Bisutella laevigata subsp. *woycickii* – the new endemic and a postglacial relic for the Polish flora

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Adaptation of plants to the presence of heavy metals in the substrate

Plants that colonize metalliferous soils must have morphological and physiological plasticity (Baker 1987, Dahmani-Muller et al. 2000). A certain level of resistance to heavy metals, so-called constitutional resistance, appears in all plant species, even those that did not come into contact with heavy metals during their growth. However, some species have exceptionally well-developed mechanisms for resistance to heavy metals, so-called induced resistance. This resistance was created through the selection of resistant ecotypes in an environment with an increased content of heavy metals in the substratum (Wierzbicka 2002, 2015). In metalliferous sites, such as zinc-lead (calamine) waste heaps, there are various taxa of vascular plants, mosses, and lichens. This is thoroughly discussed in the monograph 'Ecotoxicology – plants, soils, metals', published in 2015 under the scientific editorship of Małgorzata Wierzbicka. A common feature of organisms found in calamine waste heaps is the ability to live on metalliferous soils without any symptoms of stress. These organisms are called metallophytes. Among these metallophytes, there are species associated only with metalliferous soils (so-called absolute or obligatory metallophytes) and also those that occur on 'clean' soils (optional metallophytes or pseudometallophytes). Absolute metallophytes are generally highly specialized endemics that have evolved on media rich in weathered ore minerals for thousands of years. Optional metallophytes,

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on the other hand, had contact with metals for a much shorter time, but these organisms also exhibit a number of adaptations which allow them to survive in metalliferous areas (Antonovics et al. 1971, Prasad 2004, Baker et al. 2010, Rostański et al. 2015, Szarek-Łukaszewska et al. 2015, Wierzbicka 2015).

Plants inhabiting metalliferous substrates, in addition to developing adaptations to high levels of heavy metals, also develop adaptations to other factors hindering growth, such as drought, strong insolation, or nutrient deficiency associated with a small amount of organic matter in the substrate. As a result of multi-directional adaptation, differences may occur after some time between individuals of the same species found in metalliferous and non-metalliferous areas. The processes leading to the emergence of genetically fixed differences within a species are referred as microevolution, which was discussed in more detail in the previous chapter of this book. Such changes have occurred, for example, in the species of metallophytes inhabiting the zinc-lead waste heap in Bolesław (mine waste from Bolesław open-pit excavation, Włodarz – Chapter 1 of this volume) near Olkusz, including in *Arabidopsis arenosa* (L.) Lawalrée, *Armeria maritima* (Mill.) Willd., *Dianthus carthusianorum* L. and *Silene vulgaris* (Moench) Garcke. Changes of this type have also occurred in the species to which this volume is dedicated – *Biscutella laevigata* L. (Kruckeberg and Kruckeberg 1990, Lefèbvre and Vernet 1990, Wierzbicka and Panufnik 1998, Wierzbicka 2002, 2015, Wierzbicka and Rostański 2002, Załęcka and Wierzbicka 2002, Wierzbicka and Pielichowska 2004, Abratowska 2006, Ernst 2006, Przedpełska and Wierzbicka 2007, Abratowska et al. 2012, Wójcik et al. 2013, Wąsowicz et al. 2014, Wierzbicka et al. 2017, 2020).

One of the mechanisms of adaptation to adverse conditions prevailing on waste heaps is the so-called r-type life strategy, developing in some species of plants from metalliferous areas. It leads to the production of individuals of small size, with a short life cycle, quickly entering the generative phase and producing large amounts of small seeds (e.g. *A. arenosa*, *D. carthusianorum*, and *S. vulgaris*) (Wierzbicka and Panufnik 1998, Załęcka and Wierzbicka 2002, Przedpełska and Wierzbicka 2007, Wierzbicka 2015). These plants produce a significant number of seeds as a result of generative reproduction, and their long viability ensures diversity of the genetic pool of the species. This is a strategy that is characteristic of pioneer plants. However, on calamine soil, the growth of seedlings formed from seeds is weak; therefore, only individuals with the best adaptation characteristics remain. It has been shown that in the case of *B. laevigata*, one of the growth adaptation mechanisms on calamine heaps is the occurrence of a clonal form of vegetative reproduction. Young plants arise from root buds and in such a large number that within one growing season one plant is able to produce up to 15 daughter plants (Pielichowska 2007, Wierzbicka et al. 2015, 2017). Vegetative reproduction is costly for plants in terms of energy, which results from high expenditures on the production of vegetative propagation plants (Falińska 1997). However, it provides breeding success in extreme habitat conditions, which includes both zinc-lead waste heaps and scree mountain slopes. Progeny, like the mother plant, also have beneficial features that enable them to grow in these difficult conditions. Thus, vegetative reproduction plays a very important role in the survival strategy of *B. laevigata* in difficult growth conditions (Wierzbicka et al. 2015, 2017), which will be discussed later in this chapter. The example described is a regularity among clonal plants that enables the preservation of a compromise between new genotypes introduced into the environment as a result of sexual reproduction, and genotype reproduction as a result of vegetative reproduction of individuals (clones) that are the most suited to the environmental conditions (Falińska 1997).

When discussing the adaptation of plants to growth on post-mining waste heaps, it is also important to mention defensive reactions to heavy metals. Defensive reactions can be divided into two groups of mechanisms: (1) avoidance of heavy metals, the aim of which is to prevent metals from entering the cell, and (2) tolerance of heavy metals, which are responses to the presence of metal in the cell (Abratowska 2006, Kacperska 2007, Verbruggen et al. 2009). In the case of avoidance mechanisms, the following strategies in plants can be distinguished: exclusion, elimination, redistribution, and compartmentation. By exclusion, we mean processes that aim to prevent the plant from absorbing heavy metals. This includes the release of chelating (binding) metal ions compounds into the rhizosphere (surrounding the roots) so that they become unavailable to plants, as well as preventing the spread of metals in the plant, thanks to barriers existing on metal transport pathways in roots and shoots (Wierzbicka 1995, Clemens et al. 2002, Baranowska-Morek 2003, Baranowska-Morek and Wierzbicka 2004; Abratowska 2006; Verbruggen et al. 2009). Plant roots can modify their surroundings by secreting compounds that are able to reduce the amount of heavy metals available to plants by binding them in soil. For example, roots can secrete pectin compounds that attach to metals. These complex compounds are not taken up by the roots, so the excess of heavy metals does not reach the cellular cytoplasm. This protects plants from accumulating high doses of metals (Olko 2009, Wierzbicka 2015, Bothe and Słomka 2017). Heavy metals can also be bound to acids, such as malic or citric acid, outside or inside the roots. Root secretion can also change the pH value of the soil, and thus change both the availability and the rate of heavy metal uptake into plant roots (Bothe and Słomka 2017).

The following structures play a key role in preventing the spread of heavy metals in the roots and shoots to particularly sensitive areas of the plant: the quiescent centre (QC zone) and the zone of meristematic cap cells in the apical part of the root, meristematic pericyclic cells, and the endoderm (Wierzbicka 2015). In the first of these areas, there are initial cells, which play a particularly important role in root regeneration processes, and therefore they require special protection against heavy metals (Wierzbicka 1987a, 1995). Meristematic cells of the pericycle (the layer of parenchyma cells around the periphery of the axial roller in the root) act as a barrier on the border between the apex and the diverse root zone. Heavy metals accumulate in the cell walls of these cells. As a result of the exocytosis process, metals are rejected from the symplast into the root apoplast. This process protects the deeper tissues, from which conductive tissues are formed (Baranowska-Morek and Wierzbicka 2004). Endodermal cell walls (cell layers located deep in the root, on the border of the primary cortex and the axial roller) have so-called Casparian strips, in which the cell wall together with the middle lamella are saturated with suberin and lignin. This prevents heavy metal ion-carrying water from diffusing. It is protection against the penetration of metals into conductive tissues, and thus the aerial parts of plants are protected. More specifically, the entire photosynthetic system is protected (Wierzbicka 1987b). Thanks to the barrier in the generative part of the flower, which is located in the receptacle, the germs remain free of heavy metals, even with significant amounts in other parts of the plant (Ernst et al. 1990). This resembles an umbilical cord barrier in mammals. Seed coats protect plant germs from heavy metals during the seed swelling process. This barrier disappears when the seed coat is torn apart by a germinating embryonic root (Wierzbicka and Obidzińska 1998). Different barriers in the leaves inhibit the spread of metals to successive layers of leaf cells, e.g. periventricular vaginal cells in monocotyledons protect conductive beams (Wierzbicka 2015).

Another strategy to avoid heavy metals is elimination. Elimination processes occur when heavy metals have already entered plant tissues, and they lead to the removal of metals from the plant. The mechanisms of elimination include: the excretion of metals from the organism through its surface, the secretion of metals by the glands and trichomes, and the discharge of entire organs containing heavy metals (Ernst 1998, Clemens et al. 2002). Trichomes on the leaves of plants growing in the presence of metals usually contain large amounts of metal. For example, *B. laevigata* specimens found on the mine waste heap in Bolesław had an increased number of trichomes accumulating large amounts of metals (thallium, cadmium, lead, and zinc). At the base of the trichome is a central, large vacuole. Studies have shown that in this part of trichome the largest amounts of metals are deposited (Pielichowska and Wierzbicka 2004, Wierzbicka et al. 2015, 2016, 2017). Thus, trichome cells are areas where excessive amounts of harmful elements are thrown away, so that other leaf cells can be protected (Ma et al. 2005). Another example of elimination is the secretion of heavy metals along with other salts by plant salt glands (e.g. *A. maritima*). Epidermal cells can also secrete heavy metals on leaf surfaces (e.g. *S. vulgaris*) (Wierzbicka 2015). As an example of elimination, it is also worth mentioning that the oldest, drying and rejected plant leaves from sites heavily contaminated with metals often contain the largest amounts of heavy metals. This protects the remaining young parts of plants from metals. For example, in *B. laevigata*, removing excess heavy metals involves their accumulation in the oldest leaves that then dry up (Godzik 1984, 1991, Pielichowska 2007, Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017).

Redistribution is also a mechanism for avoiding heavy metals. The term refers to processes that are aimed at reducing the presence of heavy metals by their transport to places where there is less risk of their toxic effects on the organism, e.g. to aging leaves (Ernst 1998). Redistribution is associated with the process of elimination, described in the previous paragraph.

The last mechanism of metal avoidance is compartmentation, which occurs at the cellular level. It involves the accumulation of heavy metals in places where they do not pose a threat to plant cells, e.g. in cell walls, intercellular spaces, and vacuoles. Metal complexes are formed in vacuoles with inorganic and organic acids, phenol derivatives, and glycosides, for example (Wierzbicka 1995, 1998, Abratowska 2006, Kacperska 2007). Other good examples of compartmentation are changes occurring in the cell wall due to excess heavy metals. It has been observed that the structure and properties of the cell wall change – primarily there is an increase in the amount of pectin compounds. Pectin compounds combine with heavy metal ions to form pectinates. Under the influence of heavy metals, cell walls also thicken by increasing the amount of pectin compounds, thanks to which it is possible to bind more heavy metals in cell walls. This phenomenon has been observed in *Allium cepa* L. root cells and *Funaria hygrometrica* Hedw. cells (Wierzbicka 1998, 1999, 2015, Krzesłowska et al. 2009, 2010). In the cell wall, with the exception of pectins, carboxyl groups of polygalacturonic acids and phenolic compounds have the ability to bind heavy metals and form complexes with metal ions (Wierzbicka 2015). Also, in *B. laevigata* it was found that lead accumulated the most in cell walls, and this was often accompanied by an increase in the thickness of the cell walls. Lead was also present in intercellular spaces, and significant amounts were also found in cell vacuoles (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

Tolerance mechanisms work when heavy metal ions are already inside the cell (in the cytoplasm), where their effect on cell metabolism is the most dangerous. In response to an increase of metal concentration in the cytoplasm, stress metabolites (e.g. proline and many carbohydrates), polypeptides and so-called stress proteins (also synthesized in response to stressors other than the presence of heavy metals) are synthesized. From the functional perspective, they are e.g. signal transducers, structural proteins, enzymes, metal chelators (e.g. glutathione and its derivatives – phytochelatin), as well as osmotins (proteins associated with osmotic stress), heat shock proteins (HSP) and RAB (ras-associated binding proteins, one of the groups of proteins responsible for vesicular transport) (Abratowska 2006, Kacperska 2007, Varbruggen et al. 2009). Most heavy metals are bound and transported as complexes with ligands with a low molecular weight. Most often it is a molecule or anion in complex compounds which is attached directly to a central atom or central cation, called the coordination center or the core of the complex. The most important of these are amino acids (e.g. histidine and nicotianamine), organic acids (e.g. citric, malic, oxalic acid), phytin, flavonoids, phytochelatin, and metallothioneins (Pohlmeier 2004, Salt et al. 1995, 1998, Krämer et al. 1996, Clemens et al. 2002, Kim et al. 2005, Haydon and Cobbett 2007, Olko et al. 2008). Increased synthesis of these compounds is the basis of the plant's tolerance to heavy metals.

As already mentioned, after the entry of metal ions into the cell, they are quickly complexed by ligands with a low molecular weight, e.g. metallothioneins, phytochelatin, organic acids (mainly citric and malic), amino acids (histidine, cysteine, nicotianamine), or phytin (phosphorus storage form) (Van Steveninck et al. 1990, Krämer et al. 1996, Hagemeyer 1999, Takahashi et al. 2003, Sharma and Dietz 2006). After complexing the metal ions, the ligand together with the metal can be transported to vacuoles, where the bound metal ions remain inactive. For example, in the vacuoles of *A. cepa* epidermis cells, cadmium was present in combination with organic acids and with histidine. Lead, in turn, was present in combination with thiol groups and histidine. Plant tissues have increased detoxification capabilities for heavy metals when they contain large amounts of phenolic compounds in cell vacuoles. This is the case, for example, with *A. maritima* (Przedpełska-Wąsowicz and Wierzbicka 2011, Wierzbicka 2015).

Metallothioneins are small cysteine-rich proteins that have a thiol group (-SH). The efficiency of binding metal ions is based on their high affinity to the -SH group, which binds heavy metals in the cytoplasm. Metallothioneins are found in animal and fungal cells, but genes encoding metallothioneins are also common in plant cells (Abratowska 2012, Bothe and Słomka 2017). Phytochelatins, ranked to the third group of metallothioneins, are thiol peptides that are characteristic of plants. The precursor of phytochelatin is glutathione, which is modified by the action of an enzyme called phytochelatin synthase (PCS). A characteristic element of phytochelatin is the repeated (2–11) motif of two amino acids: glutamic acid and cysteine. The carboxyterminal amino acid in the phytochelatin molecule is glycine, and in phytochelatin homologues called isophytochelatins, instead of glycine, there is β-alanine, serine, glutamic acid, or glutamine. Phytochelatin participates in the detoxification of metals by binding them to -SH cysteine groups (Clemens et al. 1999, Agrawal et al. 2011, Abratowska 2012, Bothe and Słomka 2017, Wójcik et al. 2017). For example, cadmium proteins arising from the combination of phytochelatins with cadmium were detected in the tissues of *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz, after the treatment of plants with this metal (Przedpełska-Wąsowicz et al. 2012, Wierzbicka 2015).

Heavy metals can also be chelated by amino acids. For example, it has been shown that *Noccaea caerulescens* (J. Presl & C. Presl) F. K. Mey. can bind metals, nickel in particular, through histidine. Nicotianamine has also been shown to be involved in the transport of zinc (as well as iron and other metals), especially in *A. halleri*, which is then deposited in a vacuole in the form of a complex (Bothe and Słomka 2017). It is known from the literature that nicotianamine plays an important role in plant tolerance to heavy metals. It has metal chelating properties and can participate in the loading and unloading of phloem (Stephan and Scholz 1993). Zinc hyperaccumulating plants, e.g. *A. halleri*, have been shown to have genes encoding nicotianamine synthase. At the cellular level, metal-nicotianamine complexes are moved from the cytoplasm to vacuoles, where they are isolated and thus detoxified. Nicotianamine plays an important role in the transport of elements such as: zinc, copper, iron, nickel, or manganese (Takahashi et al. 2003, Weber et al. 2004, Kim et al. 2005). Other studies conducted using the latest analytical chemistry methods have shown that zinc is found in *B. laevigata* in a complex with nicotianamine (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

Other metal ion binding molecules in the cytoplasm are glutathione and polyamines. Plant cell wall components, in particular pectins and cellulose fractions, can also be used for the adsorption of heavy metals (Bothe and Słomka 2017), which was mentioned earlier in discussion of the mechanism of compartmentation. For example, it was shown that *D. carthusianorum* root cells (Baranowska-Morek 2008) can bind lead inside dictiosomal vesicles carrying pectins, the building components of the cell wall. These vesicles then attach to the plasmalemma, and their content is removed to the cell wall by exocytosis, where large lead deposits are formed (Baranowska-Morek 2008, Abratowska 2012). In turn, in the cells of the roots and epidermis of the granary leaves of *A. cepa* after treatment with lead, the formation of a bulge in the plasmalemma (so-called plasmotubules) was observed. At the top of the plasmotubules, the accumulation of lead was visible, stored away in the form of deposits in the cell wall (Wierzbicka 1998, Wierzbicka et al. 2007).

Stress caused by heavy metals is often accompanied by other types of stress, such as salinization, drought, food deficits, or soil acidity. Plants in these situations induce the expression of genes responsible for the stress response. As a result, among others, S-glutathione transferase, superoxide dismutase, cytochrome P450, and thioredoxin are produced. Such proteins (enzymes) can be used to detoxify the ROS (reactive oxygen species) generated, among others, by excess heavy metals. In addition, heat shock proteins (HSP) can also be produced, which are formed not only at high temperatures, but are also under the influence of other stress factors, including exposure to excess heavy metals. HSPs not only function as chaperones in protein folding and assembly but can also be used to protect and repair proteins damaged by oxidative stress. Metals are also bound by other chaperone proteins, so-called metallochaperones (Huffman and O'Halloran 2001, Hall 2002, Ouziad et al. 2005, Hildebrandt et al. 2007, Kacperska 2007, Luo et al. 2016, Bothe and Słomka 2017).

After discussing the main mechanisms of plant protection against heavy metals, it is worth mentioning that the membrane transporters and aquaporins present in plasmalemma also contribute to the emergence of metal tolerance. For example, it has been shown that modified cellular water transport, resulting from differences in aquaporin activity, can increase the level of tolerance to heavy metals (Przedpełska-Wąsowicz and Wierzbicka 2011, Wierzbicka 2015). Ectomycorrhizal fungi (EMF) are also important in moderating stress associated with heavy metals, especially in trees. EMF has been shown to reduce heavy metal accumulation, e.g. by *Populus alba* L., *Pinus sylvestris* L., or *Betula* sp. (Hrynkiewicz et al. 2015, Bothe and Słomka 2017).

The taxonomy of two *B. laevigata* subspecies occurring in Poland will be presented later, followed by a discussion of the adaptation of the species to heavy metals in detail.

Taxonomy of *B. laevigata* from the Tatra Mountains and Bolesław

According to the literature (Pawlus 1985, Guinea and Heywood 1964, Wąsowicz 2015, Rostański et al. – Chapter 3 of this volume), *B. laevigata* is the only member of the *Biscutella* genus in the Polish flora. The plant is a perennial reaching the height of 10 to 40 cm. It is a very variable species. In the whole of Europe only two subspecies have been characterized until now (Guinea and Heywood 1964). For many years it has been reported that in Poland, in the Tatra Mountains, two subspecies occur: *B. laevigata* subsp. *kerneri* Mach.-Laur. and subsp. *gracilis* Mach.-Laur. However, morphological differences between the two subspecies were not obvious and distinguishing them had seemed rather unlikely for a long time. In the end, it was ascertained that in the mountainous region of central Europe, including the Tatra Mountains, *B. laevigata* subsp. *gracilis* occurs (Guinea and Heywood 1964, 1993, Pawlus 1985, Mirek et al. 2002). Moreover, the recent morphological and genetic studies, carried out in the Department of Ecotoxicology in the Faculty of Biology, University of Warsaw (UW) indicate the occurrence of only one subspecies in the Tatra Mountains, *B. laevigata* subsp. *gracilis* (Pielichowska 2007, Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017, 2020).

Based on the morphological studies of the *B. laevigata* individuals from three different localities in the Tatra Mountains: Czerwone Wierchy, Mała Łąka Valley and Jaworzynka Valley. It was stated that the plants occurring there differed greatly from each other, both in their appearance and size. This was connected with the insolation degree and moisture of the location in which they occurred. From the plants of the three localities, seeds were collected, and plants were grown in garden soil in a glasshouse in uniform light and humidity conditions. From the seeds of *B. laevigata* occurring in the Tatra localities studied, plants equal in size and habit were grown (Pielichowska 2007). This indicates that there are no genetically preserved morphological differences between the plants studied.

During further studies, the genetic analysis of *B. laevigata* from three localities in the Tatra Mountains (Jaworzynka Valley, Mała Łąka Valley and Stoły) was performed using molecular markers as part of the AFLP method (amplified fragment length polymorphism). As a result of these studies, the genetic structure of the populations was discovered. Very small genetic distances between the different Tatra populations were shown, so all individuals of these populations were grouped within one area (Fig. 2 in Bemowska-Kałabun et al. – Chapter 5 of this volume). The result obtained testifies to the lack of genetic differences and to the substantial gene flow between the Tatra populations studied (Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017, 2020). Therefore, the study results indicate unequivocally that in the Tatra Mountains only *B. laevigata* subsp. *gracilis* occurs.

At the same time, genetic studies of *B. laevigata* growing on the post-mining waste heap in Bolesław were carried out. The level of genetic diversity between the *B. laevigata* populations from Bolesław and the Tatra Mountains was studied. The results of these studies are shown in Figure 2, in Chapter 5. A very large genetic distance was found between the plants from the waste heap and those from the Tatra Mountains – it was over 30 times bigger than the genetic distance estimated solely between the Tatra populations. In addition, in the plant population from the waste heap no genotypes from the Tatra Mountains were found. It is thought that the gene flow between the Tatra populations and the waste heap population ceased at least several thousand years ago (Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017, 2020). It seems, therefore, that the distinctiveness of the waste heap plant population is the result of the evolutionary process, which definitely exceeds the time frame determined by the first literature reports on the occurrence of *B. laevigata* on post-mining sites in the Olkusz region (Uechtritz 1877, Wierzbicka et al. 2015).

The famous Polish botanist, Władysław Szafer, who studied lowland locations of alpine plants, was convinced that *B. laevigata* occurring in the Olkusz region is a glacial relic (Szafer 1930). This idea was reinforced by the fossil data that indicated the species' presence during the last glaciations. Our genetic studies enable approximate dating of the beginning of *B. laevigata* occurrence in the Olkusz region – the genetic isolation between the populations from the Tatra Mountains and Olkusz probably took place during the Middle-Polish glaciation, about 120,000 years ago (Wąsowicz et al. 2014, Wierzbicka et al. 2015).

One may wonder how *B. laevigata* could have survived in this area for such a long period of time. The answer is simple. The bedrock geology of the Olkusz region indicates that there have been always grounds rich in zinc-lead ores. On the ground surface, outcroppings of zinc-lead ores deposits were formed, and they existed long before humans began industrial exploitation in this area. The rocks with ore outcroppings could have been the place of the *B. laevigata* occurrence due to its high tolerance to heavy metals, especially zinc. On the other hand, following the excavation of metal ores, new habitats (e.g. heaps of mine waste contaminated with metal from ores) that are very suitable for growth of this pioneer species were formed.

As it was shown, the *B. laevigata* populations from the Olkusz region have evolved in isolation from other populations for thousands of years. There are significant morphological, anatomical, and physiological differences between these groups of plants (Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017, 2020). Therefore, the *B. laevigata* population from the waste heaps may be given the rank of a subspecies. For this newly discovered taxon we propose the name of *B. laevigata* L. subsp. woycickii M. Wierzb., Pielich. & Wasowicz (in Polish – *pleszczotka Wóycickiego*) to honor the memory of Professor Zygmunt Wóycicki (1871–1941), who was one of the founders of the Faculty of Biology, University of Warsaw and who initiated research on the calamine flora of the Olkusz region. Recently, a nomenclatorical publication has been published, presenting this new subspecies of *B. laevigata* (Wierzbicka et al. 2020).

In conclusion, we would like to emphasize the uniqueness of the *B. laevigata* subsp. *woycickii* plants from Bolesław. These plants are extremely valuable for science because they are both a 'postglacial relic', given that they are a remnant of glaciations, and they are also an 'endemic' – a subspecies occurring only in the Olkusz region (endemic = taxon unique to a given region). Plants classified as endemic are usually of a higher taxon (i.e. it is usually classified as a species), thus in this case the *woycickii* subspecies has an endemic character. These plants have great value, and they deserve to be widely known and given full protection in the Olkusz region.

Impact of the habitat on the morphology of *B. laevigata* from Bolesław and the Tatra Mountains

The natural habitat of plants has a decisive impact on plant morphology. Plants which colonize dry environments, saline marshes, or acidic or metalliferous soils must be characterized by morphological and physiological plasticity, as discussed in Chapter 5 of this volume. Generally, plasticity characterizes varieties, ecotypes and species of plants found in habitats difficult to colonize. Good examples are species characteristic for the flora of metalliferous sites, e.g. *S. vulgaris*, *D. carthusianorum*, *A. maritima*, *N. caerulescens* and *B. laevigata* (Bemowska-Kałabun et al. – Chapter 5 of this volume). We will follow both the morphology of *B. laevigata* subsp. *woycickii* from the post-mining calamine waste heap in Bolesław, as well as the morphology of *B. laevigata* subsp. *gracilis* from the Tatra Mountains.

Biscutella laevigata **– a facultative metallophyte**

Before we discuss the morphology of *B. laevigata*, it is worth reviewing the habitats where this species occurs. In Poland, both in the Tatra Mountains and on the zinc-lead (calamine) waste heaps in the Olkusz region, the metal content in the soil is increased, with the metal concentrations on the heaps being two to three orders of magnitude higher than the metal concentration in the Tatra Mountains. In the Olkusz region, the average concentration of heavy metals in the tested substrates (mainly calamine) are: 3,657 mg/kg lead, 9,764 mg/kg zinc and 76 mg/kg cadmium (Kapusta et al. 2015). In contrast, soil studies in the Tatra National Park have shown that the rendzinas occurring there also belong to soils with a relatively high content of heavy metals, which are: 38–411 mg/kg lead, 81–745 mg/kg zinc and 0.4–17.0 mg/kg cadmium (Miechówka et al. 2002). In the soil from *B. laevigata* sites in these regions of the Tatra Mountains, the metal concentrations fall within the above ranges (Rostański et al. – Chapter 3 of this volume). *B. laevigata* was found in France at similar sites on a zinc-lead heap in Les Malines (Las Aviniēres) near Montpellier, where the following metal concentrations were determined: 15,000 mg/kg zinc, 5,000 mg/kg lead, 40 mg/kg thallium. In the Austrian Alps, the presence of *B. laevigata* was found at a metalliferous site by the Gailitz river, where the metal concentrations in the soil were: up to 75,000 mg/kg zinc, up to 4,000 mg/kg lead, up to 44 mg/kg cadmium. *B. laevigata* has also been described to occur in areas with non-elevated heavy metal contents. For example, in the Hochobir region of the Austrian Alps where metal concentrations were determined at the level of the geochemical background of non-metalliferous areas: 63 mg/kg zinc, 33 mg/kg

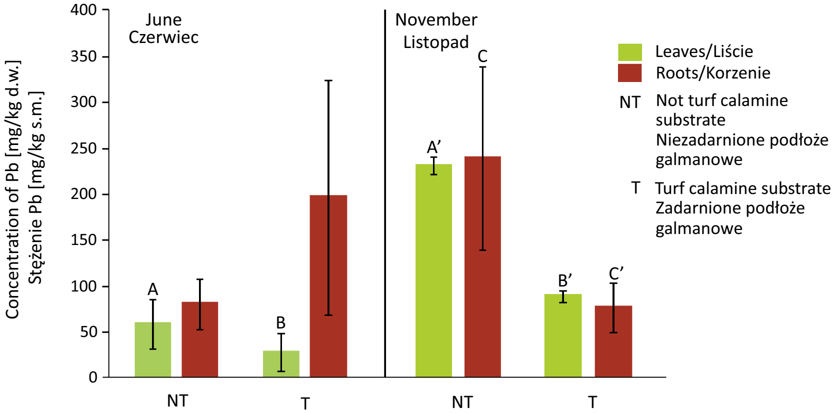

Fig. 1. Lead concentration in leaves and roots of *Biscutella laevigata* subsp. *woycickii* from the calamine waste heap in Bolesław. The plants grew in unvegetated calamine soil and vegetated calamine soil. The plants were collected in June and November. The results were given as means with standard deviation (n = 5). AA'/BB'/CC' – stiatistically significant differences (base on Pielichowska 2007, modified)

Ryc. 1. Stężenie ołowiu w liściach i korzeniach *Biscutella laevigata* subsp. *woycickii* z hałdy w Bolesławiu. Rośliny rosły na niezadarnionym i zadarnionym podłożu galmanowym. Rośliny zebrano w czerwcu i listopadzie. Wyniki podano jako średnie arytmetyczne wraz z odchyleniem standardowym (n = 5). AA'/BB'/CC' – różnice istotne statystycznie (za Pielichowska 2007, zmienione)

lead, 0.03 mg/kg cadmium (Anderson et al. 1999a, Wenzel and Jockwer 1999, Wierzbicka et al. 2015, 2017).

Plant species found on metalliferous soils are called metallophytes. They can be either obligatory metallophytes – if they occur only on metalliferous soils, or facultative metallophytes – if they occur on both metalliferous and normal soils. Considering the fact that *B. laevigata* occurs, in Poland and other European countries, on soils characterized by low, high and very high concentration of heavy metals, it should be classified as a facultative metallophyte (Wierzbicka et al. 2015, 2017). At the same time, research that has been conducted so far (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Brzost 2005, Owczarz 2006, Pielichowska 2007, Wierzbicka et al. 2015, 2017) indicates that *B. laevigata* has a strong preference for growth on metalliferous soils.

In previous studies, it has also been shown that the quantity of heavy metals taken up by *B. laevigata* is influenced by two factors: the vegetation period and the degree of soil sodding. For example, the highest concentrations of lead in the tissues of *B. laevigata* subsp. *woycickii* from Bolesław was found in individuals growing on non-turfy calamine substratum at the end of the growing season (November). In this case, the concentration of lead in plant roots increased 3 times, while in leaves it was 4 times compared to individuals found on turfy calamine. In addition, a significant increase (3 to 4 times) in the concentration of lead in leaves was observed in November, compared to June, regardless of whether the plants came from turfy or non-turfy soil (Fig. 1). A similar relationship was observed for zinc and cadmium (Pielichowska 2007).

Biscutella laevigata – prefers calcium rich soils

Biscutella laevigata from Bolesław and the Tatra Mountains occurs on substrates with a high calcium content and an alkaline

Table 1. The element concentrations [mg/kg d.w.] and pH of the calamine soil from Bolesław and the garden soil (Pielichowska 2007). Standard deviations are marked with '±'

Tabela 1. Stężenia pierwiastków [mg/kg s.m.] oraz pH podłoża galmanowego z Bolesławia i ziemi ogrodniczej (Pielichowska 2007). Znakiem „±" oznaczono odchylenia standardowe

Parameter Parametr Variant Wariant	Zn	Pb	Cd	Tl	Ca	Mg	K	Fe	N	C	pH
	mg/kg d.w mg/kg s.m.										
Calamine substrate Podłoże galmanowe	127317 ±9417	8832 ±804	536 ±34	43 ±5	73873 ±5895	44256 ±10718	3004 ±104	77444 ±6742	6400 ±100	73820 ±2879	7.81 ±0.06
Horticultural substrate Ziemia ogrodnicza	65 ±32	10 ±5	1 ±2	< LoD	16325 ±2977	1653 ±406	417 ±61	579 ±303	12140 ±716	440000 ±22497	4.97 ±0.08

LoD – the limit of detection
LoD – granica wykrywalności

pH. The geochemical background for soils in Poland is 1,700 mg/kg for calcium at pH 5.9 (Lis et al. 1995), whereas in soil from the Bolesław waste heap, the calcium content is very high (73,873 mg/kg d.w., at pH 7.81) (Table 1). In the Tatra Mountains, the content of this element in the substrate is even higher and amounts to 250,000 mg/kg (at pH 7.3) according to Godzik (1984), or 118,000 mg/kg according to Niklińska and Szarek-Łukaszewska (2002). Therefore, the presence of high calcium content in the substrate seems to be very important for *B. laevigata* as a whole species. It is important to understand that the high calcium content in the substrate and its high pH causes a lower toxicity of heavy metals to plants. In such conditions, smaller amounts of heavy metals are found in soil-soluble forms, which are available to plants (Pielichowska 2007, Wierzbicka et al. 2015). *B. laevigata* prefers habitats rich in calcium, with an alkaline pH, and this is an important feature of the species which allows it to colonize areas with elevated metal concentrations.

***Biscutella laevigata* – dry and wet habitats**

Studies have shown that (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015, 2017) the growth of *B. laevigata* subsp. *woycickii* occurs on waste heaps, most often in conditions that are characteristic of xerothermic habitats. *B. laevigata* occurs in waste heap soil that is dry, skeletal, and poor in organic matter, on the calamine substratum (Fig. 2A). Plants reach a different size in these conditions, depending on the water content of the soil, but their leaves have an average length of 4 ± 1.34 cm and width of 0.8 ± 0.3 cm (Fig. 3A, B). However, in the Tatra Mountains (Fig. 2B) two main habitat types of *B. laevigata* subsp. *gracilis* were distinguished: the wet ones, in which plants grew abundantly and were much bigger than the plants occurring on the post-mining waste heap; and the dry and rocky ones, in which plants have got a very similar habit to that of the plants of *B. laevigata* subsp. *woycickii* from Bolesław (Fig. 3C, D). *B. laevigata*

Fig. 2. Examples of locations of *Biscutella laevigata* in Poland: the old mine waste heap (calamine waste heap) in Bolesław – *B. laevigata* subsp. *woycickii* (A), the Western Tatra Mountains – *B. laevigata* subsp. *gracilis* (B). (photo A – O. Bemowska-Kałabun, B – M. Pielichowska)

Ryc. 2. Przykładowe stanowiska *Biscutella laevigata* w Polsce: hałda odpadów górniczych (hałda galmanowa) w Bolesławiu – *B. laevigata* subsp. *woycickii* (A), Tatry Zachodnie – *B. laevigata* subsp. *gracilis* (B). (fot. A – O. Bemowska-Kałabun, B – M. Pielichowska)

Fig. 3. Morphological diversity of two *Biscutella laevigata* subspecies depending on the place of occurrence. The individuals of *B. laevigata* subsp. *woycickii* growing on the calamine waste heap in a stony and dry location (A) or in a wet location (B). The individuals of *B. laevigata* subsp. *gracilis* in the Western Tatra Mountains growing on dry rock debris (C) or in a wet location (D) (base on Pielichowska 2007, Wierzbicka et al. 2015, 2017, modified)

Ryc. 3. Zróżnicowanie morfologiczne dwóch podgatunków pleszczotki górskiej w zależności od miejsca występowania. Osobniki *Biscutella laevigata* subsp. *woycickii* rosnące na hałdzie galmanowej na stanowisku kamienistym i suchym (A) lub na stanowisku wilgotnym (B). Osobniki *B. laevigata* subsp. *gracilis* w Tatrach Zachodnich rosnące na suchym rumoszu skalnym (C) lub na stanowisku wilgotnym (D) (za Pielichowska 2007, Wierzbicka i in. 2015, 2017, zmienione)

in these localities was characterised by a very large size and habit variability. Depending on the habitat conditions (dry or wet habitat), the mean leaf length was equal to 5 ± 2 cm and 9 ± 3.7 cm respectively, and the mean leaf width was 0.9 ± 0.3 cm and 1.0 ± 0.4 cm respectively. It was shown, however, that differences in the size of the Bolesław and Tatra plants (Fig. 4) are not genetically preserved. In the first plant generation (F1), cultivated in uniform glasshouse conditions with optimal humidity for growth, the sizes of the plants from both regions became unified (Fig. 5). Therefore, it was concluded that the smaller sizes of the calamine waste heap plants in comparison with the Tatra plants was a result of the adverse humidity conditions or presence of heavy metals (Pielichowska 2007). This is illustrated in Figure 6.

Biscutella laevigata – flowering period

The next features differentiating plants from waste heaps in Bolesław and the Tatra

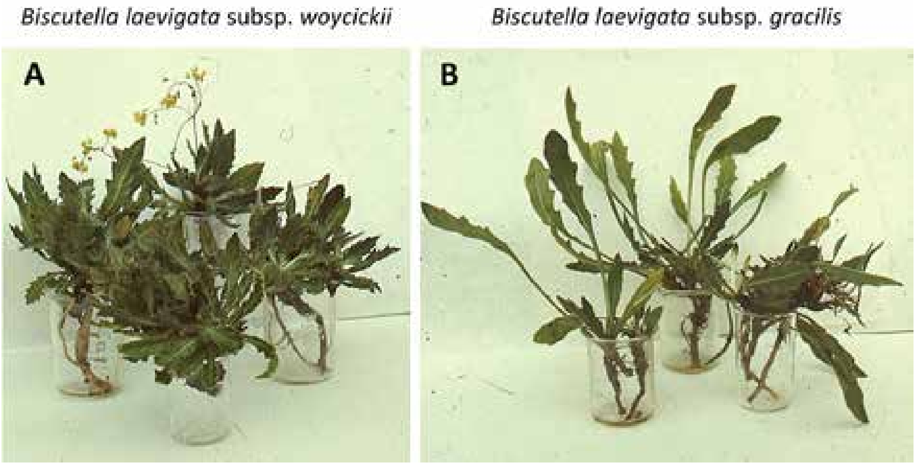

Fig. 4. *Biscutella laevigata* subsp. *woycickii* from Bolesław (A) and *B. laevigata* subsp. *gracilis* from the Tatra Mountains (B), collected in the field. The parental generation (F0). The difference is visible in plants' habit of both subspecies when they occur in the field

Ryc. 4. *Biscutella laevigata* subsp. *woycickii* z Bolesławia (A) oraz *B. laevigata* subsp. *gracilis* z Tatr (B), zebrane w terenie. Pokolenie rodzicielskie (F0). Widoczna jest różnica w pokroju roślin obu podgatunków gdy występują w terenie

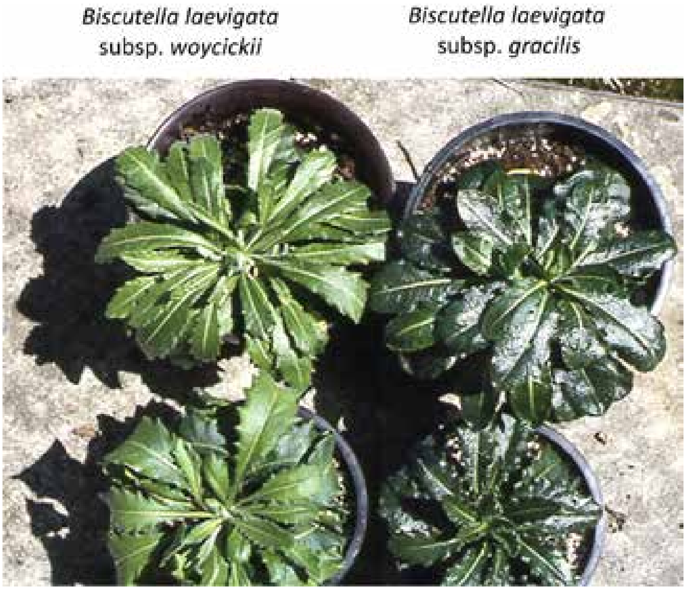

Fig. 5. *Biscutella laevigata* subsp. *woycickii* and *B. laevigata* subsp. *gracilis*, grown in garden soil, in the same glasshouse conditions. The first offspring generation (F1) (from seeds of plants growing in the field), the plants are five months old. The difference is visible in the leaf colour between the subspecies of *B. laevigata* (base on Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020, modified)

Ryc. 5. *Biscutella laevigata* subsp. *woycickii* oraz *B. laevigata* subsp. *gracilis*, wyhodowane w ziemi ogrodniczej, w jednakowych warunkach szklarniowych. Pierwsze pokolenie potomne (F1) (z nasion roślin z terenu), wiek roślin – pięć miesięcy. Widoczna jest różnica w kolorze liści pomiędzy badanymi podgatunkami pleszczotki górskiej (za Pielichowska 2007, Wierzbicka i in. 2015, 2017, 2020, zmienione)

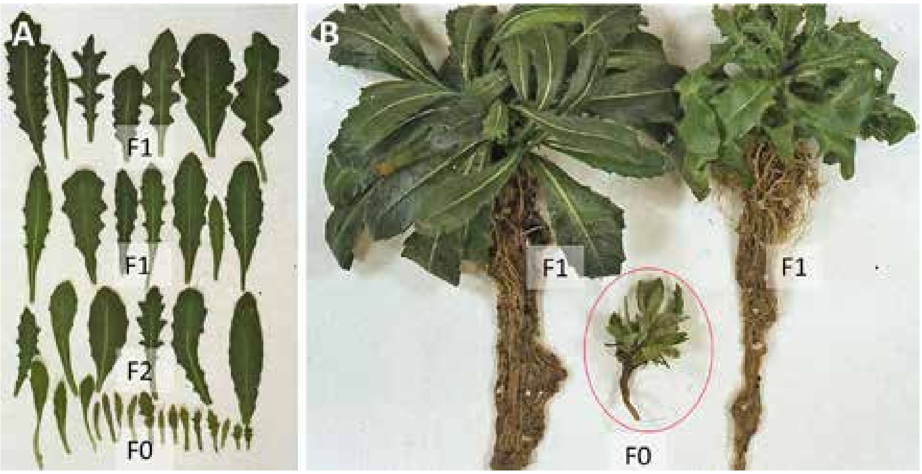

Fig. 6. Leaves (A) and whole plants (B) of *Biscutella laevigata* subsp. *woycickii* from Bolesław. The plants collected in the field (the parental generation, F0) or grown in the glasshouse under the same conditions (the first and second offspring generations, F1 and F2). The photos show that smaller size of the plants from the waste heap in Bolesław (the generation F0 marked with a red circle) did not occur in the next generation (F1), thus size is not a genetically preserved feature (B)

Ryc. 6. Liście (A) oraz całe rośliny (B) *Biscutella laevigata* subsp. *woycickii* z Bolesławia. Rośliny zebrane w terenie (pokolenie rodzicielskie F0) lub wyhodowane w szklarni w jednakowych warunkach (pierwsze i drugie pokolenie potomne – F1 i F2). Na zdjęciach widać, że małe rozmiary roślin z hałdy w Bolesławiu (pokolenie F0 oznaczone czerwonym okręgiem) nie wystąpiły w następnym pokoleniu (F1), czyli nie były cechą utrwaloną genetycznie (B)

Mountains, depending on the habitat conditions, were the periods of flowering and fruiting. On the waste heaps, *B. laevigata* subsp. *woycickii* usually entered the generative phase quickly (May to June). A certain number of individuals repeated flowering in September to October. On the other hand individuals of *B. laevigata* subsp. *gracilis* in the Tatra Mountains tended to enter the generative phase later, while the flowering period of the plants depended on the altitude. The following relationship was observed: the higher the plants were above sea level, the later they bloomed. In the higher parts of the mountains, the plants bloomed in the period from August to September, and the plants from the valleys in the period from June to July. However, this property did not persist in progeny plants when growing *B. laevigata* subsp. *woycickii* and subsp. *gracilis* in a greenhouse under the same conditions. All tested plants entered the period of flowering simultaneously, in June (Pielichowska 2007). Therefore, the date when plants enter the generative phase is only a feature caused by different habitat conditions, not a genetically fixed feature.

Genetically established features of *B. laevigata* subspecies

As a part of the research on *B. laevigata* (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015, 2016, 2017, 2020), a number of genetically fixed characteristics differentiating individuals of *B. laevigata* subsp. *woycickii* from the calamine waste heap in Bolesław and individuals of *B. laevigata* subsp. *gracilis* from the Tatra Mountains were identified. These differences were observed

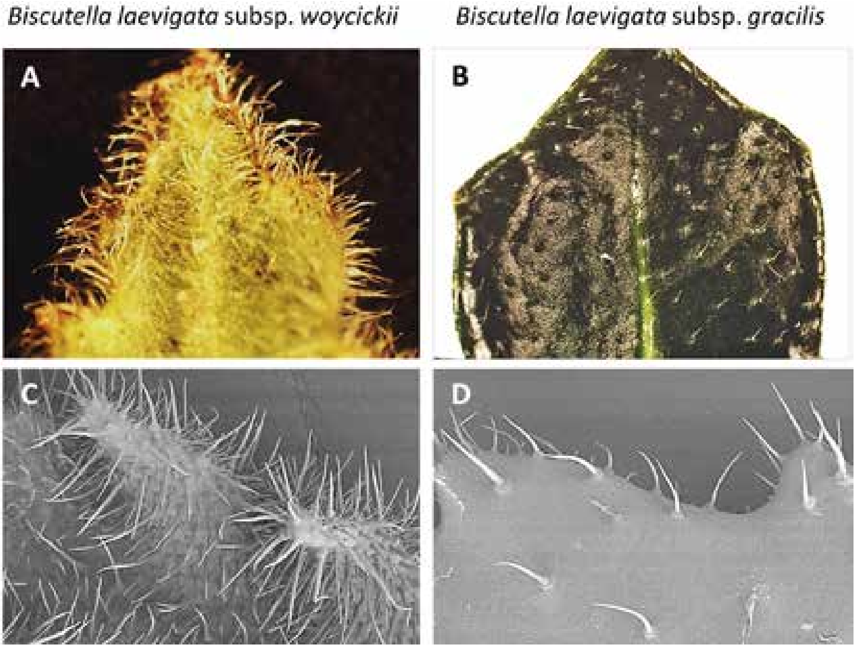

Fig. 7. Epidermal trichomes on the leaves of *Biscutella laevigata* subsp. *woycickii* (A, C) and *B. laevigata* subsp. *gracilis* (B, D). View of leaves in the stereoscopic microscope with magnification 4× (A, B) and the scanning electron microscope with magnification 400× (C, D). First offspring generation (F1), 5-month-old plants. The difference in a number of trichomes on leaves between the studied subspecies of *B. laevigata* is visible (base on Pielichowska 2007, Wierzbicka et al. 2015, 2017, modified)

Ryc. 7. Włoski na liściach *Biscutella laevigata* subsp. *woycickii* (A, C) i *B. laevigata* subsp. *gracilis* (B, D). Widok liści spod mikroskopu stereoskopowego z powiększeniem 4× (A, B) oraz skaningowego mikroskopu elektronowego z powiększeniem 400× (C, D). Pierwsze pokolenie potomne (F1), rośliny pięciomiesięczne. Widoczna jest różnica w ilości włosków na liściach pomiędzy badanymi podgatunkami pleszczotki górskiej (za Pielichowska 2007, Wierzbicka i in. 2015, 2017, zmienione)

both in plants from natural sites of the parental generation (F0) and plants from the first generation of offspring (F1), which were grown in the greenhouse. The following differences were found between representatives of both subspecies:

- density of leaf coverage with epidermal trichomes – epidermal trichomes covered a large leaf area in *B. laevigata* subsp. *woycickii* from Bolesław (usually 75–100% coverage), and a low leaf area in *B. laevigata* subsp. *gracilis* from the Tatra Mountains (usually 5–50% coverage) (Fig. 7),
- layer of wax covering the leaves – smaller for plants from Bolesław and larger for plants from the Tatra Mountains,
- leaf blade thickness – the leaf blade was thinner in *B. laevigata* subsp. *woycickii* from Bolesław (about 0.03 mm) than in *B. laevigata* subsp. *gracilis* from the Tatras (about 0.045 mm). The average difference of 0.015 mm was statistically significant. It was observed that the increased thickness of the leaf blade in the *B. laevigata* from the Tatra Mountains comes from the increase in the size of the palisade mesophyll cells with a con-

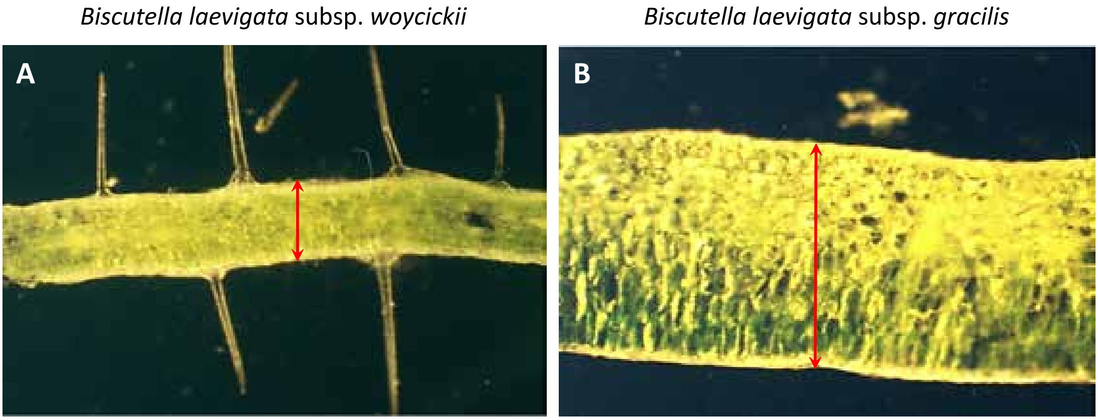

Fig. 8. Cross sections of leaf blades in *Biscutella laevigata* subsp. *woycickii* (A) and *B. laevigata* subsp. *gracilis* (B). Photos under the light microscope, in dark field (magnification 200×). First offspring generation (F1), 5-month-old plants. Thickness of leaf blades is shown by red arrows. The difference in leaf blade thickness between the studied subspecies of *B. laevigata* is visible (base on Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020, modified)

Ryc. 8. Przekroje poprzeczne przez blaszki liściowe *Biscutella laevigata* subsp. *woycickii* (A) i *B. laevigata* subsp. *gracilis* (B). Zdjęcia spod mikroskopu świetlnego, w ciemnym polu (powiększenie 200×). Pierwsze pokolenie potomne (F1), rośliny pięciomiesięczne. Czerwonymi strzałkami pokazano grubość blaszek liściowych. Widoczna jest różnica w grubości blaszki liściowej pomiędzy badanymi podgatunkami pleszczotki górskiej (za Pielichowska 2007, Wierzbicka i in. 2015, 2017, 2020, zmienione)

stant number of cells in the palisade mesophyll of plants from both subspecies (Fig. 8),

- color of leaves – light green in *B. laevigata* from Bolesław (52% plants) and dark green in *B. laevigata* from the Tatra Mountains (74% plants) (Figs 3, 5, 9). Chlorophyll content was the same in both subspecies, and the reason for the differences in the color of leaves was the difference in leaf blade thickness,
- seed size and germination strength – the seeds were 50% smaller in *B. laevigata* from Bolesław (average weight 1.8 ± 0.1 mg) than in the *B. laevigata* from the Tatra Mountains (average weight 3.0 ± 0.2 mg), which was shown for both plants from the parental generation (natural positions) and progeny plants (greenhouse) (Fig. 10). The germination strength of plant seeds from Bolesław and the Tatra Mountains was about 70% and

Fig. 9. Leaves of *Biscutella laevigata* subsp. *woycickii* from the waste heap in Bolesław and *B. laevigata* subsp. *gracilis* from the Tatra Mountains, collected in the field. Parental generation (F0). The difference in leaf colour and the number of trichomes between the studied subspecies of *B. laevigata* is shown

Ryc. 9. Liście *Biscutella laevigata* subsp. *woycickii* z hałdy w Bolesławiu oraz *B. laevigata* subsp. *gracilis* z Tatr, zebrane w terenie. Pokolenie rodzicielskie (F0). Widoczna jest różnica w kolorze liści oraz ilości włosków pomiędzy badanymi podgatunkami pleszczotki górskiej

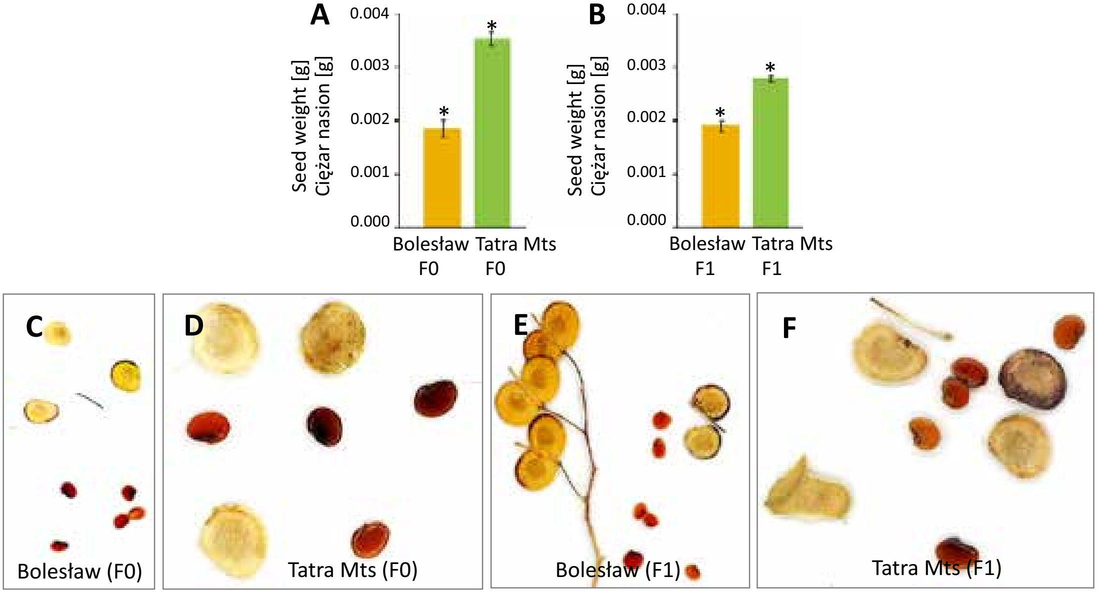

Fig. 10. Seed weight of the Bolesław and Tatra plants, collected in the field (parental generation, F0) (A) and from the individuals cultivated in a glasshouse (first offspring generation, F1) (B). Fruit and seeds of *Biscutella laevigata* subsp. *woycickii* from the waste heap in Bolesław (C, E) and *B. laevigata* subsp. *gracilis* from the Tatra Mountains (Jaworzynka Valley) (D, F) collected from individuals growing in the field (parental generation, F0) (C, D) and from individuals cultivated in a glasshouse (first offspring generation F1) (E, F). Magnification 1 : 1 (base on Pielichowska 2007, modified). Seeds of the Bolesław waste heap plants are significantly smaller than seeds of the Tatra plants – the feature is genetically preserved, and it occurred in generations F0 and F1

Ryc. 10. Ciężar nasion roślin z Bolesławia i Tatr, zebranych w terenie (pokolenie rodzicielskie, F0) (A) i z osobników wyhodowanych w szklarni (pierwsze pokolenie potomne, F1) (B). Owoce i nasiona *Biscutella laevigata* subsp. *woycickii* z hałdy w Bolesławiu (C, E) oraz *B. laevigata* subsp. *gracilis* z Tatr (Dolina Jaworzynki) (D, F) zebrane z osobników występujących w terenie (pokolenie rodzicielskie, F0) (C, D) i z osobników wyhodowanych w szklarni (pierwsze pokolenie potomne, F1) (E, F). Powiększenie 1 : 1 (za Pielichowska 2007, zmienione). Nasiona roślin z hałdy w Bolesławiu są istotnie mniejsze od nasion roślin z Tatr – cecha utrwalona genetycznie, wystąpiła w pokoleniu F0 i F1

85% respectively, i.e. it was similar. In both cases, seed germination remained at a similar level for the next three years,

- tolerance to heavy metals – for lead and cadmium it was higher by about 50%, and for zinc up to 90% in *B. laevigata* subsp. *woycickii*, than in *B. laevigata* subsp. *gracilis* (Fig. 11),
- vegetative reproduction (clonal forms) (Fig. 12A, B) – the ability to produce daughter rosettes (so-called ramets) from root buds (Fig. 12C–F). One plant from Bolesław produced on average 4 ± 2 rosettes, while one plant from the Tatra Mountains produced on average 2 ± 2 rosettes.

For *B. laevigata* subsp. *woycickii* from Bolesław and *B. laevigata* subsp. *gracilis* from the Tatra Mountains some similarities, which are species traits, were also observed:

- potential for hyperaccumulation of thallium – was at a similar level in both subspecies,
- plants size and leaves shape – in progeny generations these traits were the same in plants of both subspecies (Fig. 5, 6).

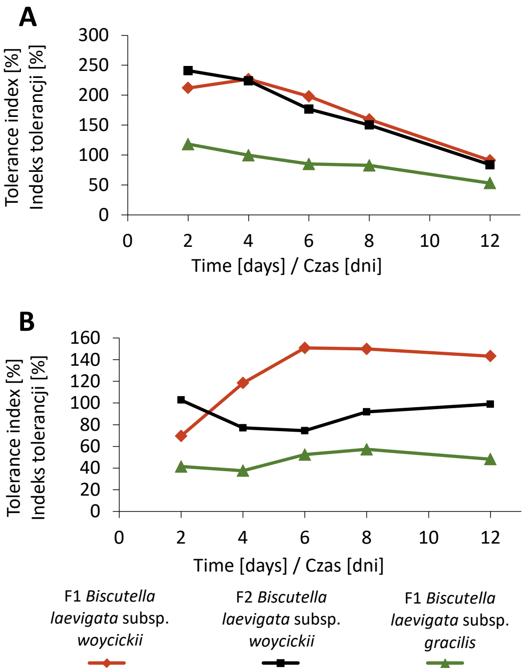

Fig. 11. Tolerance index [%] for the seedlings of *Biscutella laevigata* subsp. *woycickii* and *B. laevigata* subsp. *gracilis* on different days of incubation in medium with the addition of 2.5 mg/l lead (A) and 10 mg/l zinc (B). The tolerance index is the ratio of root length of plants treated with metal to root length of control plants multiplied by 100%. F1 – first offspring generation, F2 – second offspring generation. The values are given as means, n = 100. The results show high tolerance to lead and zinc of *B. laevigata* subsp. *woycickii*, in comparison with *B. laevigata* subsp. *gracilis* (base on Pielichowska 2007, modified)

Ryc. 11. Indeks tolerancji [%] dla siewek *Biscutella laevigata* subsp. *woycickii* oraz *B. laevigata* subsp. *gracilis* w kolejnych dniach inkubacji w pożywce z dodatkiem: ołowiu 2,5 mg/l (A) oraz cynku 10 mg/l (B). Indeks tolerancji to stosunek długości korzeni roślin traktowanych metalem w stosunku do długości korzeni roślin kontrolnych pomnożony przez 100%. F1 – pierwsze pokolenie potomne, F2 – drugie pokolenie potomne. Wartości podano jako średnie arytmetyczne, n = 100. Wyniki pokazują wysoką tolerancję na ołów i cynk *B. laevigata* subsp. *woycickii*, w porównaniu do *B. laevigata* subsp. *gracilis* (za Pielichowska 2007, zmienione)

The thickness of the leaf blade and the intensity of leaves' coverage with epidermal trichomes is associated with adaptation to xerothermic habitats. At the same time, plants from the calamine waste heap in Bolesław and those from the mountains have created different strategies of 'rub along' with the conditions of these habitats, including lack of water and strong sunlight. Leaves of *B. laevigata* subsp. *woycickii* are thin and covered with numerous trichomes. Intensive coverage of leaves with trichomes protects plants from waste heaps against excessive transpiration. In turn, the leaves of *B. laevigata* subsp. *gracilis* are thick, with few trichomes, but are covered with a thicker wax layer. Studies show that thicker leaf blades in *B. laevigata* subsp. *gracilis*, compared to subsp. *woycickii*, were the result of an increase in the size of the palisade mesophyll cells. At the same time, the number of cells in the palisade mesophyll remained constant in both subspecies of *B. laevigata*. Larger palisade mesophyll cells with large vacuoles (accumulation of water in tissues) and covering of leaves with a thick layer of wax protects plants from the Tatra Mountains against excessive transpiration and high UV radiation, which we deal with in the mountains (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020).

Both the habit and size of plants on calamine mine waste heaps are less diverse than in Tatra plants. This can be explained by the dominant xerothermic conditions throughout the waste heap area. In turn, the very large morphological diversity of the *B. laevigata* in the Tatra Mountains is caused by large differences in habitat conditions between its sites (among others, air temperature and insolation), resulting mainly from differences in altitude, the steepness of the slope and its exposure, as well as the variability in water availability. As

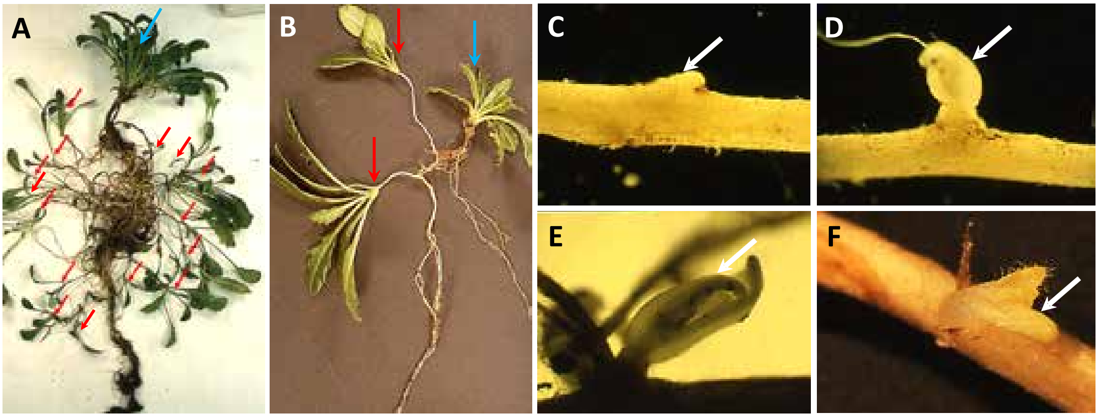

Fig. 12. Vegetative propagation in *Biscutella laevigata* subsp. *woycickii*, using the example of plants cultivated for 7 months in garden soil in a glasshouse. The mother individual (marked with a blue arrow) and 15 daughter rosettes (ramets), originating from root buds (marked with red arrows) (A). The mother individual (marked with a blue arrow) and 2 daughter rosettes (marked with red arrows) (B). Next stages of root bud development (marked with white arrow) (C–F): images from the light microscope at 25× magnification in the dark field (C, D) and bright field (E) and photos from a stereo microscope at 5× magnification (F) (base on Pielichowska 2007, Wierzbicka et al. 2015, modified)

Ryc. 12. Rozmnażanie wegetatywne u *Biscutella laevigata* subsp. *woycickii* na przykładzie roślin hodowanych przez 7 miesięcy w szklarni w ziemi ogrodniczej. Osobnik macierzysty (oznaczony niebieską strzałką) oraz 15 rozet potomnych (ramet), pochodzących z pączków korzeniowych (oznaczone czerwonymi strzałkami) (A). Osobnik macierzysty (oznaczony niebieską strzałką) oraz 2 rozety potomne (oznaczone czerwonymi strzałkami) (B). Kolejne etapy rozwoju pączka korzeniowego (oznaczonego białą strzałką) (C–F): zdjęcia spod mikroskopu świetlnego przy powiększeniu 25× w ciemnym polu (C, D) i jasnym polu (E) oraz zdjęcia spod mikroskopu stereoskopowego, przy powiększeniu 5× (F) (za Pielichowska 2007, Wierzbicka i in. 2015, zmienione)

already mentioned, differences in the habit and size of plants did not persist in the next generation of plants, which were growing under equal conditions. Also, differences in flowering dates are related to the habitat type in which *B. laevigata* occurs. On calamine waste heaps, the air temperature during the year is higher than in the mountains, so the vegetation period begins earlier; the first flowering occurs in April and can be repeated in September/October. High in the mountains, the growing season begins later (July to September) (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015, 2016, 2017). The flowers of plants from Bolesław and the Tatra Mountains were the same – they had four yellow petals of corolla about 4–7 mm long. Alternating with the petals of corolla were four yellow-green epicalyces that were shorter than them. The flowers were bisexual with a single pistil with a style and rod-like stigma and several stamens (Fig. 13) (Pielichowska 2007). In turn, the difference in seed size between *B. laevigata* from Bolesław and the Tatra Mountains was already a feature independent of the habitat, significantly differentiating plants from both subspecies. Plant seeds from Bolesław were 50% smaller than plant seeds from the Tatra Mountains. It was a genetically fixed trait (Fig. 10) (Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020). These differences between subspecies are very important from the point of view of their taxonomy.

The ability to produce clonal forms of reproduction ensures breeding success for the

Fig. 13. The single flower of *Biscutella laevigata* (A) and the whole inflorescence (B) (base on Pielichowska 2007, modified)
Ryc. 13. Pojedynczy kwiat pleszczotki górskiej (A) oraz cały kwiatostan (B) (za Pielichowska 2007, zmienione)

entire *B. laevigata* species on calamine waste heaps and in the mountains. Epidermal trichomes in *B. laevigata* play an important role in the detoxification of excess zinc, lead, cadmium, and thallium. It should be noted, however, that the increased amount of trichomes in plants from Bolesław, compared to those from the Tatra Mountains is one of many possible explanations for the greater tolerance of *B. laevigata* subsp. *woycickii* to heavy metals.

Metal tolerance in *B. laevigata* (especially subsp. *woycickii* from the waste heap in Bolesław) is associated with a number of mechanisms enabling detoxification. At the whole organism level, these mechanisms involve the removal of metals with the oldest leaves or metal accumulation in epidermal trichomes of leaves, as mentioned previously. At the cell level, however, it is metal deposition in vacuoles, cell walls, and intercellular spaces, as well as the synthesis of chelating compounds (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Brzost 2005, Owczarz 2006, Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020). These issues will be discussed in detail later in this chapter.

Tolerance to heavy metals of *B. laevigata* from Bolesław and the Tatra Mountains

Understanding the adaptations developed by plants growing on calamine waste heaps, such as *B. laevigata* subsp. *woycickii* from Bolesław, which enable them to grow in metalliferous areas, may facilitate the use of these plants in phytoremediation processes of heavily contaminated soils with heavy metals in the future (Muszyńska et al. – Chapter 7, Wiszniewska et al. – Chapter 8 of this volume). However, this requires experiments under strictly controlled conditions and a comparison of individuals with higher and lower tolerance to heavy metals. At the Department of Ecotoxicology UW, such experiments were carried out (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015, 2017, 2020). They allowed the observation of differences between *B. laevigata* subsp. *woycickii* from Bolesław and *B. laevigata* subsp. *gracilis* from the Tatra Mountains. The most important results of these works are presented below.

The level of tolerance to heavy metals of *B. laevigata* seedlings from Bolesław and the Tatra Mountains was tested using the Wilkins' root tolerance test and a comparison of their biomass. The tolerance index (IT) is the increase in root length of metal treated plants, expressed as a percentage of the control (Wilkins 1957). Plants were grown in Knop liquid medium, and their growth in solutions containing the given metal lasted 12 days. The following heavy metals concentrations were used: 2.5 mg/l lead, 10 mg/l zinc and 4 mg/l cadmium. It was shown that seedlings of *B. laevigata* from Bolesław and the Tatra Mountains significantly differed in their level of tolerance to lead, zinc and cadmium. An increased level of tolerance by about 50% was found for lead and cadmium and by 90% for zinc, and this occurred in two successive generations of progeny (F1, F2). Very high tolerance to lead in the F1 and F2 generation of *B. laevigata* subsp. *woycickii* was shown, therefore it was genetically fixed. The tolerance of plants seedlings from the Bolesław waste heap for zinc found in F1 also remained high, compared to plant seedlings from the Tatra Mountains, although at a lower level than in the case of tolerance to lead (Fig. 11). The cadmium tolerance was similar in plant seedlings from both subspecies. Biomass measurement is another indicator of plants tolerance to heavy metals. After 12 days of treating seedlings with the heavy metals mentioned above, their biomass was measured. It was found that the weight of seedlings from the first (F1) and second generation (F2) *B. laevigata* subsp. *woycickii* treated with heavy metals was higher than in *B. laevigata* subsp. *gracilis* (Pielichowska and Wierzbicka 2004, Wierzbicka and Pielichowska 2004, Pielichowska 2007, Wierzbicka et al. 2015).

Fig. 14. The root system of an adult plant of *Biscutella laevigata* subsp. *woycickii* during growth in medium with addition of lead. Young roots (marked with the red arrow) appeared during the 12-week cultivation in the solutions with a lead concentration of 8 mg/l and 16 mg/l (base on Pielichowska 2007, modified)

Ryc. 14. System korzeniowy dojrzałej rośliny *Biscutella laevigata* subsp. *woycickii* podczas wzrostu w pożywce z dodatkiem ołowiu. Młode korzenie (oznaczone czerwoną strzałką) pojawiały się podczas 12-tygodniowej hodowli, w roztworach o stężeniu 8 mg/l i 16 mg/l ołowiu (za Pielichowska 2007, zmienione)

Lead and cadmium tolerance of adult *B. laevigata* from Bolesław and the Tatra Mountains were also examined. Their growth (root and leaf length, emergence of new roots, number of alive and withered leaves) in the long-term test was compared. During this experiment, lead or cadmium concentrations were increased every 3 weeks: 2 mg/l, 4 mg/l, 8 mg/l and 16 mg/l. After a 12-week incubation period of plants with the metals, they were left for 3 weeks in a metal-free medium to see if they were able to regenerate. Within these studies it was shown that individuals of *B. laevigata* subsp. *woycickii* produced new roots throughout the experiment and in all tested concentrations of lead (Fig. 14). In contrast, plants from the Tatra Mountains produced new roots for the first two weeks of the experiment only. Then root development was inhibited. Plants from Bolesław treated with lead were also characterized by more intense growth of new leaves compared to plants from the Tatra. Dried leaves also appeared on plants from both subspecies, with more of them on the plants from the Tatra Mountains, which indicates their gradual death. In the case of treatment with cadmium, *B. laevigata* plants from both Bolesław and the Tatra Mountains produced new roots in the first weeks of the experiment, where there were low concentrations of this element (2 mg/l and 4 mg/l cadmium). Then, with the increase in cadmium concentrations in the medium (8 mg/l and 16 mg/l), the plants from Bolesław saw a decrease in the number of new roots, while plants from the Tatra had a complete growth inhibition of new roots. It was also shown that after cadmium treatment, the increase in the number of new, live leaves was higher in *B. laevigata* subsp. *woycickii*, in comparison with plants of *B. laevigata* subsp. *gracilis*. After stopping the incubation of plants with the metals, it was found that those from calamine waste heaps have a stronger ability to regenerate than those from the Tatra Mountains.

In summary, it was shown that *B. laevigata* subsp. *woycickii* from Bolesław, both in the seedling and vegetative maturity stages, are characterized by an increased tolerance to heavy metals (lead, zinc and cadmium), compared to *B. laevigata* subsp. *gracilis* from the Tatra Mountains (Pielichowska 2007).

Metal concentration and translocation in *B. laevigata*

During experimental cultivation of plants in liquid medium with the addition of metals, the concentration of heavy metals in the seedlings and adult plants of *B. laevigata* subsp. *woycickii* from waste heaps in Bolesław and subsp. *gracilis* from the Tatra Mountains was measured. The studies showed the intensity with which the plants are able to take up and translocate metals. In the experiments, seedlings (generations F1 and F2) and 2.5-month-old adult plants (generation F1) were used; they were incubated for 12 days (a short-term experiment) in the solutions with the following heavy metal concentration: 2.5 mg/l lead, 10 mg/l zinc and 4 mg/l cadmium.

The uptake of metals by the seedlings and adult plants of *B. laevigata* from Bolesław and the Tatra Mountains and the metal concentrations in the plants were compared. It was found that lead was taken up in smaller quantities by the seedlings than adult plants. In the seedling roots, the lead content reached the level of 2,500 mg/kg d.w., and in the adult plants 10,000 mg/kg d.w. In the aboveground parts of seedlings, the lead concentration was higher (about 180 mg/kg d.w.) in comparison with the concentration of this element in the adult plants (about 20 mg/kg d.w.). Furthermore, zinc was accumulated differently in seedlings and adult plants. It was found that the seedling roots of *B. laevigata* subsp. *woycickii* had a significantly higher zinc concentration than the seedling roots of *B. laevigata* subsp. *gracilis* – about 9,000 and 5,000 mg/kg d.w respectively. As for the plants from Bolesław, the zinc concentration found in the aboveground parts of seedlings was significantly higher (about 3,000 mg/kg d.w.) than in the aboveground parts of adult plants (about 1,800 mg/kg d.w.). It was shown that the metal transport to the aboveground parts is higher in the seedlings than in the adult plants (Pielichowska 2007).

In the case of the adult plants (F1), lead, zinc and cadmium were mostly accumulated in their roots. No significant differences in the accumulation of these metals in roots between the Bolesław and Tatra plants were found. The roots accumulated lead the most, then zinc, and cadmium the least. It was also observed that *B. laevigata* accumulated metals both in the adult leaves and the old, withering ones. Transport of the studied elements to aboveground parts was varied and dependent on both the given element and the plants' origin. More intensive translocation of zinc and cadmium to the aboveground parts was observed in the plants of *B. laevigata* subsp. *woycickii* than those of subsp. *gracilis* from the Tatra Mountains. As for lead, transport to the aboveground parts was very weak and equal in both subspecies (Pielichowska 2007).

Calamine substrate characteristics

The substratum from the zinc-lead waste heap in Bolesław is not homogeneous. It is mine waste, which is a mixture of variously fragmented and weathered wastes from the extraction of ores with a variable content of elements (Brunarska and Szarek-Łukaszewska – Chapter 2 of this volume). For experiments carried out in our laboratory, we used calamine substratum taken from the mine waste heap in Bolesław in three places. Then, the collected substratum was mixed, and stones were removed from it. The concentration of elements in the calamine substrate is shown in Table 1. For comparison, horticultural soil was also tested. The concentration of both heavy metals and nutrients were tested (Table 1).

When comparing the composition of the calamine substratum with the horticultural soil, there are very high concentrations of heavy metals in the calamine substratum. In the case of zinc, the differences between calamine substratum and horticultural soil are higher by four orders of magnitude. In the case of lead, cadmium, and thallium these differences are two orders of magnitude higher (Table 1). It should be emphasized that the amount of metal available to plants was within a few percent of their total content. The concentrations of nutrients are rather interesting. Compared to horticultural soil, calamine substratum contained elevated amounts of calcium (5 times more), magnesium (3 times), potassium (7 times) and iron (133 times). However, the low nitrogen content in the calamine substratum is significant; it was 19 times less than in horticultural soil (Table 1). Nitrogen in the soil is necessary for the proper growth and development of plants. Undoubtedly, the insufficient amount of nitrogen in the calamine substratum was one of the basic factors limiting the composition of calamine flora and limiting plants growth. The amount of carbon was also 6 times smaller in calamine substratum than in horticultural soil. However, this is due to the presence of a low amount of plant biomass in the calamine substrate. The calamine substrate pH was 7.81, whereas the horticultural soil pH value is usually around 5 (4.97) (Table 1).

The results of the analysis show how unusual the composition of calamine substratum is compared to horticultural soil. Considering these results, it is amazing that the plants are able to colonize substrate which is so difficult to grow on. As the next subchapter shows, *B. laevigata* has such abilities, and it even shows a tendency to stimulate growth on such substrates.

Growth of *B. laevigata* subsp. *woycickii* and subsp. *gracilis* in calamine substrate

When we were researching this extremely interesting species, we often asked ourselves whether the plants originating from the Tatra Mountains would also grow on the substratum from the waste heap in Bolesław. To investigate this, we carried out breeding of both subspecies of *B. laevigata* in calamine substrate (F1 generation). The control group consisted of plants grown in horticultural soil. Plants of different ages were transplanted to both types of substrates: 2-week-old seedlings, 1-, 2-, and 10-month-old mature plants. Plant cultivation

Fig. 15. A comparison of the appearance of *Biscutella laevigata* subsp. *woycickii* and subsp. *gracilis*, from the generation F1, after 5 months of cultivation in the calamine soil. Adult plants (10-month-old) were replanted to the calamine soil. The photo illustrates good growth of both subspecies in the calamine soil (base on Pielichowska 2007, modified)

Ryc. 15. Porównanie wyglądu *Biscutella laevigata* subsp. *woycickii* i subsp. *gracilis*, z pokolenia F1, po pięciu miesiącach hodowli w podłożu galmanowym. Do podłoża galmanowego przesadzono dojrzałe rośliny (10-miesięczne). Zdjęcie ilustruje dobry wzrost obu podgatunków na podłożu galmanowym (za Pielichowska 2007, zmienione)

was carried out for a period of 5 months. It turned out that both individuals of *B. laevigata* subsp. *woycickii* and *B. laevigata* subsp. *gracilis* grow well in calamine substrate, reaching similar sizes (Fig. 15).

The youngest plants, i.e. transplanted to this medium in the seedling phase, were the most sensitive to growth in calamine substrate. The growth of seedlings in calamine substrate was almost completely inhibited, equally in both subspecies of the *B. laevigata*. After a period of 2.5 months, most seedlings in calamine substrate began to die off. In both cases, only 10% of seedlings survived. 1-month-old plants, after a period of 5 months growing in calamine substrate, reached the size of about 20–50% of the control plants. Plants of *B. laevigata* subsp. *woycickii* from Bolesław were characterized by better growth than subsp. *gracilis* from the Tatra Mountains. In this phase of growth, differences in tolerance to calamine substrate between both subspecies were most apparent (Fig. 16A). On the other hand, transplanted plants at the age of 2 months, after 5 months of growth in calamine substrate, achieved a size between 70% to 160% of the control plants. When the oldest, 10-month-old, mature plants were transplanted into calamine substrate, these plants obtained on average larger sizes than the control plants – from 105% to 160% of the control plants after 5 months of cultivation. Usually, plants of *B. laevigata* subsp. *woycickii* were larger. Thus, the research showed a very large difference in the sensitivity of *B. laevigata* plants of both subspecies, depending on their age, to growth in calamine substrate. For plants in the seedling phase (from both subspecies), calamine substrate was toxic for most of the

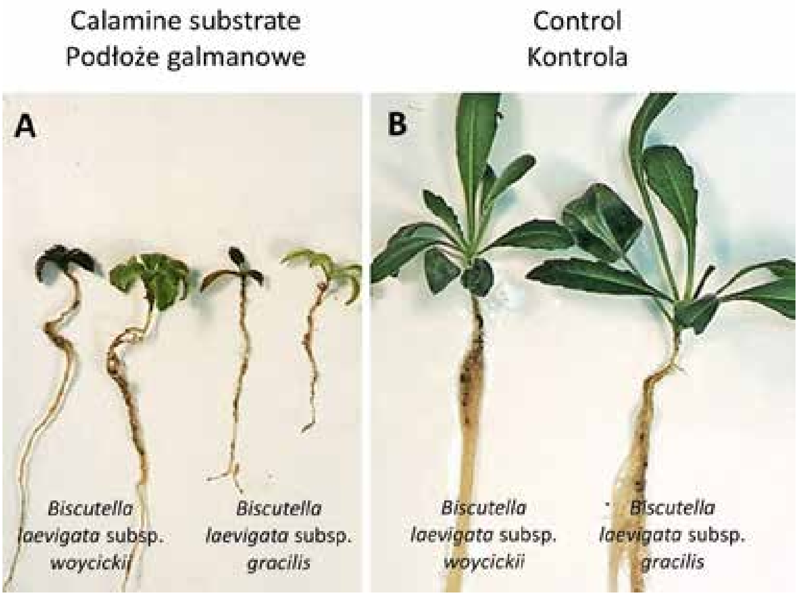

Fig. 16. A comparison of the size of *Biscutella laevigata* subsp. *woycickii* and subsp. *gracilis*, from the generation F1 cultivated for 5 months in the calamine soil (A) and the garden soil (control) (B). The 1-month-old plants were replanted to the calamine soil. The photo illustrates weaker growth of the plants of *B. laevigata* from the Tatra Mountains in comparison with the plants from Bolesław (A) (base on Pielichowska 2007, modified)

Ryc. 16. Porównanie wielkości *Biscutella laevigata* subsp. *woycickii* i subsp. *gracilis*, z pokolenia F1 hodowanych przez pięć miesięcy w podłożu galmanowym (A) i ziemi ogrodniczej (kontrola) (B). Do podłoża galmanowego przesadzono rośliny w wieku jednego miesiąca. Zdjęcie ilustruje słabszy wzrost roślin pleszczotki górskiej z Tatr, w porównaniu do roślin z Bolesławia (A) (za Pielichowska 2007, zmienione)

seedlings. In contrast, the older the plants were, the easier their growth in calamine substrate was. In the case of the oldest plants (2- and 10-month-old), even their growth was stimulated on calamine substrate, but to a greater extent in the *woycickii* subspecies (Fig. 15). This result indicates a high tolerance of mature *B. laevigata* subsp. *woycickii* and subsp. *gracilis* for growth in calamine substrate. In this way, it was shown that plants from the Tatra Mountains have sufficient tolerance to metals and can grow on calamine substrate if they survive the seedling phase (natural selection). The *woycickii* subspecies, however, shows better adaptation to growth in the calamine substrate (Pielichowska 2007, Wierzbicka et al. 2015).

Uptake of metals from calamine substrate by *B. laevigata* subsp. *woycickii* and subsp. *gracilis*

As part of the above experiment, in plants grown in laboratory conditions in calamine substrate, the content of metals that are usually found in calamine (lead, zinc, cadmium and thallium) was also checked. After 5 months of cultivation, plants growing on calamine substrate, both those from Bolesław and from the Tatra Mountains, had accumulated the following elements in their tissues: about 700 mg/kg d.w. lead, 8,000 mg/kg d.w. zinc, 75 mg/kg d.w. cadmium, and 70 mg/kg d.w. thallium. These high concentrations of metals are indicative of the species' high ability to accumulate these elements in tissues without symptoms of their toxic effects, i.e. high tolerance of plants to heavy metals (Wierzbicka et al. 2015).

Comparing the above results with the uptake of metals by *B. laevigata* subsp. *woycickii*, growing in natural conditions on the calamine waste heaps in Bolesław, a surprising difference can be observed. Plants growing on the calamine waste heap contained 2–3 times less metals in their tissues compared to plants grown in the laboratory, also in calamine substrate. For example, plants from the waste heap contained up to 240 mg/kg d.w. lead, up to 2,618 mg/kg d.w. zinc, and up to 35 mg/kg d.w. cadmium (Pielichowska 2007). We did not expect such a result, because the time of growth of *B. laevigata* plants on the waste heap was much longer (perennial plant) in comparison to our experiment (5 months). We thought that plants growing on a waste heap contain more metals than plants grown in a laboratory. However, the reverse turned out to be true. The calamine substrate in both cases had a similar composition. So, what was the reason for a such significant increase in metal uptake during their cultivation in calamine in an experimental context? There was only one difference. Under experimental conditions, the plants were constantly watered to ensure optimal growth. However, in natural conditions (waste heap), water deficit conditions are most common. Therefore, drought reduces the availability of metals for plants. This means that in conditions of excessive rainfall, a much larger amount of metals will be taken up by the plants, and thus they will be incorporated into the biological cycle, which is a very unfavorable phenomenon. Lowering the calamine substrate pH will significantly increase the availability of metals for plants.

There is also a difference in the uptake of metals depending on the age of the plants. This was tested in plants of different ages which were transplanted to the calamine substrate (2-week-old seedlings, 1-, 2-, and 10-month-old mature plants) and grown there for a period of 5 months. In *B. laevigata* subsp. *woycickii* and *B. laevigata* subsp. *gracilis*, the uptake and transport of zinc, lead, and cadmium was higher in young plants (seedlings and 1- and 2-month-old plants), compared to the oldest (10-month-old) plants. These differences were 3–4 times higher and statistically significant. The translocation of lead, zinc, and cadmium from roots to overground parts of plants was relatively high in all combinations tested. For example, for 10-month-old plants, after 5 months of growing in calamine soil, the translocation of metals from the roots to the overground parts was 60–70% for lead, 80% for zinc, and 40–50% for cadmium.

In the case of the fourth of the studied metals, thallium, it was taken up by both the *B. laevigata* from Bolesław and from the Tatra Mountains. Thallium was accumulated to the greatest extent by seedlings (reaching an average concentration of 219 mg/kg d.w.), and to a much lesser extent by older plants – 1-, 2- and 10-month-old (reaching an average concentration of 43 mg/kg d.w.). Among these older plants, no significant differences were found in the amount of accumulated by the roots and transported to the overground parts, depending on their age. Thallium concentration in the roots was low, with the concentration of this element in the overground parts, where about 85% of the total thallium content in the plant was found in all age groups of plants from Bolesław and the Tatra Mountains (Pielichowska 2007, Wierzbicka et al. 2015).

The studies discussed so far show that the greater sensitivity of seedlings to metals than adult plants is due to the fact that they took up several times the amount of metal than adult plants. Therefore, it was questioned how the *B. laevigata* species is maintained and how mature individuals develop, given that they are so abundant on the calamine waste heap in Bolesław, if the seedling phase of these plants is so sensitive to growth in calamine substrate. It seems that the answer to this question is the ability of this species to create clonal forms of vegetative reproduction (Fig. 12). As a result of seedling selection, few plants (5–10%) are able to grow further. This issue was widely discussed in the subsection 'Adaptation of plants to the presence of heavy metals in the substrate'. It is worth emphasizing here again that the production of a clonal form of vegetative reproduction was more effective in individuals of *B. laevigata* subsp. *woycickii*, than in individuals of *B. laevigata* subsp. *gracilis* (Pielichowska 2007, Wierzbicka et al. 2015, 2017). This means that when attempting to remediate fresh zinc-lead waste heaps, the most beneficial modes of action will be sowing a very large number of seeds, because only a few percent of plants will survive the seedling phase, or replanting older plants which are at least 2-months-old.

Tolerance to heavy metals of the *B. laevigata* subsp. *woycickii* and subsp. *gracilis* plants compared to tolerance of other plant species

Another interesting question is whether tolerance to heavy metals in *B. laevigata* is exceptionally high in comparison to the tolerance of other plant species. In the case of plants growing on waste heaps, characterised by induced (acquired) tolerance to heavy metals, the studies (Wierzbicka 1999) showed that plant species with the highest tolerance to lead

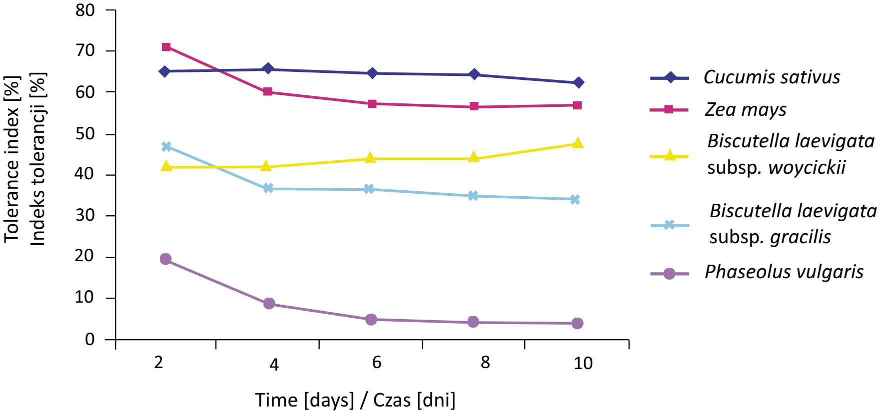

Fig. 17. The tolerance index in different days of plants' incubation in the cadmium solution (2.5 mg/l) for the seedlings of *Cucumis sativus*, *Zea mays*, *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis* and *Phaseolus vulgaris*. The tolerance index is the ratio of root length increase of plants treated with cadmium [cm] to the root length increase of control plants [cm], multiplied by 100%, N = 70 (base on Brzost 2005, modified). The cadmium tolerance of *B. laevigata* is average in comparison with other cultivated plant species

Ryc. 17. Indeks tolerancji w kolejnych dniach traktowania roślin roztworem kadmu (2,5 mg/l), dla siewek *Cucumis sativus*, *Zea mays*, *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis* i *Phaseolus vulgaris*. Indeks tolerancji to stosunek przyrostu korzeni roślin traktowanych kadmem [cm] do przyrostu korzeni roślin kontrolnych [cm] pomnożony przez 100%. N = 70 (za Brzost 2005, zmienione). Tolerancja na kadm *B. laevigata* jest przeciętna w porównaniu z innymi gatunkami roślin uprawnych may be ranked in the following order, beginning with the least tolerant species: *Leontodon hispidus* < *B. laevigata* < *S. vulgaris*.

However, what about constitutive (innate) tolerance? Comparative studies for a number of cultivated and wild plant species were conducted. They showed that the highest tolerance to lead was demonstrated by barley and maize, as well as the waste heap plants *S. vulgaris* and *B. laevigata* (Wierzbicka 1999). A lower level of tolerance was shown by wheat, pea, cucumber, lupine, and radish, and the lowest by bean, rape, and soya. In the Department of Ecotoxicology, in the Faculty of Biology UW, studies were also undertaken that aimed to compare the tolerance level to cadmium in the plants of *B. laevigata* subsp. *woycickii* and subsp. *gracilis*, alongside three cultivated plant species: cucumber (*Cucumis sativus* L.), bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.) (Brzost 2005). It was shown that as little as a 2.5 mg/l cadmium dose causes inhibition of root elongation of the plants treated with this metal, compared with the control plants. However, the inhibitory action of cadmium was varied for different species. The calculated values of the Wilkins's tolerance index (Wilkins 1957) enabled one to determine the tolerance level to cadmium in the studied species and to divide the species into three groups: of the highest tolerance – *C. sativus* and *Z. mays* (the tolerance index of the plants to cadmium was maintained at the level of about 60%), of moderate tolerance – *B. laevigata* subsp. *woycickii* and subsp. *gracilis* (the tolerance index of the plants to cadmium was maintained at the level of about 40%) and the one of low tolerance – *P. vulgaris* (the tolerance index of the plants to cadmium was equal to 10%, on average) (Fig. 17). The cadmium concentration in the roots of the studied plant species was the highest in the case of *Z. mays* and amounted to 1,936 mg/kg d.w., and less cadmium was accumulated in *C. sativus*. Moderate values were noted in *B. laevigata* subsp. *woycickii* and subsp. *gracilis*. The least cadmium accumulated in roots was in *P. vulgaris* – only 1,056 mg/kg d.w. In the aboveground parts of plants, the cadmium content was the highest in *B. laevigata* subsp. *woycickii* – 2,039 mg/kg d.w., and half of this cadmium amount was accumulated by *B. laevigata* subsp. *gracilis* – 1,048 mg/kg d.w.

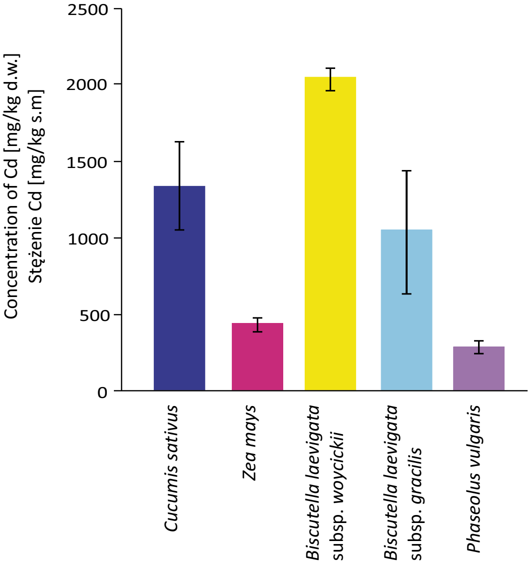

Fig. 18. The cadmium concentration [mg/kg d.w.] in aboveground parts of plants after 10 days of incubation in the cadmium solution (2.5 mg/l), for the seedlings of *Cucumis sativus*, *Zea mays*, *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis* and *Phaseolus vulgaris*. A mean cadmium concentration (a bar) with a standard deviation (a vertical line). N = 70 (base on Brzost 2005, modified). The cadmium concentration in plants does not coincide with their cadmium tolerance (cf. Figure 17)

Ryc. 18. Stężenie kadmu [mg/kg s.m.] w częściach nadziemnych po 10 dniach traktowania roślin roztworem kadmu (2,5 mg/l), dla siewek roślin *Cucumis sativus*, *Zea mays*, *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis* i *Phaseolus vulgaris*. Przedstawiono średnie stężenie kadmu (słupek) wraz z odchyleniem standardowym (pionowa linia). N = 70 (za Brzost 2005, zmienione). Stężenie kadmu w roślinach nie pokrywa się z ich tolerancją na kadm (por. Rycina 17)

In other species, the cadmium concentration in aboveground parts amounted from 290 mg/kg d.w. to 1,337 mg/kg d.w. (Fig. 18). The results indicate that the biggest transport of cadmium to aboveground parts occurred in the seedlings of *B. laevigata* subsp. *woycickii* from Bolesław. This proves that differences in the tolerance of these species were not connected with the amount of cadmium in plants. So they resulted from internal processes, specific for a given species.

The species studied can be ranked according to the level of their tolerance from the least to the most cadmium tolerant ones: *P. vulgaris* < *B. laevigata* subsp. *gracilis* < *B. laevigata* subsp. *woycickii* < *Z. mays* < *C. sativus*. It is also important to note the difference in cadmium tolerance between *B. laevigata* from Bolesław and the Tatra Mountains. The *B. laevigata* subsp. *woycickii* plants indicated about 20% higher tolerance to cadmium compared with *B. laevigata* from the Tatra Mountains (Wierzbicka 1999, Brzost 2005).

The results obtained show that tolerance to lead and cadmium is rather moderate in the buckler mustard plants in comparison with other plant species. We were surprised by these results. Why does maize, with the highest tolerance to the studied metals, not grow on the waste heaps?

The zinc-loving properties, as a secret of *B. laevigata*

Metallophilic plants, which develop unhindered by an environment where the concentration of heavy metals in the soil exceeds the permissible values, are the subject of many research works. As we already know, such plants include the main subject of this book – *B. laevigata*, which occurs in large numbers at the post-mining waste heap, from the extraction of zinc and lead ores in Bolesław. In many studies, attention has been paid to the amazing occurrence of this species in Poland. This species is found in the Tatra Mountains, but also in the lowlands, only on the heaps in Bolesław. According to Szafer (1927) it is a Pleistocene relic of the glacial age in the Polish lowland. This fact was confirmed by genetic tests (Wąsowicz et al. 2014), discussed in the previous chapter (Bemowska-Kałabun et al. – Chapter 5 of this volume). However, the puzzling distribution of this plant species in our country remains unexplained. For what reason do these plants occur in isolated positions, at a large distance from each other (about 100 km)? For many years this question was asked.

It is known that *B. laevigata* is characterized by adaptations to dry and very sunny (xerothermic) habitats, which was discussed at the beginning of this chapter. It is a light- and thermophilic plant, which tolerates water deficits well. It prefers substrates rich in calcium (Dobrzańska 1955). Therefore, what prevented the wider range of occurrence of this species both in Poland and in Europe?

The main reason seems to be the presence of zinc in the substrate at high concentrations. In the soil from the heap in Bolesław, the zinc concentration is very high, over 50,000 mg/kg. Iron concentration is similarly high, over 55,000 mg/kg. However, the concentration of lead here is about 3,100 mg/kg, and of cadmium about 180 mg/kg (Niklińska and Szarek-Łukaszewska 2002). The origin of these metals is easy to explain. Waste heaps arise from mining activities, these are wastes remaining after the extraction of zinc and lead ores (Włodarz – Chapter 1 of this volume, Brunarska and Szarek-Łukaszewska – Chapter 2 of this volume). But in the Tatra Mountains, where *B. laevigata* also occurs, elevated heavy metal concentrations were not expected. Godzik (1984) and later Babst-Kostecka et al. (2016) found that at the site in the Tatra Mountains (e.g. in the Mała Łąka Valley) the substrate contains a surprisingly high concentration of zinc, over 300 mg/kg. In addition to excessive amounts of zinc, there is also lead at a concentration of over 200 mg/kg.

It turned out that the main source of increased heavy metal concentration in the Tatras was the mining of metal ores in previous centuries (Miechówka et al. 2002). There were ironworks in the Tatra Mountains, which were open, with breaks, until the year 1878. The legacy of these ironworks is seen in some of the names of the districts of Zakopane (the main city at the foot of the Tatra Mountains), e.g. 'Kuźnice' – this name comes from the word smithy (pl. *kuźnia*), i.e. a building for blacksmith's works. The former name of this district is 'Huty Hamerskie'. It appeared in historical records in 1766. It should now be explained why we consider zinc as the main reason for such an interesting occurrence of *B. laevigata* plants in Poland.

In the laboratory tests carried out by the authors of this chapter, the zinc tolerance of *B. laevigata* plants was compared with the tolerance of other plant species: beans (*P. vulgaris*), cucumber (*C. sativus*) and maize (*Z. mays*) (Owczarz 2006). Plants in the seedling phase were tested. They were grown for 11 days in a mineral medium with the addition of zinc in the form of $ZnCl_2$ (8 mg/l). During this time, the plants' growth (roots and shoots) was measured. Control plants (without adding excess zinc to the medium) were grown in an identical manner. Figure 19 shows the final results. *B. laevigata* subsp. *woycickii* from Bolesław tolerated excess zinc extremely well. The tolerance of these plants as a percentage of the control plants was 200%. This means there was a strong stimulation of their growth in the presence of zinc. In contrast, *B. laevigata* subsp. *gracilis* from

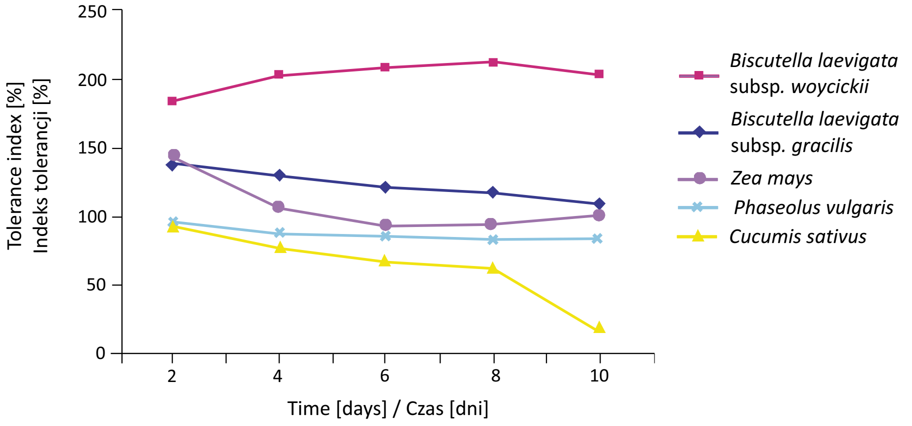

Fig. 19. The tolerance index on different days of the incubation of plants in zinc solution (8 mg/l), for the seedlings of *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis*, *Zea mays*, *Phaseolus vulgaris* and *Cucumis sativus*. The tolerance index is the ratio of root length increase [cm] of plants treated with zinc to the root length increase [cm] of control plants, multiplied by 100%. N = 70 (base on Owczarz 2006, modified). The tolerance to zinc is extraordinarily high in *B. laevigata* subsp. *woycickii*, the growth stimulation was over double the amount seen in other studied plants

Ryc. 19. Indeks tolerancji w kolejnych dniach traktowania roślin roztworem cynku (8 mg/l), dla siewek *Biscutella laevigata* subsp. *woycickii*, *B. laevigata* subsp. *gracilis*, *Zea mays*, *Phaseolus vulgaris* i *Cucumis sativus*. Indeks tolerancji to stosunek przyrostu korzeni [cm] roślin traktowanych cynkiem do przyrostu korzeni [cm] roślin kontrolnych pomnożony przez 100%. N = 70 (za Owczarz 2006, zmienione). Tolerancja na cynk jest wybitnie wysoka u *B. laevigata* subsp. *woycickii*, stymulacja wzrostu była tu ponad dwukrotna w porównaniu do pozostałych badanych roślin the Tatra Mountains showed slightly lower tolerance – around 100–150% of the control. This value also means that zinc stimulates plants growth. The other three plant species tolerated the presence of excess zinc in the medium much less. Their tolerance levels ranged from 20 to 80% of the control, which means that the dose of zinc was toxic to them, but not lethal. Test species in terms of zinc tolerance can be ranked in the following order, starting with the least tolerant plants: *C. sativus* < *P. vulgaris* < *Z. mays* < *B. laevigata* subsp. *gracilis* < *B. laevigata* subsp. *woycickii* (Owczarz 2006).

The data presented above (as well as other data that is not yet published) clearly indicates an unusually high level of tolerance for *B. laevigata*, and especially *B. laevigata* subsp. *woycickii* from Bolesław was strongly stimulated to grow in the presence of this element. It was characterized by twice bigger root growth compared to the control. *B. laevigata* subsp. *gracilis* from the Tatra Mountains also reached larger root length, however, the roots were maximally 50% longer comapred to the control. Crops, which were the reference points for *B. laevigata* plants, were selected due to their known tolerance to heavy metals. It is known that corn tolerates excess heavy metals in soil well, and cucumber and beans worse (Wierzbicka 1999). In this way, a comparative scale of zinc tolerance of plants could be created. This comparison showed that *B. laevigata* species has an extremely high tolerance to zinc, especially individuals of *B. laevigata* subsp. *woycickii* from the waste heap in Bolesław. The high tolerance to zinc in the whole *B. laevigata* species was also confirmed in studies by Babst-Kostecka et al. (2016).

The question of how such a large amount of zinc can be taken up into the plant tissues remained. The results clearly indicated the zinc-loving of *B. laevigata* from Bolesław and the Tatra Mountains. The zinc concentration in the tissues of the plants was very high. Zinc concentration in the roots of plants treated with zinc was the highest in *B. laevigata* subsp. *woycickii* from Bolesław and amounted to 20,287 mg/kg d.w. Less than half of this amount of zinc was accumulated in the roots of *B. laevigata* subsp. *gracilis* from the Tatra Mountains (10,005 mg/kg d.w.) and *Z. mays* (10,445 mg/kg d.w.). The lowest concentration values were found in *P. vulgaris* (4,167 mg/kg d.w.) and *C. sativus* (4,488 mg/kg d.w.). In turn, in aboveground parts of plants treated with zinc, the content of this element was highest in *C. sativus*, at a value of 3,752 mg/kg d.w. Lower levels were found in *B. laevigata* from Bolesław and the Tatra Mountains (2,093 and 2,278 mg/kg d.w. respectively) and *P. vulgaris* (2,147 mg/kg d.w.). The lowest concentration of zinc, 1,306 mg/kg d.w., was found in the aboveground parts of *Z. mays*. These results indicate that the largest transport of zinc to aboveground parts occurs in *C. sativus* plants. Transport in *B. laevigata* from Bolesław and Tatra also remained at a high level (Owczarz 2006, Wierzbicka i in. 2015, 2017).

In this way, it was shown that the growth of *B. laevigata* from heaps in Bolesław and the Tatra Mountains was stimulated by the presence of zinc in the medium, and that it accumulated in their tissues in very large amounts. However, this element had an inhibitory effect on the growth of the remaining species tested, even though these plants contained lower concentrations of zinc in the tissues. It can therefore be concluded that *B. laevigata* is not only a zinc-tolerant plant (i.e. well tolerant of excess zinc) (Przedpełska-Wąsowicz et al. 2012), but even zinc-loving (i.e. better developing with excess zinc) (Owczarz 2006, Przedpełska-Wąsowicz i in. 2012, Wierzbicka i in. 2015, 2017). This feature occurred most strongly in *B. laevigata* subsp. *woycickii*. Under natural conditions, such a feature is

Fig. 20. The fruiting plant of *Biscutella laevigata* subsp. *gracilis* during its growth in the garden of the University of Warsaw. The photo shows, the size that *B. laevigata* may reach during cultivation in garden soil, where no surplus of zinc occurs (photo M. Wierzbicka)

Ryc. 20. Owocująca roślina *Biscutella laevigata* subsp. *gracilis* podczas wzrostu w ogrodzie Uniwersytetu Warszawskiego. Zdjęcie to pokazuje jak duże rozmiary może osiągać pleszczotka górska podczas hodowli w ziemi ogrodniczej, gdzie nie występuje nadmiar cynku (fot. M. Wierzbicka)

extremely useful. The presence of zinc in the substrate limits the growth of many plant species. This is not the case with *B. laevigata*. In the conditions of the excess of this element in the substrate, it even stimulates its growth like a 'super vitamin', while for other species it is simply a poison. Therefore, our title species can win against other plants in the competition for habitat. In this way, as a pioneer species, it can colonize a zinc-enriched substrate. This feature may explain the occurrence of *B. laevigata* in Poland, in the Tatra Mountains and on zinc-lead heaps in Bolesław. Excess zinc in the substrate was found at both of these locations. Whereas in other areas, where no elevated zinc concentrations were found in the substrate, *B. laevigata* loses competition for habitat. For the same reason, i.e. limited tolerance to zinc, species mentioned earlier, such as maize, will not be able to grow on zinc-lead heaps.

The next question arises – is excess zinc in the soil is necessary for the proper growth and development of *B. laevigata*? It turns out that this species grows well and blooms well in horticultural soil with a typical zinc content. Figure 20 shows the substantial dimensions (height 80 cm, width 40 cm) that *B. laevigata* can reach in comfortable conditions when growing in horticultural soil (culture in the garden) (Pielichowska 2007). Other researchers also indicate that *B. laevigata* can occur in the Tatra Mountains in sites with 'normal' zinc content (Babst-Kostecka et al. 2016). Therefore, the zinc-loving of *B. laevigata* is an asset in competition with other plant species.

The question still remains: what lies at the heart of the zinc-loving properties of *B. laevigata*? The results of research on zinc speciation – i.e. chemical compounds in which zinc was present in the tissues of *B. laevigata* – bring us closer to answering this question. The research was carried out using the latest analytical chemistry methods, using a complex chromatographic system – multidimensional chromatography SEC/HILIC ICP-MS. Based on the obtained spectra, it was found that zinc occurs in plants in a complex combination with nicotianamine (Fig. 21) (Pielichowska 2007, Wierzbicka et al. 2015, 2017). This is one of the mechanisms of tolerance for heavy metals, discussed more broadly at the beginning of this chapter. It is significant that no other mechanism of metal tolerance was found, namely the presence of metal complexes with phytochelatin, which was expected based on the results of other researchers (Przedpełska-Wąsowicz et al. 2012). Nicotianamine plays an important role in plant tolerance to metals, in particular

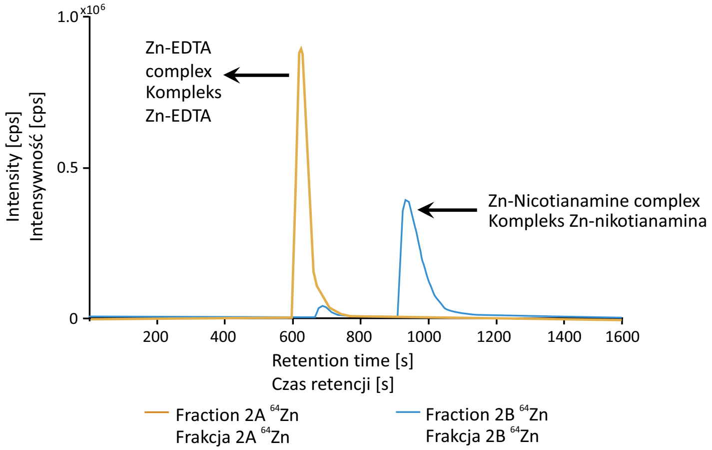

Fig. 21. The chromatogram for the leaf extract from *Biscutella laevigata* incubated for 10 days in the medium with 10 mg/l Zn. The SEC/HILIC ICP-MS method, the second phase of chromatographic separation. A single peak marked with an orange line indicates the 2A ^{64}Zn fraction, in that the complex of zinc with EDTA was detected. A single peak marked with a violet line indicates the 2B ^{64}Zn fraction, in that the complex of zinc with nicotianamine was detected (after Pielichowska 2007, modified)

Ryc. 21. Chromatogram dla ekstraktu z liści *Biscutella laevigata* inkubowanej przez 10 dni w pożywce z 10 mg/l Zn. Metoda SEC/HILIC ICP-MS, drugi etap rozdziału chromatograficznego. Pojedynczy pik oznaczony pomarańczową linią przedstawia frakcję 2A ^{64}Zn, gdzie wykryto kompleks cynku z EDTA. Pojedynczy pik oznaczony fioletową linią przedstawia frakcję 2B ^{64}Zn, gdzie wykryto kompleks cynku z nikotianaminą (za Pielichowska 2007, zmienione)

zinc. It has metal binding properties, it can participate in loading and unloading phloem (Stephan and Scholz 1993). Zinc hyperaccumulating plants, among others *A. halleri*, have been shown to have genes encoding nicotianamine synthase (Weber et al. 2004). At the cellular level, metal-nicotianamine complexes are removed from the cytoplasm to vacuoles, where they are isolated and thereby detoxified. Nicotianamine also plays an important role in the transport of such elements as: zinc, copper, iron, nickel, or manganese (Takahashi et al. 2003, Kim et al. 2005). The presence of nicotianamine in a complex combination with zinc in response to the elevated concentration of zinc in *B. laevigata*, is undoubtedly associated with the phenomenon of zinc-loving of this species and may be the key to the mystery of *B. laevigata* occurrence in the calamine heaps in Bolesław, and in the Tatra Mountains (Wierzbicka et al. 2015, 2017).

Detoxification of heavy metals in cells – the compartmentation mechanism

As shown by the research of the authors of this chapter, among all organs, the roots of *B. laevigata* subsp. *woycickii* from Bolesław and subsp. *gracilis* from the Tatra Mountains uptake and accumulate the largest amounts of heavy metals in their tissues. When lead, zinc, or cadmium salts were given to adult plants in liquid medium for 12 days, 99% of the lead uptake, 75% zinc uptake and 68% cadmium uptake remained in the roots. The remaining amount of metal taken up was transported to the aboveground parts of the tested plants. The metal content in the roots has repeatedly exceeded the concentration of soluble metal forms in the surrounding environment. For example, lead in liquid medium was at a concentration

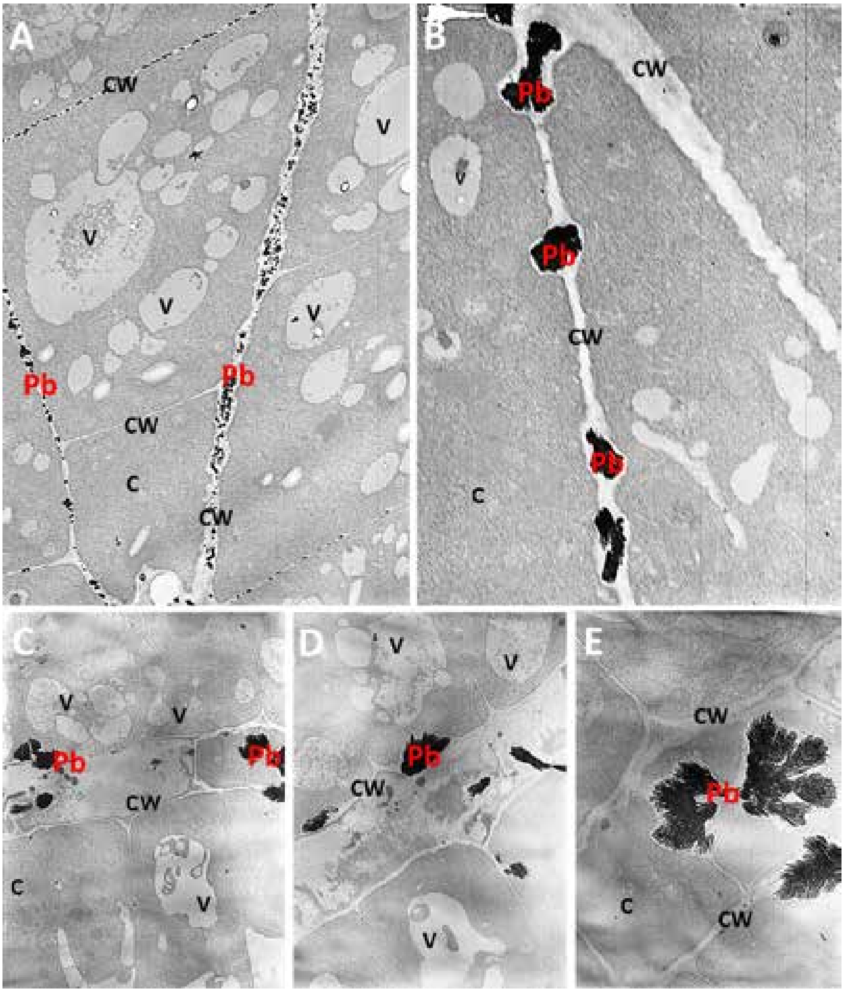

Fig. 22. Lead in the cells of the meristematic zone in the seedling roots of *Biscutella laevigata* subsp. *woycickii* after 1 day of incubation (A, B) and 5 days of incubation (C, D, E) in the solution with 8 mg/l of lead. The black deposits are visible in the cell walls of: proparenchyma at a magnification of 3,400× (A), on the border of proparenchyma and procambium zones (the cell walls are much thickened in the places of lead accumulation) at a magnification of 10,000× (B), on the border of the root cap and the meristematic zone at a magnification of 4,600× (C), on the border of proparenchyma and procambium at a magnification of 4,600× (D) and 8,000× (E). Pb – lead, V – vacuole, CW – cell wall, C – cytoplasm.. The observations were made using a transmission electron microscope. The microscopic slides were not contrasted. Lead is clearly visible because it has got sufficient density to reflect electrons reaching the sample (after Pielichowska 2007, modified)

Ryc. 22. Ołów w komórkach strefy merystematycznej korzeni siewek *Biscutella laevigata* subsp. *woycickii* po 1 dniu inkubacji (A, B) oraz 5 dniach inkubacji (C, D, E) w roztworze 8 mg/l ołowiu. Czarne złogi ołowiu widoczne w ścianach komórkowych: pramiękiszu w powiększeniu 3400× (A), na granicy strefy pramiękiszu i pramiazgi (ściany komórkowe są mocno pogrubione w miejscach gromadzenia ołowiu) w powiększeniu 10 000× (B), na pograniczu czapeczki i strefy merystematycznej korzenia w powiększeniu 4600× (C), na pograniczu pramiękiszu i pramiazgi w powiększeniu 4600× (D) i 8000× (E). Pb – ołów, V – wakuola, CW – ściana komórkowa, C – cytoplazma.. Obserwacje były prowadzone w transmisyjnym mikroskopie elektronowym. Preparaty nie były kontrastowane. Ołów jest dobrze widoczny ponieważ posiada wystarczającą gęstość, aby odbić elektrony docierające do próbki (za Pielichowska 2007, zmienione) of 2.5 mg/l, but after 12 days of the plants' treatment with this metal in *B. laevigata* tissues (depending on the development phase of the plant and its organ) was found at concentrations from 20 to 10,000 mg/kg (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

The question then arises: in what places are metals accumulated in root cells, since *B. laevigata* retained the opportunity for growth and development with such a high toxic metal contents? The answer to this question was given by the visualization of lead

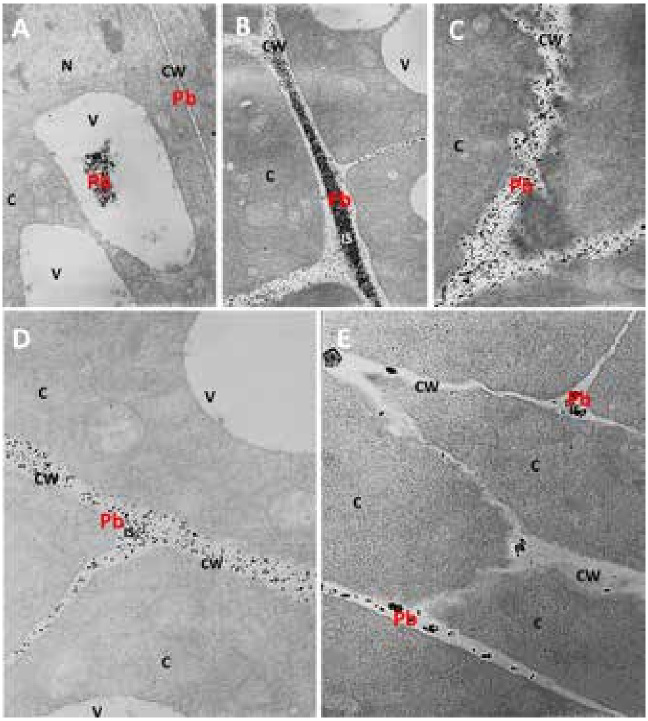

Fig. 23. Lead in the meristematic zone cells of adult plant roots of *Biscutella laevigata* subsp. *woycickii* after 1 day of incubation (A, B, C) and 5 days of incubation (D, E) in the solution with 8 mg/l of lead. The black lead deposits are visible in: cell walls and vacuole of proparenchyma cells at a magnification of 3,400× (A), cell walls and intercellular spaces on the border of proparenchyma and procambium (many deposits) at a magnification of 8,000× (B), cell walls of parenchyma cells at a magnification of 14,000× (C), cell walls of procambium cells (tiny deposits) at a magnification of 14,000× (D) and 10,000× (E). Pb – lead, V – vacuole, CW – cell wall, C – cytoplasm, N – cell nucleus, IS – intercellular space. The observations were done in the transmission electron microscope. The microscopic slides were not contrasted. Lead is clearly visible because it has got sufficient density to reflect electrons reaching the sample (after Pielichowska 2007, modified)

Ryc. 23. Ołów w komórkach strefy merystematycznej korzeni dorosłych roślin *Biscutella laevigata* subsp. *woycickii* po 1 dniu inkubacji (A, B, C) oraz 5 dniach inkubacji (D, E) w roztworze 8 mg/l ołowiu. Czarne złogi ołowiu widoczne w: ścianach komórkowych i wakuoli komórek pramiękiszu w powiększeniu 3400× (A), ścianach komórkowych i przestworach międzykomórkowych na granicy pramiękiszu i pramiazgi (liczne złogi) w powiększeniu 8000× (B), ścianach komórkowych komórek miękiszu w powiększeniu 14 000× (C), ścianach komórkowych komórek pramiazgi (drobne złogi) w powiększeniu 14 000× (D) i 10 000× (E). Pb – ołów, V – wakuola, CW – ściana komórkowa, C – cytoplazma, N – jądro komórkowe, IS – przestwór międzykomórkowy. Obserwacje były prowadzone w transmisyjnym mikroskopie elektronowym. Preparaty nie były kontrastowane. Ołów jest dobrze widoczny ponieważ posiada wystarczającą gęstość, aby odbić elektrony docierające do próbki (za Pielichowska 2007, zmienione) deposits in the root growth apex, using electron microscopy techniques. Seedlings and adult plants were treated with an 8 mg/l lead solution for one day and for five days (Pielichowska 2007, Wierzbicka et al. 2015, 2017). Observations of the root apex of *B. laevigata* seedlings from Bolesław and the Tatra Mountains after one day of incubation with lead showed the presence of this element mainly in cell walls, but also in intercellular space and vacuoles. The accumulation of lead in cell walls was often accompanied by an increase in their thickness. Lead was not found in the cell cytoplasm (Fig. 22A, B). In turn, observations of *B. laevigata* subsp. *woycickii* and subsp. *gracilis* seedlings after a five-day incubation in lead, showed that large lead deposits were mainly found in cell walls and that there were more present than after one day of incubation (Fig. 22C, D, E) (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

Observations of the root apex of mature plants from both subspecies after a one-day incubation with lead showed the presence of lead in cell walls and intercellular spaces. These deposits were numerous but small. Accumulation of lead in vacuoles was also found (Fig. 23A, B, C). In contrast, observations of root apex after a five-day incubation with lead showed a small number of lead deposits. This element sporadically appeared in the cell walls and intercellular spaces (Fig. 23D, E) (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

Thus, it was shown that metal detoxification in *B. laevigata* cells of both subsp. *woycickii* and subsp. *gracilis* involves the accumulation of metals in vacuoles, cell walls and intercellular spaces. No differences between the two subspecies in lead accumulation were observed. However, fewer lead deposits were found in the root apex of older plants, compared to seedlings (Pielichowska 2007, Wierzbicka et al. 2015, 2017). Therefore, we are dealing with the compartmentation mechanism discussed earlier. As a result, even toxic elements, if retained in the cell areas listed above, will not be included in cell metabolism. They remain non-toxic to the whole organism because they do not have contact with the cytoplasm, where important metabolic processes take place. This is an important defense mechanism of *B. laevigata* as a whole species against metal toxicity that allows plants to grow in areas contaminated with heavy metals.

The role of epidermal trichomes in heavy metal tolerance – the mechanism of elimination

One of the mechanisms for avoiding heavy metals is elimination. As mentioned at the beginning of this chapter, heavy metal accumulation in epidermal trichomes is an example of elimination. This mechanism occurs in *B. laevigata* subsp. *woycickii* from the post-mining area in Bolesław.

Elemental analysis of epidermal trichomes on the leaves of *B. laevigata* subsp. *woycickii*, which grew on calamine substrate in laboratory conditions, was conducted. The method of laser evaporation combined with mass spectrometry (LA-ICP-MS) was used. After directing the laser beam parallel to the leaf edge, where epidermal trichomes occurred on the leaf surface, large amounts of calcium and manganese, average amounts of magnesium, thallium and lead, and small amounts of zinc and cadmium were found in them (Fig. 24). Therefore, the presence of all metals present in high concentrations in the calamine substrate of Bolesław was demonstrated. This result proves that epidermal trichomes actually constitute a ‘catchment’ of heavy metals from plant leaves (Pielichowska 2007, Wierzbicka et al. 2015, 2017).

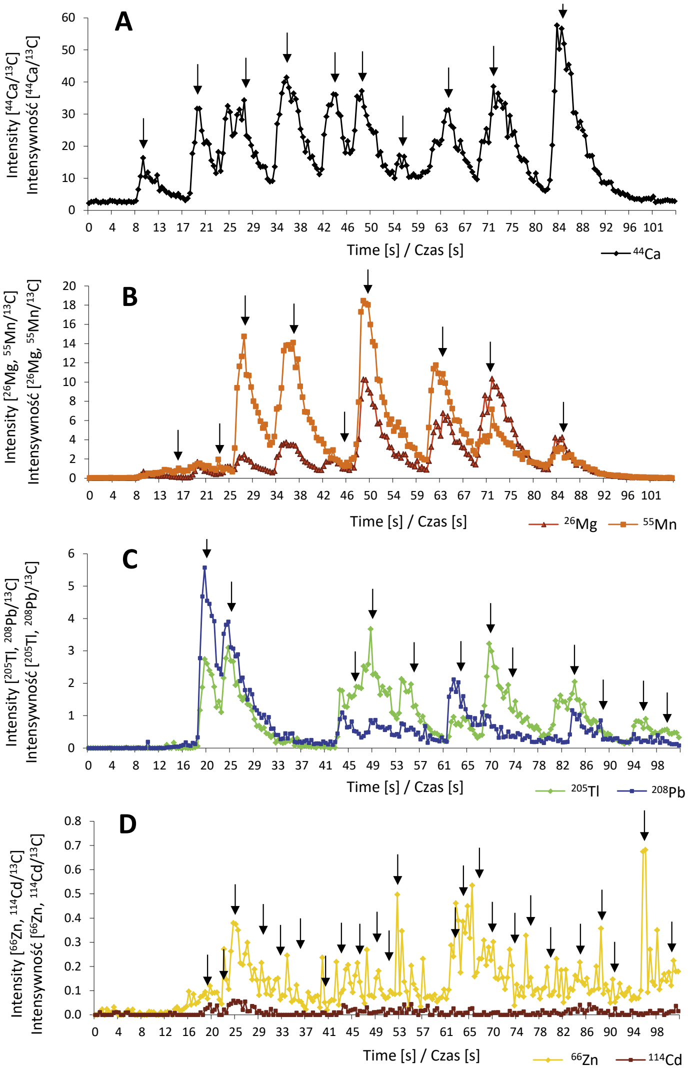

Fig. 24. The signal intensity of the isotopes of calcium (A), magnesium and manganese (B), thallium and lead (C), zinc and cadmium (D) in the leaf epidermal trichomes of *Biscutella laevigata* subsp. *woycickii* from the first offspring generation (F1) cultivated in calamine soil with 1.5 mmol EDTA added per kilogram of substrate. In the figure, every peak marked with an arrow corresponds with a single trichome. The LA-ICP-MS method was used, and the signal intensity of the metal isotope was expressed in relation to the carbon isotope (^{13}C) (base on Pielichowska 2007, Wierzbicka et al. 2015, 2016, 2017, modified). The results indicate the presence of calcium, magnesium, manganese, thallium, lead, zinc, and cadmium in epidermal trichomes

Ryc. 24. Intensywność sygnału izotopów wapnia (A), magnezu i manganu (B), talu i ołowiu (C), cynku i kadmu (D) we włoskach epidermalnych liścia *Biscutella laevigata* subsp. *woycickii* z pierwszego pokolenia potomnego (F1) hodowanego w glebie galmanowej z 1,5 mmol EDTA dodanym na kilogram podłoża. Na wykresie każdy pik oznaczony strzałką odpowiada pojedynczemu włoskowi. Metoda LA-ICP-MS, intensywności sygnału izotopu metalu wyrażona w odniesieniu do izotopu węgla (^{13}C) (za Pielichowska 2007, Wierzbicka i in. 2015, 2016, 2017, zmienione). Wyniki przedstawione na wykresach wskazują na obecność we włoskach epidermalnych wapnia, magnezu, manganu, talu, ołowiu, cynku i kadmu

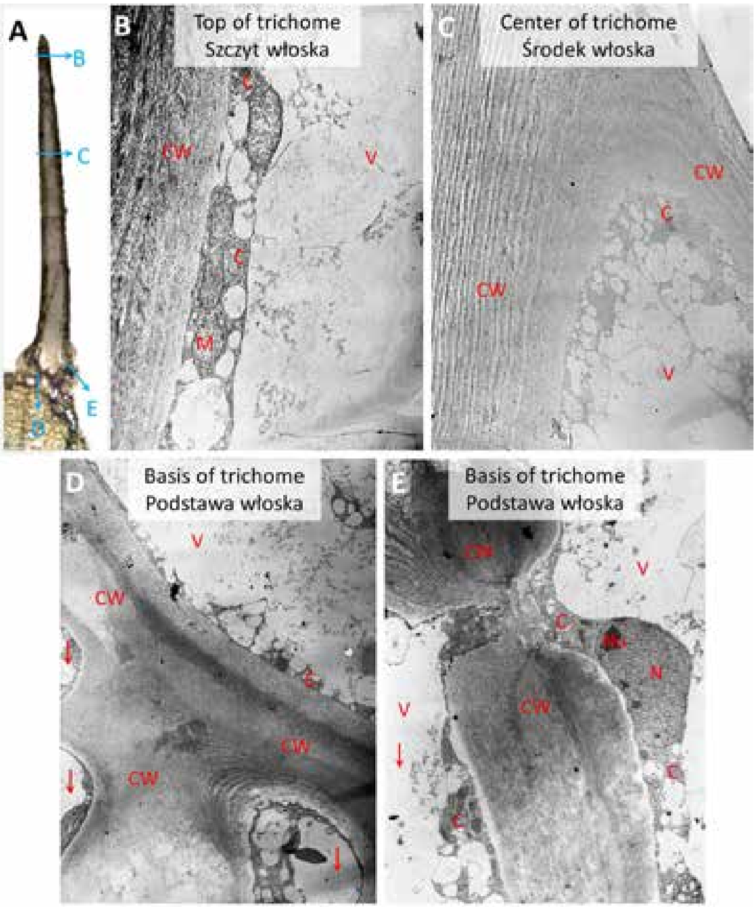

Fig. 25. The structure of a trichome of *Biscutella laevigata* subsp. *woycickii*. The places seen in the transmission electron microscope are marked with blue arrows. The trichome is single-celled, with a cell node at the base, edged with a thick cell wall. The trichome lumen is almost entirely filled with a vacuole, cytoplasm only at the cell wall; the trichome cell has got a symplastic contact with neighbour cells through plasmodesmata located in the straight lacunae (A). The view of trichome parts under the transmission electron microscope (B–E): the fragment of the trichome cell wall and cytoplasm at a magnification of 10,000× (B), the fragment with bubbles in a vacuole and cell wall at a magnification of 4,000× (C), the lower part of the trichome cell and neighboring cells at a magnification of 3,000× (D); the straight lacuna between the trichome cell and a neighbouring cell at a magnification of 4,000× (E). V – vacuole, CW – cell wall, C – cytoplasm, M – mitochondrium, N – cell nucleus, Nu – nucleolus, the neighbouring parenchyma cells of the trichome are marked with a red arrow (after Owczarz 2006, modified)

Ryc. 25. Budowa włoska *Biscutella laevigata* subsp. *woycickii*. Niebieskimi strzałkami zaznaczono miejsca oglądane w transmisyjnym mikroskopie elektronowym. Włosek jest jednokomórkowy, ze zgrubieniem komórki u podstawy, otoczony grubą ścianą komórkową; światło włoska prawie w całości wypełnione wakuolą, cytoplazma tylko przyścienna; komórka włoska ma kontakt symplastyczny z komórkami sąsiednimi przez plazmodesmy znajdujące się w jamkach prostych (A). Widok części włoska spod transmisyjnego mikroskopu elektronowego (B–E): fragment ściany komórkowej i cytoplazmy włoska w powieszeniu 10 000× (B), fragment z pęcherzykami w wakuoli i ściana komórkowa w powiększeniu 4000× (C), dolna część komórki włoska i komórek sąsiednich w powiększeniu 3000× (D); jamka prosta pomiędzy komórką włoska a komórką sąsiednią, w powiększeniu 4000× (E). V – wakuola, CW – ściana komórkowa, C – cytoplazma, M – mitochondrium, N – jądro komórkowe, Nu – jąderko, czerwoną strzałką oznaczono sąsiadujące z włoskiem komórki miękiszu (za Owczarz 2006, zmienione)

Trichomes in *B. laevigata* are alive. Accurate observations carried out using light microscopy and transmission electron microscopy have shown that a single trichome on the leaves of *B. laevigata* is made of one cell, surrounded by a thick cell wall. This cell is almost completely filled with vacuole (Fig. 25). The excess of heavy metals taken up by the plant can be accumulated in the central vacuole of the trichome cell or in its thick walls. Thanks to these processes, other leaf cells are protected (Pielichowska and Wierzbicka 2004, Owczarz 2006, Wierzbicka et al. 2015, 2016, 2017). For example, the presence of cadmium deposits in leaf trichomes in *B. laevigata* subsp. *woycickii* from Bolesław were observed very quickly, after just 24 hours of incubation of plants with this metal in culture in mineral medium. Particularly large amounts of cadmium were deposited primarily in the bases of epidermal trichomes of leaves and stems (Brzost 2005, Wierzbicka et al. 2015, 2016, 2017). Dense leaf trichome coverage of *B. laevigata* subsp. *woycickii*, discussed earlier (Fig. 7A, C), allows the detoxification of significant amounts of heavy metals. Thus, the detoxification process in this subspecies is more efficient than in *B. laevigata* subsp. *gracilis* from the Tatra Mountains, where there are a small number of epidermal trichomes on the leaf blades (Fig. 7B, D), (Wierzbicka 2002, Wierzbicka et al. 2015, 2016, 2017).

Metal removal by the oldest and drying leaves – the mechanisms of redistribution and elimination

Further mechanisms for avoiding heavy metals occurring in *B. laevigata* subsp. *woycickii* are: the mechanism of redistribution, consisting of the transport of metals to aging leaves, and the mechanism of elimination – the release of aging leaves with a high content of heavy metals in tissues. It has been shown that in natural conditions the uptake of heavy metals from a substrate contaminated with metals by *B. laevigata* is a process that increases over time. This is evidenced by a double increase in the amount of zinc in plants in the autumn, compared to the beginning of the growing season. This is also indicated by the detection of the maximum concentration of metals in the oldest tissues (Wierzbicka et al. 2015, 2017). Similar relationships for *B. laevigata* were also described by Godzik (1984, 1991), and for other plant species by Ernst (1995).

The above-mentioned studies were conducted for individuals found in the field, which makes it difficult to determine their age. *B. laevigata* is a perennial plant, therefore both annual and perennial plants occur in the field. It is therefore not known how long plants accumulated heavy metals in their tissues. Therefore, tests were carried out for 5-month-old plants grown in calamine substrate, in a greenhouse under uniform conditions. It was shown that in the oldest and drying leaves, the amount of heavy metals was very high – 277 mg/kg d.w. lead, 2,787 mg/kg d.w. zinc, while in green leaves these values were 150 mg/kg d.w. and 1,520 mg/kg d.w. respectively. Thus, the lead content in drying leaves was significantly higher than in green leaves (Pielichowska 2007, Wąsowicz et al. 2014, Wierzbicka et al. 2015, 2017).

It has also been shown that cadmium uptake by leaf and root cells affects mineral metabolism, especially calcium and to a lesser extent magnesium. It has been observed that the average concentration of calcium and magnesium in leaves increases with their age. However, it has also been shown that a higher concentration of cadmium in leaf tissues (and other organs) reduces the percentage of calcium and magnesium in them. Thus, we are dealing with competition in transport between cadmium ions and calcium and magnesium ions. These regularities were found for plants from both subspecies. Therefore, the mentioned mechanisms for avoiding heavy metals are a species feature (Brzost 2005). Thanks to the above research, it was finally proved that *B. laevigata* plants remove excess heavy metals by accumulating them in the oldest leaves – this is the redistribution mechanism. The oldest leaves with accumulated metals in their tissues then dry up and are removed from the plant, which is an example of the mechanism of elimination.

Biscutella laevigata as a thallium hyperaccumulator

Hyperaccumulators are a particular group among metallophytes. The plants are distinguished by the ability to take up from the soil and accumulate extremely large amounts of heavy metals in their aboveground parts without a visible negative influence on metabolic processes (Baker and Brooks 1989, Branquinho et al. 2007, Roosens et al. 2008, Przedpełska-Wąsowicz 2015). Different levels of the elements content in plants are reported, above which the plants are considered as being able to hyperaccumulate them. As an example, for plants growing in their natural habitats, van der Ent et al. (2013) proposed the amount of a given element, expressed in milligrams per kilogram of shoot dry mass, as: 100 for cadmium, selenium and thallium, 300 for cobalt, copper and chromium, 1,000 for nickel and lead, 3,000 for zinc and 10,000 for manganese. In the case of thallium, the hyperaccumulation level of 500 mg/kg is also given (Anderson et al. 1999a, Leblanc et al. 1999). Therefore, we define plants as hyperaccumulators when they grow in soils enriched in metals and are able to accumulate amounts of metals in their shoots over 100 times more than other plants (i.e. for cadmium over 100 mg/kg d.w., for lead over 1,000 mg/kg d.w., for zinc over 10,000 mg/kg d.w., and for thallium 100 or 500 mg/kg d.w. (Anderson et al. 1999a, Leblanc et al. 1999, van der Ent et al. 2013). Hyperaccumulators are constantly sought because of the practical possibility of using them in the remediation of heavy metal-contaminated soils (phytoremediation) (Salt et al. 1998, Lasat 2002, Muszyńska et al. – Chapter 7 of this volume).

It is worth noticing that most species hyperaccumulating heavy metals have been found in the *Brassicaceae* family so far (Baker and Brooks 1989, Reeves 2003, Krämer 2010), the family that *B. laevigata* belongs to. Thus, the question arises as to whether or not *B. laevigata* is able to hyperaccumulate heavy metals, since it has got such efficient heavy metal detoxification mechanisms.

The studies carried out on the *B. laevigata* plants in western Europe showed that plants of the populations from metalliferous areas in the Austrian Alps are able to hyperaccumulate lead (over 1,000 mg/kg d.w. of shoots) (Wenzel and Jockwer 1999, Reeves 2003, Krämer 2010), and also in southern France (Les Malines) are able to hyperaccumulate thallium (up to 15,200 mg/kg d.w.), (Anderson et al. 1999a, Leblanc et al. 1999). *B. laevigata* from the Alps also contained an elevated amount of cadmium in its shoots (up to 78 mg/kg d.w.), which is below the level of hyperaccumulation (Wenzel and Jockwer 1999). Therefore, *B. laevigata* was identified as a thallium and lead hyperaccumulator. On the other hand, past studies on *B. laevigata* subsp. *woycickii* from zinc-lead waste heaps in Bolesław indicated that the plants have a much higher tolerance to the presence of lead, cadmium, and zinc in the soil, compared with the plants of the *gracilis* subspecies from the Western Tatra Mountains region (Wierzbicka and Pielichowska 2004, Wąsowicz et al. 2014). However, the metal levels that would clearly indicate hyperaccumulation were not exceeded in tissues of these plants.

Biscutella laevigata along with two other wild species, *Iberis intermedia* L. and *Melandrium album* (Mill.) Garcke, as well as the cultivated one, *Brassica oleracea* (*acephala* group), were commonly regarded as the only known thallium hyperaccumulators until now (Anderson et al. 1999a, LaCoste et al. 1999, Leblanc et al. 1999, Al-Najar et al. 2003, Scheckel et al. 2004, 2007, Sheoran et al. 2009, Escarré et al. 2011, Pošćić et al. 2013, 2015). It is worth stressing that in *B. laevigata* studied in southern France (in the area of post-flotation ponds around the zinc and lead work in Les Malines, Les Avinières, near Montpellier), the thallium concentration in shoots was noted to be much higher than the threshold for hyperaccumulation i.e. 15,200 mg/kg d.w. (Anderson et al. 1999a, Leblanc et al. 1999, van der Ent et al. 2013). In developed leaves of *I. intermedia*, the thallium content was determined to be between about 2,810 mg/kg d.w. in the shoots according to LaCoste et al. (1999) and Leblanc et al. (1999) to about 13,000 mg/kg d.w. according to Scheckel et al. (2004). Whereas in the case of other species, the thallium content ranged from about 1,000 and 3,000 mg/kg d.w. of shoots: in *S. latifolia* it amounted to about 1,500 mg/kg d.w. (Escarré et al. 2011), in *B. oleracea acephala* to about 12,000 mg/kg d.w. and in *I. intermedia* to about 29,000 mg/kg d.w. (Al-Najar et al. 2003). It should be noted that the thallium concentration in plants growing in the noncontaminated environment do not usually exceed the values from 0.02 mg/kg d.w. (van der Ent et al. 2013) to 0.05 mg/kg d.w. (Wierzbicka et al. 2004).

Based on this, the assumption was made that *B. laevigata* subsp. *woycickii* occurring in the region of excavation and processing of zinc-lead ores in the surroundings of Bolesław can also hyperaccumulate thallium, given that an elevated amount of thallium in the soil was confirmed in this area (Dmowski et al. 1998, Dmowski 2000, Dmowski and Badurek 2001, 2002, Wierzbicka et al. 2004). The excavation and processing of heavy metal ores containing traces of thallium as well as the functioning of heat and power plants and refineries contribute the most to the contamination of the environment with thallium (Sager 1994). In the soil around the zinc smelter at the Bolesław Mining and Metallurgical Plant in Bukowno (Zakłady Górniczo-Hutnicze 'Bolesław' – ZGH Bolesław), the thallium content was as high as 149 mg/kg d.w., and 30–40 mg/kg d.w. in post-flotation wastes (Dmowski and Badurek 2002). For comparison, the mean thallium content in noncontaminated soils falls within the range of 0.02–2.8 mg/kg d.w. (Kabata-Pendias 2011).

Thallium toxically impacts plants, animals, and humans, mainly due to its chemical similarity to potassium, with which it competes and disturbs cell metabolism (Scheckel et al. 2007). Many biomonitoring studies in the industrialized regions of Upper Silesia and Lesser Poland have shown that thallium is a real threat to different groups of organisms (Dmowski et al. 1998, Dmowski 2000, Dmowski and Badurek 2001, 2002, Wierzbicka et al. 2004). For example, in feathers of magpies (*Pica pica* Linnaeus) from Bukowno, in the vicinity of the zinc smelter of ZGH Bolesław, the thallium concentration was several hundred times higher than in the feathers of magpies from other industrialized regions in Poland (Dmowski 2000). On the other hand, thallium is an important element in many branches of industry (Scheckel et al. 2007, Sheoran et al. 2009). Therefore, studies on the ability to hyperaccumulate thallium in *B. laevigata* bring not only knowledge on this mechanism but also aim at the potential for the use of the plants in soil phytoremediation (Anderson et al. 1999a, LaCoste et al. 1999, Bini 2010, Ali et al. 2013; Muszyńska et al. – Chapter 7 of this volume) and phytomining, i.e. using metals from plants able to accumulate significant amounts of them (Anderson et al. 1999a, LaCoste et al. 1999, Leblanc et al. 1999, Sheoran et al. 2013).

In studies on *B. laevigata*, for the analysis of thallium content, plants originating from the thallium-contaminated area were taken (Anderson et al. 1999a, LaCoste et al. 1999, Leblanc et al. 1999, Pošćić et al. 2015), whereas plants from other populations of this species were cultivated in standardized glasshouse conditions (Pošćić et al. 2013). Therefore, the conclusions on thallium hyperaccumulation drawn from field studies should have been accepted with some caution, because many factors could have influenced the thallium level in plants. Firstly, thallium may not only be taken from the soil by roots, but also it may be present in airborne dust fall, which may be most visible in densely haired plants. Secondly, the soil that plants grow in may have a non-homogenous composition (Vaněk et al. 2010). Thirdly, the period of thallium accumulation in aboveground parts of plants is unknown since *B. laevigata* is a perennial and in natural populations plants are of different ages and in different growth phases. In the studies carried out in the area of the waste heap in Bolesław, the thallium content was also assayed in the calamine soil sampled in habitats differing in vegetation cover. It was shown that this substrate had a different thallium content depending on the place from which it was taken; the soil from the unvegetated places contained 43 mg/kg d.w. and from the vegetated ones it contained 15.2 mg/kg d.w. (Pielichowska 2007, Wierzbicka et al. 2016). Diversified vegetation cover is another factor which indirectly influences the possibility of thallium accumulation by *B. laevigata* because in more densely vegetated places, where there is less thallium in the calamine soil, plants take up a smaller amount as well.

The preliminary studies by the authors of this chapter, on *B. laevigata* subsp. *woycickii* collected in the field from the calamine waste heap in Bolesław, showed that the thallium content in shoots indicated not only a lack of hyperaccumulation of the element, but that even its overall accumulation was very poor (at relatively high thallium content in soil, i.e. 43 mg/kg d.w. on average and a maximum of 78 mg/kg d.w., and in *B. laevigata* shoots thallium was on average 0.1 mg/kg d.w. and a maximum of 0.5 mg/kg d.m), (Wierzbicka et al. 2004). Therefore, taking into account earlier reports from France on thallium hyperaccumulation by *B. laevigata* (Anderson et al. 1999a, Leblanc et al. 1999), it was decided to check this in the standardized cultivation conditions and on two subspecies: *woycickii* from Bolesław and *gracilis* from the Western Tatra Mountains (Wierzbicka et al. 2016). Plants of different ages: the 2-week-old, 1-, 2- and 10-month-old ones were cultivated in the soil taken from the calamine waste heap in Bolesław and in horticultural soil (control). The cultivation of plants lasted 5 months. It should be stressed that thallium in a form available for plants occurred in very small amount (1.1 mg/kg). Nevertheless, the plants of *B. laevigata* subsp. *woycickii* from Bolesław and *B. laevigata* subsp. *gracilis* from Tatra took up a considerable amount of thallium during the whole cultivation period, 98.5 mg/kg d.w. on average. Most of the thallium was located in the leaves, 164.9 mg/kg d.w. on average (in particular individuals, the thallium amount ranged from 59.8 to 588.2 mg/kg d.w.). The amount of thallium in roots was much lower, 32 mg/kg d.w. on average. Amongst the

Bolesław and Tatra plants, most of the thallium was taken up by seedlings (219 mg/kg d.w.), and the least of it by the oldest plants (43.4 mg/kg d.w.), thus the thallium concentration decreased with plant age. After the compound increasing thallium availability (EDTA) had been added to the calamine soil, plants took up even more thallium, 108.9 mg/kg d.w. on average, most of which was located in the leaves, e.g. 138.4 mg/kg d.w. (up to a maximum of 260 mg/kg d.w.), (Pielichowska 2007, Wierzbicka et al. 2016).

The ability to hyperaccumulate thallium in plants can be determined by a characteristic distribution of this metal seen across many organisms. In the *B. laevigata* roots, only 16% of the thallium taken up was retained in its roots, 84% was transported to the leaves. Thallium was distributed in plants according to the following pattern: the most in green leaves, less in roots, the least in the oldest withering leaves. The translocation factor (being a quotient of metal content in a shoot to its content in a root), (Anderson et al. 1999a, b) was high and equal to 6.1, whereas the bioconcentration factor (referring to metal content in a shoot compared to its content in the soil), (Anderson et al. 1999a, b) amounted to 10.9. After adding EDTA to the calamine soil, the translocation factor was equal to 2.2, and the bioconcentration factor 5.8 (Pielichowska 2007, Wierzbicka et al. 2016). It should be noted that values of both factors above 1 are characteristic of metal hyperaccumulators and indicate significantly larger transport of metals to aboveground parts compared to their content in the roots of plants (van der Ent et al. 2013). The laboratory studies showed that thallium content in *B. laevigata* did not exceed the threshold value for the hyperaccumulation of this element, 500 mg/kg d.w., reported by Anderson et al. (1999a) and Leblanc et al. (1999). However, it was higher than the threshold of 100 mg/kg d.w. given by van der Ent et al. (2013), (Pielichowska 2007, Wierzbicka et al. 2016). Therefore, it seems possible that *B. laevigata* is a facultative thallium hyperaccumulator, or at least it has potential to hyperaccumulate this element. This is a species-specific feature that is not dependent on whether plants come from the zinc-lead waste heap in Bolesław or the Tatra Mountains.

This draws attention to the fact that other authors observed the ability to hyperaccumulate thallium in the *B. laevigata* populations occurring in metalliferous areas, rather than in populations from uncontaminated areas, thus they attributed it to particular populations and not to the whole species (Pošćić et al. 2013, 2015). This disagreement in different studies on whether metal hyperaccumulation is a feature of a species or is specific to a population was highlighted by Pollard et al. (2014). The authors emphasized the need for further studies and further explanation of this issue. According to the authors of this chapter, it may be a species feature which does not depend on the place of the plants' origin.

It is worth pointing out that in the work by Pošćić et al. (2015), the only *B. laevigata* population which demonstrated a very high thallium content that is specific to hyperaccumulators was the one from the areas rich in zinc and lead ores (Cave de Predil, Italian Alps). The population was also genetically distinct in comparison with populations from other areas. In the work by Wąsowicz et al. (2014), the distinctiveness of the *B. laevigata* population indigenous to the old zinc-lead waste heap in Bolesław was also confirmed with use of genetic studies. Here, the population was given the rank of subspecies, which has been already mentioned in Chapter 5 of this volume. However, it was shown that both *B. laevigata* subsp. *woycickii* from Bolesław and *B. laevigata* subsp. *gracilis* from the Tatra Mountains, in spite of being

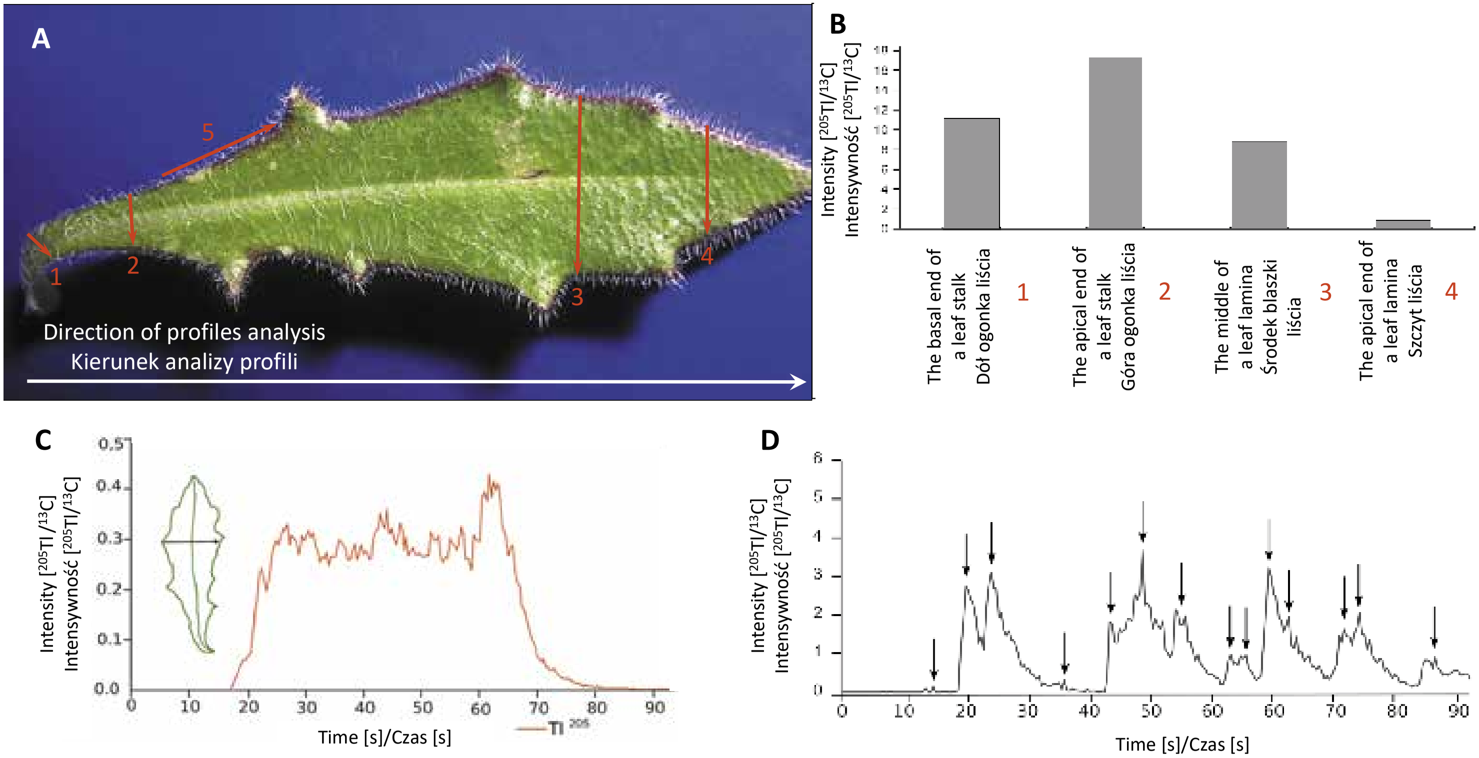

Fig. 26. Thallium in the leaves of *Biscutella laevigata* subsp. *woycickii* cultivated in the calamine soil with 1.5 mmol EDTA added per kilogram of substrate. The measurements were made using the LA-ICP-MS method. The red arrows on the leaf indicate lines along which the laser beam was led: across the leaf (lines 1–4), and across the trichomes on the leaf edge (line 5) (A). The thallium distribution in the leaf along lines 1–4 (B). The signal intensity of thallium in the leaf blade (in the leaf scheme, the direction of the laser beam passage is marked with the black arrow) (C) and in the leaf trichomes (along line 5), every peak marked with an arrow corresponds with a single trichome on the leaf surface (D). The signal intensity of the thallium isotope (^{205}Tl) expressed in relation to the carbon isotope (^{13}C) (after Pielichowska 2007, Wierzbicka et al. 2016, modified)

Ryc. 26. Tal w liściach *Biscutella laevigata* subsp. *woycickii* hodowanej na podłożu galmanowym z 1,5 mmol EDTA dodanym na kilogram podłoża. Pomiary wykonane metodą LA-ICP-MS. Na liściu czerwone strzałki pokazują linie wzdłuż których prowadzona była wiązka lasera: przez liść (linie 1–4), przez włoski na brzegu liścia (linia 5) (A). Rozmieszczenie talu w liściu wzdłuż linii 1–4 (B). Intensywność sygnału talu w blaszce liściowej (na schemacie liścia czarną strzałką oznaczono kierunek przejścia wiązki lasera) (C) oraz we włoskach liścia (wzdłuż linii 5), każdy pik oznaczony strzałką odpowiada pojedynczemu włoskowi na powierzchni liścia (D). Intensywności sygnału izotopu talu (^{205}Tl) wyrażona w odniesieniu do izotopu węgla (^{13}C) (za Pielichowska 2007, Wierzbicka i in. 2016, zmienione)

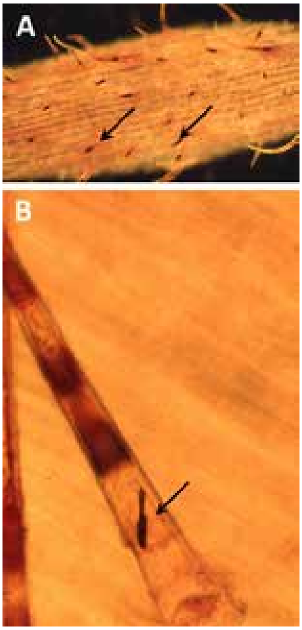

Fig. 27. The epidermal trichomes on the leaf surface of *Biscutella laevigata* subsp. *woycickii*. The trichomes include heavy metal deposits (black arrows). The visualisation of metals with the use of a histochemical method (ditizone). The light microscope: the cross section of the leaf, magnification 10× (A); the epidermal trichome, magnification 50× (B). The plants were cultivated in the calamine soil for 5 months, with 1.5 mmol EDTA added per kilogram of substrate (after Pielichowska 2007, Wierzbicka et al. 2015, 2016, 2017, modified)

Ryc. 27. Włoski epidermalne na powierzchni liścia *Biscutella laevigata* subsp. *woycickii*. Włoski zawierają złogi metali ciężkich (czarne strzałki). Wizualizacja metali przy użyciu metody histochemicznej (ditizon). Mikroskop świetlny: przekrój poprzeczny liścia, powiększenie 10× (A); włosek epidermalny, powiększenie 50× (B). Rośliny były hodowane w podłożu galmanowym przez 5 miesięcy, z 1,5 mmol EDTA dodanym na kilogram podłoża (za Pielichowska 2007, Wierzbicka i in. 2015, 2016, 2017, zmienione)

spatially and genetically isolated (Wąsowicz et al. 2014, Wierzbicka et al. 2020), both have the ability to hyperaccumulate thallium (Pielichowska 2007, Wierzbicka et al. 2016). It is worth pointing out that *B. laevigata* in the Tatra Mountains also occurs in metal-enriched substratum and it was classified in the Tatra locations as a metallicolous species (Godzik 1991, 1993), which indicates its high tolerance to metals regardless of its place of origin. Therefore, one may conclude that the ability to hyperaccumulate thallium is a species-wide feature.

Using the method of laser ablation inductively coupled plasma mass spectrometry, LA-ICP-MS, to analyse elemental composition, the way in which thallium was distributed in leaves of *B. laevigata* cultivated in the calamine soil with the addition of EDTA was shown (Pielichowska 2007, Wierzbicka et al. 2016). After the passing of the laser beam at different heights across a leaf and along its edge, the profiles for thallium distribution were obtained. It turned out that the biggest amount of thallium occurred in the upper part of a petiole, and the smallest amount in the top part of a lamina (Fig. 26). The presence of thallium, along with other heavy metals such as lead, zinc, and cadmium, was also detected in the epidermal trichomes of leaves. The deposits with thallium, redly stained with dithizone, were located in a big vacuole at the base of a single-celled trichome (Fig. 27). The presence of thallium in trichomes was detected for the first time (Pielichowska 2007, Wierzbicka et al. 2016). As already mentioned, trichomes on the leaves of *B. laevigata* may play a significant role in protecting the metabolism of other cells against the toxic influence of heavy metals, including thallium. The ability to accumulate a substantial amount of metal in trichomes is considered to be a feature of different hyperaccumulating species, e.g. zinc and cadmium in *A. halleri* (Küpper et al. 2000) or nickel in the *Alyssum* genus (Broadhurst et al. 2004a, b).

Finally, it was shown that the *B. laevigata* individuals, regardless of their place of origin – the zinc-lead waste heap in Bolesław or the Tatra region, are able to hyperaccumulate thallium and transport it from roots mainly to green leaves. This is a feature common to the whole species. Thallium is accumulated most intensively by young plants, and its content in leaves decreases in older plants. For the first time, the presence of thallium was confirmed, using the LA-ICP-MS method in epidermal trichomes of *B. laevigata* leaves. Storing thallium in epidermal trichomes should be considered as a mechanism to protect sensitive cells from the toxic effects of thallium. In view of the fact that there are more epidermal trichomes on leaves of the waste-heap plants of *B. laevigata* subsp. *woycickii* in comparison with the Tatra plants of *B. laevigata* subsp. *gracilis*, the plants from Bolesław are thought to be better adapted to living in the metalliferous substratum. One should expect that if thallium availability in the soil is elevated, plants will take up this element and transport it to above-ground parts more effectively. This means that *B. laevigata* might be suitable for use in phytomining.

Summary – the new knowledge we have presented on *B. laevigata*

To sum up this chapter, we want to indicate all of the novelties our studies have brought to the existing knowledge on *B. laevigata*.

A. The most important result was showing that on the calamine waste heap in Bolesław near Olkusz, the unique subspecies, *B. laevigata* subsp. *woycickii* (in Polish *pleszczotka Wóycickiego*), occurs. The subspecies originated 120 thousand years ago, thus it should be regarded as a postglacial relic. This means that the taxon is a remnant of the glacial period. The subspecies is unique to the Olkusz region and therefore it should be accepted that it has an endemic character. 'Endemic' and 'relict' taxa are extremely rare in flora and that is why *B. laeviagta* from the area of Bukowno, Bolesław and Olkusz is remarkably valuable for science.

B. For over 120 thousand years, plants of this subspecies have had appropriate conditions for growth and development on the outcroppings of zinc-lead ores above the ground surface. Over the last 800 years, due to human activity, specifically zinc and lead thermal metallurgy, which in the surroundings of Olkusz began as long ago as the 13th century, new habitats have appeared, creating suitable growth conditions for this plant. These habitats are post-mining waste heaps and wastes with a high content of heavy metals.

C. A specific feature of *B. laevigata* is its extraordinarily strong 'inclination' towards zinc. It is a zinc-loving plant, and the subspecies *woycickii* is particularly zinc-loving. A high zinc concentration in the soil inhibits the growth of other plant species but stimulates growth of *B. laevigata* at the same time. Such a strategy enables *B. laevigata* to settle in zinc-enriched localities and win in competition with other plant species. This is, therefore, a pioneer plant, especially in metalliferous zinc-enriched soils.

D. However, a high zinc content in the soil is not essential for the plant. *B. laevigata* also grows and blooms well in ordinary garden soil. Thus, the species is a facultative metallophyte – able to grow both in metalliferous and non-metalliferous soils.

E. *B. laevigata* is a typical montane species, in Poland it occurs in the Tatra Mountains at an elevation of 800 to over 2,000 m a.s.l. The other place of its occurrence is the Olkusz region which is located at an elevation much closer to sea level (300 m a.s.l.). The distance between the two localities is about 100 km. The extraordinary occurrence of *B. laevigata* in Poland is due to the ability of this species to grow in soils with a high zinc content. This very element is present both in the substratum in the Tatra Mountains (the remnants of the old-time iron works) and in the Olkusz region (zinc-lead soils). By extension, this led to the strong affinity to zinc that is responsible for such a surprising distribution of *B. laevigata* in Poland. High concentrations of zinc in both localities are accompanied by high concentrations of calcium, which is also of great importance for the growth of *B. laevigata*.

F. In the Tatra Mountains, only one subspecies of *B. laevigata* occurs. This is *B. laevigata* subsp. *gracilis*, which was confirmed using genetic research.

G. The main morphological features that are genetically preserved and enable one to distinguish both subspecies are the colour and covering of leaves with trichomes. In subsp. *woycickii*, the leaves are thin, light green, and densly covered with trichomes. Whereas in subsp. *gracilis*, the leaves are dark green, thicker, often glossy, and covered with fewer trichomes. The second feature distinguishing the two subspecies is the size of seeds. In subspecies *woycickii*, the seeds are about 30–50% smaller than in subsp. *gracilis*.

H. The level of tolerance to lead and cadmium is different in both subspecies of *B. laevigata*. The tolerance to these metals is about 50% higher in subsp. *woycickii* than in subsp. *gracilis.* However, the comparison with other plant species showed that the tolerance level to lead and cadmium of both *B. laevigata* subspecies falls within the range of their constitutional (innate) tolerance.

I. *B. laevigata* subsp. *gracilis* from the Tatra Mountains is able to grow and develop in the calamine soil from Bolesław. However, in both subspecies a 'critical point' is survival into the seedling phase. At this phase of plant development, about 80–90% of seedlings die, meaning that only 10% of plants grow to the adult phase. This feature is important to know for the future use of *B. laevigata* for vegetating new waste heaps. One should sow a huge number of seeds, bearing in mind that only a few percent of seedlings will survive. It is also possible to replant older plants, which have already been cultivated for 2–5 months, on a waste heap.

J. If only a small percentage of germinating seeds have the ability to grow and develop further, then in this very phase strong selection of the most resistant plants, best fitted to growth in pioneer and difficult conditions of waste heaps, takes place. After these plants become adults, they may further propagate vegetatively. Consequently, only the most resistant plant population settle in the waste heap area.

K. The calamine soil that is vegetated by calamine flora has very high concentrations of zinc, lead, cadmium, and thallium, but an insufficient content of nutritional elements. Very small amounts of nitrogen may be an inhibitory factor for the species composition of calamine flora and may also inhibit plant growth. The lack of nitrogen is one of the factors inhibiting the development of plants on the waste heap.

L. Another factor that also limits growth of *B. laevigata* on the waste heap is water deficiency. When more water was added and the *B. laevigata* plants were optimally watered, they were many times bigger (which is understandable), however, they contained over 2–3 times more metal in their tissues. This means that during intense rainfalls, a much greater quantity of heavy metals may be taken up into the plant tissues, and thus to the biological cycle.

M. When the heavy metals' availability from the calamine soil was increased, both *B. laevigata* subspecies showed the potential to hyperaccumulate thallium. Therefore, it seems that this is a feature of the whole species.

N. High concenctrations of heavy metals in the *B. laevigata* tissues, with no signs of toxicity, undergo detoxification and thus become non-toxic to plants. Metals are accumulated in cell walls, intercellular spaces, and vacuoles, meaning that the cell cytoplasm and nucleus, where significant metabolic processes take place, are protected. In addition, metals are removed to the epidermal trichomes on leaves, and as a result, the amount of metals in other leaf cells decreases. The excessive amount of metals is also transported to the oldest, withering leaves, which reduce the amount of metal in a plant after they fall off. Most metals that are taken up (lead, zinc, cadmium) are retained in the largest amounts in roots of plants. This protects the green parts, the aboveground parts of a plant, against their toxic action, and due to this, the whole photosynthetic apparatus is protected.

O. The seeds of *B. laevigata* subsp. *woycickii* are very valuable, because the subspecies has got the most effective adaptations to growth on the zinc-lead waste heaps. Seeds of these plants may be used for recultivation of new metalliferous substrates.

P. Natural localities of *B. laevigata* may be used as indicators of zinc-enriched soils by geologists. The species may be acknowledged as an indicator of zinc ore deposits.

Taking into account the very limited occurrence area of *B. laevigata* subsp. *woycickii* (post-mining areas of Bolesław, Bukowno and Olkusz) and the unique features of the subspecies, which is an endemic, a postglacial relic, and a facultative metallophyte characterised by strong affinity to zinc, one should consider it remarkably valuable for science.

At present, only two sites of this plant are legally protected within the Natura 2000 European Ecological Network. We strongly advocate for species protection of *B. laevigata* subsp. *woycickii* within the whole Olkusz region. It is worthy to note that this unusual plant may be acknowledged as a 'natural sensation' and it may become a symbol and one of the natural attractions in the whole area of Bolesław, Bukowno and Olkusz.

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