). In turn, tissue cultivated in the dark was yellowish in color and more friable than callus from explants cultivated in light (Fig. 6c, d). Independent of light conditions, root regeneration was only sporadically observed in both ecotypes.

The major difference in morphology of the cultivated tissue resulted from its internal organization. Histological investigation revealed the presence of many primordium-like areas with well-formed xylem vessels. The vessel number was higher in CAL than in SER callus clumps cultivated in light (Fig. 6e, f). Around these organized structures in CAL, callus regions of sloughed-off cells were ascertained that separated the interior tissue from the peripheral mass of parenchyma cells (Fig. 6e), while in SER callus such areas were absent (Fig. 6f). The anatomical features of callus grown in darkness differed significantly from those described above. In this case, CAL tissue showed the greatest tendency for inner organization regardless of the examined treatment (Fig. 6g). On the contrary, SER callus was characterized by homogenous unorganized tissue formed mainly via proliferation of parenchyma cells (Fig. 6h). By anatomical analysis, we could confirm the observed rate of proliferation which was promoted by light in the case of SER callus, while in darkness callus development from CAL explants was more intensive.

Discussion

Field research

The plant communities spontaneously occurring on both naturally or secondarily polluted with HMs areas have attracted considerable attention due to their unique ability to tolerate enormous amounts of toxic ions. Adaptation to HMs-contaminated environment can be manifested in a range of phenotypic traits such as specific morphological, anatomical and physiological alterations that enable plants to thrive and colonize even extremely harsh habitats. Dependently on mineral composition of soils, calamine and serpentine flora could be distinguished with various species among which *Silene vulgaris* has been chosen as a model plant for the

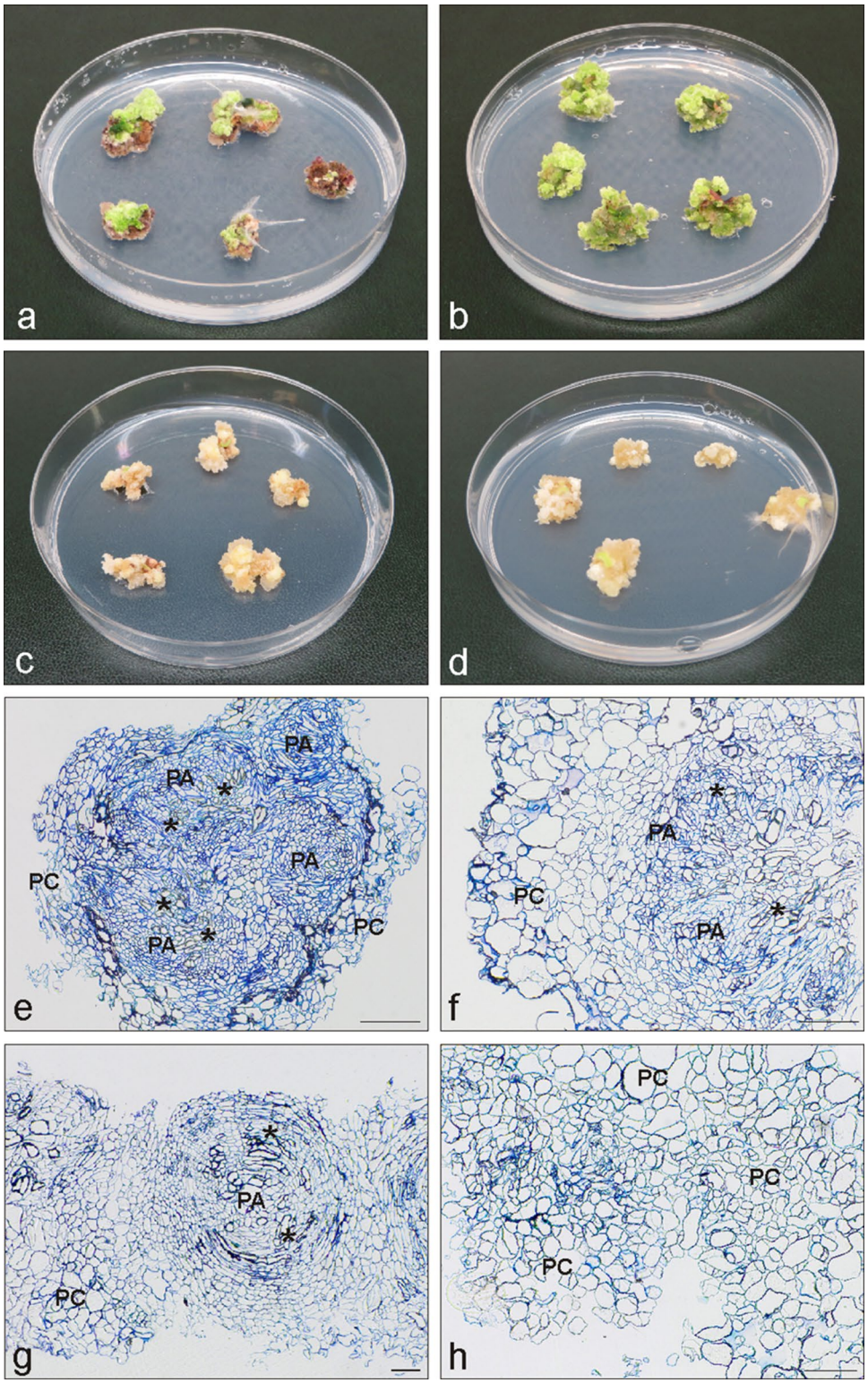

Fig. 6 The development of *Silene vulgaris* callus from leaf explants on MS medium enriched with 0.5 mg l^{-1} NAA and 5.0 mg l^{-1} BA. **a**–**d** Morphology of calamine (**a**, **c**) and serpentine (**b**, **d**) callus clumps cultivated in light (**a**, **b**) or in darkness (**c**, **d**). **e**–**h** Anatomy of calamine (**e**, **g**) and serpentine (**f**, **h**) callus tissue growing in light (**e**, **f**) or in darkness (**g**, **h**). Bar = 100 µm. *PC* parenchyma cell, *PA* primordium-like area, *xylem vessel

present research. Considering the diversity of heavy metals present in metalliferous waste heaps, our study deals with the comparative assessment of *S. vulgaris* ecotypes, originating from different populations growing on terrains non-contaminated with HMs, as well as those rich in Zn, Pb and Cd (calamine) or in Ni, Cr and Co (serpentine).

Differences in leaf blade structure

Taking into account that leaf structure and functioning reflect its essential role in plant growth and development, their modifications might help to understand plant adaptations to stressful environmental conditions (Karnaukhova 2016). Our research revealed that leaves of *S. vulgaris* taken from the CAL population presented a distinct anatomical pattern compared to leaves of SER and NM specimens. One of the most pronounced differences was concerned with the clearly developed layers of palisade parenchyma. An intensive development of this tissue indicates the adaptation mainly to high insolation and protects inside parts of leaves from unfavorable light influence (Karnaukhova 2016). Such conditions occur on HMs-polluted areas where not only excess amount of HMs, but also high insolation, water deficiency and strong winds occurred (Wierzbicka and Panufnik 1998). Plants from sunny and dry sites are usually characterized by greater leaf thickness than plants from other habitats (Karnaukhova 2016). Anatomical adaptations to minimize water loss were also shown in the present research. Both metallicolous ecotypes had thicker leaves and bigger-sized parenchyma cells in comparison to the NM ecotype. These features are in accordance with findings observed by Wierzbicka and Panufnik (1998) on calamine ecotype of *S. vulgaris*. Thickening leaves under HMs contamination have also been reported for many other tolerant plants (Luković et al. 2012; Pereira et al. 2016). Another feature of water stress resistance is the lower number of stomata in leaves of metallicolous ecotypes than in non-metallicolous ones. Such adaptation might play a crucial role in regulation of HMs uptake and translocation, closely related to the transpiration rate (Gomes et al. 2011). According to Bertel et al. (2017), the reduced stomatal number on adaxial leaf surfaces prevents evapotranspiration, while more and smaller stomata on abaxial leaf surfaces enable a more precise regulation of gas exchange. Despite the indirect influence on metal exclusion from shoots, changes in stomata density that regulate CO_2 flux to the mesophyll might lead to significant limitation of photosynthetic efficiency. Although this process was outside the field of the present research, based on microscopic comparative analysis of the leaf blade anatomy of the tested specimens, it might be assumed that the way by which photosynthesis is enhanced depends on the ecotype. In CAL specimens, an appearance of typical palisade parenchyma layers resulted probably in an increased RuBisCO activity—the main enzyme enhancing photosynthesis. This hypothesis could be supported by research of Pereira et al. (2016) on *Schinus molle* treated with Cd ions in which leaf thicknesses was strongly correlated with increased palisade parenchyma cells and RuBisCO activity. On the contrary, leaves of SER ecotype contained bigger intercellular spaces due to more round cell shapes in the parenchyma that may facilitate the contact between diffused CO_2 and chloroplasts, and thereby recompense the limited CO_2 uptake (Evans et al. 1994).

Pigment content in contrasting ecotypes

Photosynthesis is closely related to the photosynthetic pigment content and its composition. Although pigment reduction under HMs is well documented (Maina and Wang 2015; Chandra and Kang 2016; Piwowarczyk et al. 2018), its degradation is much more complex. Therefore, it is difficult to point any one culprit of such state of affairs. According to Aarti et al. (2006), the oxidative stress impeded key steps in chlorophyll synthesis through directly or indirectly inhibiting the enzyme activity of Mg-chelatase, Fe-chelatase and protoporphyrinogen IX oxidase. Hendry and Price (1993) indicated that the chlorophyll $a+b$/carotenoid ratio is a good indicator of sensitivity of chlorophyll to photooxidative damage. In our study, the degradation of chlorophyll *a, b* and their total amounts was observed suggesting the decreased photosynthesis potential. It is worth emphasizing that the values of chlorophyll *a/b* between SER and CAL as well as SER and NM ecotypes were maintained, although metallicolous specimens are exposed to higher insolation than non-metallicolous ones. Results on *S. vulgaris* ecotypes are not in accordance with Maina and Wang (2015) which postulated that the chlorophyll a/b ratio may be termed as species specific and varies depending on the irradiance and leaf morphology. According to Reed et al. (2012), plant species have a certain range of tolerance to light intensity, over which the acclimatization process should occur, otherwise the photooxidative damage may lead to organism's death. Thus, we suppose that the tested ecotypes have a photosynthetic apparatus acclimatization associated with changes at the chloroplast level. We cannot exclude that anatomical and physiological plasticity regarding pigment parameters and parenchyma arrangement in metallicolous plants allows these ecotypes to achieve a higher efficiency of photosynthesis and better cope with stress conditions during growth on HMs-contaminated grounds (Maina and Wang 2015). A loss of carotenoids in CAL and SER ecotypes in comparison with NM was also noted and this was reflected in the decline of the chlorophyll/carotenoid ratio in both metallicolous specimens. It might imply the minor role of carotenoids as antioxidant-protective pigments in metallicolous ecotypes, yet further research must clarify this point.

Considering the factors significantly influencing pigment content in plants growing in their natural habitats, one cannot overlook the fact that the availability of nitrogen in the soil may affect the profiles (Pereira et al. 2016; Piwowarczyk et al. 2018). As nitrogen is an indispensable macronutrient for formation of efficiently operating photosynthetic machinery (Evans and Poorter 2001), the decrease in chlorophyll content noted by us in CAL and SER ecotypes may

be a physiological response to the low content of nitrogen available for plants in calamine and serpentine substrates (Koszelnik-Leszek 2007; Ciarkowska et al. 2017). Taking all information together, the three populations of *S. vulgaris* differ from each other in leaf anatomy and these differences strongly suggest a physiological distinction of the tested ecotypes, regarding among others the accumulation and composition of photosynthetic pigments.

Antioxidant response of tested ecotypes

The stressful environmental conditions stimulate the formation of reactive oxygen species (ROS) and their inactivation is controlled by various plant enzymatic and non-enzymatic mechanisms to prevent oxidation damage. Many experiments have highlighted the potential role of phenolic metabolites in ROS defense (Maestri et al. 2010; Labudda et al. 2016; Muszyńska et al. 2018a; Piwowarczyk et al. 2018). Phenols protect not only from oxidative stress by direct ROS scavenging or modification of lipid membrane fluidity, but might also chelate metal ions and prevent Fenton's reaction. In our experiments, the measurement of total secondary metabolites and particular phenol groups revealed differences in their accumulation between the tested ecotypes. Phenols presented in *S. vulgaris* shoots had a rather low ability of DPPH$^{\cdot}$ scavenging (9–11%). Similarly, the studies of Keilig and Ludwig-Müller (2009) on *Arabidopsis thaliana* seedlings treated with HMs showed that naringenin, the flavonoid from the flavones group, did not react with the DPPH radical, while quercetin and kaempferol, which belong to the flavonols, were characterized by high reactivity with DPPH. Perhaps, similar radical scavenging activity of the tested extracts might be related to the heterogeneous structure of accumulated phenolic compounds (Leong et al. 2012), as well as their lower concentrations in metallicolous specimens than in the non-metallicolous one. The observed decrease of phenols resulted probably from their consumption during defense mechanisms against environmental stress conditions. Interestingly, phenols accumulated in leaves of CAL and SER ecotypes belonged to the same groups, although metallicolous habitats were spatially isolated and ecologically differed from each other. It implicates the existence of analogous pathways of synthesis/degradation of these phenolic compounds functioning in both metallicolous populations that have been developed independently through microevolutionary changes. Hereby, phenylpropanoids and flavonols could be treated as markers in studies on plant adaptation to HMs and a secondary appearing increase in ROS production. On the contrary, anthocyanins proved to be the most variable group of phenols. This fact is consistent with numerous reports suggesting that anthocyanin accumulation may be induced by various abiotic and biotic factors including visible and UVB radiation, cold temperature, drought or pathogens (Trojak and Skowron 2017; Labudda et al. 2018).

Apart from phenolic compounds, antioxidant enzymes participate in ROS detoxification. Superoxide dismutases (SODs) are ubiquitous enzymes in plants and play an essential role in ROS scavenging mechanisms. As a result of their activity, the content of superoxide and hydrogen peroxide molecules, the two Haber–Weiss cycle substrates, is controlled. All this causes the production of extremely reactive hydroxyl radicals to be inhibited, so cell-building molecules are protected against oxidation (Pilon et al. 2011). In the present study, increased SOD activity was found in two metallicolous ecotypes, similar to the effect of Ni^{2+} or Cu^{2+} on basil plants under controlled metal treatment (Georgiadou et al. 2018). The SOD activity seems to be strongly correlated with the dose of metal in the growth medium and/or with the plant species as observed within *Alyssum* genus in the hyperaccumulating plants of *A. argenteum* and *A. maritimum* treated with Ni or Cd ions (Schickler and Caspi 1999). The enhancement in SOD activity in both metallicolous ecotypes indicated that the ROS scavenging function of SOD is not impaired and such increase leads to better protection against effects of oxidative stress. The three types of plant SOD are described: MnSOD (Mn cofactor), FeSOD (Fe cofactor) and Cu/ZnSOD (Cu and Zn as cofactors with the proviso that Cu is the redox active catalytic metal) and it has been confirmed that SODs are regulated on the transcriptional and translational level in multiple ways (Pilon et al. 2011). For example in *Arabidopsis thaliana* plants exposed to Cu, Fe or Zn, Cu/ZnSOD mRNA content was significantly enhanced (Herald et al. 2003). Since some divalent metal cations are crucial for enzyme functioning and cell signaling, its level *in planta* must be tightly controlled. Thus, the anomalous concentrations of Ni, Cr and Co in serpentine soils as well as Zn, Pb and Cd in calamine soils lead to their higher amounts in plant organs, which may result in the increase of divalent cation-dependent enzyme activity. Taking into account that HMs in serpentine soils are accompanied by a high content of Fe and Mg, whereas calamine soils are enriched with an admixture of Cu and S, the activity of different SOD isoforms in metallicolous *S. vulgaris* ecotypes cannot be ruled out.

H_2O_2 molecules, the products of SOD activity, are still reactive and must be removed from cells. In plants, a few enzymes are responsible for the H_2O_2 catabolic process, but catalase (CAT) and guaiacol-type peroxidase (GOPX) are considered as the most important ways (Bhaduri and Fulekar 2012). CAT breaks down H_2O_2 into water and molecular oxygen. CAT has a very fast turnover rate, but its substrate affinity is less than that of peroxidases (Mhamdi et al. 2010). Siedlecka and Krupa (2002) suggested that as long as the stress conditions do not exceed the plant antioxidant capacity, key enzymatic response to HMs is an increase in

SOD and GOPX activities. In SER ecotype, GOPX activity was very strong enhanced in comparison to NM and CAL ecotypes. However, it was noted that their relatively low activity in CAL ecotype is compensated by elevated CAT activity. We postulate that enzymatic ROS scavenging mechanism in CAL ecotype is based on the joint action of SOD and CAT, but in SER ecotype on SOD and GOPX. These findings reinforce our conviction that the two metallicolous ecotypes vary considerably also on the antioxidant enzymes level.

Genetic diversification

Estimation of genetic diversity and relatedness via molecular markers is an important approach in plant evolutionary biology and modern breeding (Garrido-Cardenas et al. 2018). DNA polymorphism revealed by RFLP band patterns is the consequence of the base-pair deletions, point mutations, duplications, inversions, transpositions and translocations (Nadeem et al. 2017). Molecular markers (including PCR–RFLP) are a widely applied tool for studying the genetic variation in plant populations under abiotic stress conditions (Forster et al. 2000; Bonilla et al. 2002). In our study, *S. vulgaris* populations exhibited some changes at the genetic level which might have been developed in a similar way, but independently in both metallicolous ecotypes subjected to different HMs in natural habitats. The expressed PCR–RFLP genetic variation (*atpA*/*Msp*I) of CAL and SER populations compared with the reference NM ecotype suggests that metallicolous specimens are not only able to acclimatize at the anatomical/physiological level, but also have been genetically adapted to growth in the permanent presence of HMs and in other harsh environmental conditions. Nevertheless, our findings are the beginning of the needed advanced molecular investigations. The diversification of *S. vulgaris* ecotypes at various levels of organism organization might be a convenient starting point for further research on plant tolerance mechanism under fully controlled laboratory conditions.

Laboratory research

The present study revealed significant differences at anatomical, physiological and genetic level between examined ecotypes from contrasting ecological niches. It contributed to the development of in vitro protocol for their standardized cultivation to conduct further research in the domain of *S. vulgaris* stress reactions and phytomanagement programs. The elaboration of an identical medium composition for more than one tested object is a difficult task, due to various nutritional and hormonal requirements of particular plant species. It constitutes an innovative approach and creates the possibility of carrying out the experiments under unified conditions that are more easily controlled than in the case of greenhouse or field studies. It refers mainly to toxic substances that can be admittedly added to soil, but simultaneously they may be adsorbed or bind with soil components, and thus be rendered unavailable to plants. In vitro techniques, such as callus, cell suspension or shoot cultures, allow genetic manipulations and/or the selection of resistant plant material, thus possibly improving plant potential for environmental remediation (Doran 2009; Chen et al. 2015; Muszyńska and Hanus-Fajerska 2017).

Optimization of shoot culture protocol

To start in vitro culture, seed samples of contrasting *S. vulgaris* populations from natural habitats were taken. Studies on plant generative processes under HMs stress show strong abnormalities in micro- and macrosporogenesis, embryo and endosperm development as well as in the seed germination phase (De Storme and Geelen 2014; Bothe and Słomka 2017). Such malformations or degenerations in male and female lines resulted in a decreased reproductive success in contaminated habitats, a situation ascertained for numerous species belonging to different families, e.g., *Chenopodium botrys* (Yousefi et al. 2011) or *Cardaminopsis arenosa* (Kwiatkowska and Izmaiłow 2014). The results of our study on seed germination indicated that plants exposed to a combination of environmental factors in natural conditions showed disturbances in embryological processes. Thus, the germination of seeds collected from specimens representing metallicolous populations could be significantly affected in comparison to non-metalliferous poopulations. In this context, the elaboration of in vitro propagation protocol seems to be even more important and is needed to be undertaken.

An innovative approach of tissue culture in studies on plant responses to different abiotic stresses is a synchronous cultivation of different specimens belonging to the same genus, but representing opposite ecological niches. Such unified culture medium enables the comparison of particular specimen reaction on applied stressors excluding the influence of diversified medium compositions. According to the protocol proposed by Hanus-Fajerska (2011), the use of 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA for the establishment of *S. vulgaris* calamine ecotype shoot culture brought a positive effect. Likewise, Corduk et al. (2018) reported that the same auxin and cytokinin type was suitable for in vitro propagation of *S. bolanthoides,* while Kritskaya et al. (2016) showed that combination with kinetin and gibberellic acid was the most effective for micropropagating shoots of *S. cretacea*. In our study, we also tested the medium with calcium gluconate to increase calcium supplementation, an excess content of which was noted in wastes obtained from Zn–Pb ores exploitation and processing (Ciarkowska et al. 2017). Although the medium with an increased amount of Ca ions was the most

effective for in vitro propagation of other calamine ecotypes such as *Gypsophila fastigiata* (Muszyńska et al. 2018b) and *Dianthus carthusianorum* (Muszyńska and Hanus-Fajerska 2017) also from Caryophyllaceae family, such an application to both metallicolous and non-metallicolous ecotypes of *S. vulgaris* did not bring satisfactory effects. In all the abovementioned cases, the morphogenetic response of cultures depended on the *S. vulgaris* ecotype. On the contrary, the best growth of tested ecotypes was observed on medium enriched with 0.1 mg l^{-1} NAA and 0.25 mg l^{-1} BA. The same plant growth regulators added to MS medium are proposed for rooting of micropropagated shoots; however, in this experimental step macro- and micronutrient reduced to 1/3 should be used. The obtained microplants were successfully transferred to ex vitro conditions confirming the usefulness of shoot culture for effective regeneration of a large amount of plant material that could be directly introduced to terrains polluted with heavy metals. The noticeable morphological traits that distinguished plants growing on non-contaminated and contaminated substratum could be the effect of unfavorable physico-chemical properties of the contaminated substratum. Such growth and development inhibition resulting from harsh environmental conditions has been observed in many others species colonizing waste heaps (Nagajyoti et al. 2010; Muszyńska et al. 2017).

Callus culture initiation

Studies reporting the callogenesis and further tissue proliferation under in vitro conditions have been widely applied to many commercially important dicots (Boamponsem and Laung 2017; Huang et al. 2017). Relatively little is known about callus culture of metallophytes that might be used as model systems to investigate the capacity of plant cells to tolerate, detoxify or store various pollutants. Most research was related to either indirect organogenesis or embryogenesis (Jack et al. 2005; Hanus-Fajerska 2011). In our experiments, we tested the influence of various plant growth regulators on long-term culture. The differences in callus proliferation of the studied ecotypes were reliant on medium composition and light treatment, as reaffirmed by serial observations of tissue cross sections. Anatomical investigation reflected the specific manner in which differentiation of *S. vulgaris* ecotypes was changed in an individual way by chemical and/or physical culture conditions. It is plausible that the differences in callus traits and its ability for indirect regeneration were attributed to the specific genotype. In future, callus culture would be used as a convenient model to explore the mechanism of tolerance resulting from microevolutionary changes between both metallicolous populations with regard to the response of the reference population taken from unpolluted sites.

Conclusions

The present study revealed significant differences between non-metallicolous and both metallicolous ecotypes in anatomical and physiological features. The observed changes resulted not only from various ecological conditions in natural divergent habitats, but also from genetic variation of the tested specimens. It points to the genetically established adaptation of metallicolous populations to excess amounts of heavy metals. Such diversification at various levels of *S. vulgaris* organization contributed to the optimization of laboratory conditions for further experiments. For the first time, in vitro approaches with regard to Polish non-metallicolous and serpentine ecotypes were carried out. A highly efficient method of clonal propagation as well as callus proliferation was exploited and the synchronous cultures for representatives of different ecological niches were obtained. It provides the opportunity to conduct research on plant stress biology and tolerance mechanisms under freely controlled conditions. The study also revealed that in vitro delivered microplants are able to survive on substrata contaminated with heavy metals. Therefore, micropropagation might be proposed as an efficient method to achieve a great amount of plant material for application in rehabilitation schemes and phytoremediation of polluted areas.

Author contribution statement EM designed the research and wrote the manuscript, obtained the funding and performed in vitro cultivation and anatomical study. EM and ML contributed to physiological data acquisition and interpreted and discussed all data. ER performed genetic analysis. EHF and AK-L provided seed samples and advised on manuscript preparation. All authors read and approved the manuscript.

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