ADAPTATIONS OF *ARABIDOPSIS HALLERI* TO HABITATS RICH IN HEAVY METALS IN SOUTHERN POLAND

by

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ABBREVIATIONS

A.: *Arabidopsis*
A: available form
a_{18}^{i}: standardized chlorotypic allelic richness
A_S: mean allelic richness
BF: bioaccumulation factor
cpDNA: chloroplast DNA
CV: coefficient of variation
d. wt: dry weight
F_{IS}: inbreeding coefficient
H_E: expected heterozygosity
H_S: chlorotype diversity
IM: isolation with migration model
M: metalliferous sites, metallicolous populations
MCMC: Markov chain Monte Carlo algorithm
MS: mean square
MST: minimum–spanning tree
n_i: number of studied individuals per population
NJ: neighbour–joining tree
NM: non–metalliferous sites, non–metallicolous populations
NMp: non–metallicolous populations from polluted region
nNM: “new” non–metallicolous populations
ns: not significant
O: organic form
PC: Principal Component
PCA: Principal Component Analysis
QTL: Quantitative Trait Loci
S/R: a ratio of metal concentration in shoots to concentration in roots
SD: standard deviation
TI: tolerance index
$\overline{X}$: average
V_E: environmental variance
V_G: genetic components of phenotypic variation
V_P: phenotypic varianc

ABSTRACT

The present study attempted to provide a better description and understanding of the origin and the evolution of Zn tolerance and accumulation ability in *Arabidopsis halleri* populations from southern Poland, and clear up the history and colonization patterns of this species in investigated area. Here, in southern Poland, metalliferous (M) and non–metalliferous (NM) sites occur within relatively short distances. This fact makes from this part of Europe the only known area where gene flow between two different edaphic types of *A. halleri* populations can be studied. In terms of research on adaptation to extreme environments and colonization of metalliferous sites in southern Poland is therefore a unique and particularly interesting area.

The research was carried out on plant material from 15 *A. halleri* populations, originated from both metalliferous (Silesia and Olkusz region) and non–metalliferous (Niepołomice Forest and Tatra Mts.) sites. Plant material collected directly from field was used in physic–chemical and genetic analyses, while plant material from seeds collected from fields and grown under controlled greenhouse conditions was used in tolerance and accumulation tests. Physic–chemical analyses of field soil and plant samples were performed in order to characterize *A. halleri* habitats and plants' behavior in southern Poland. Then, genetic analyses using neutral molecular markers (performed on nuclear and chloroplast DNA) aimed to resolve genetic structure and to reconstruct phylogeography of *A. halleri* populations from investigated area as well as allowed to reconstruct migration routes which enabled *A. halleri* to colonize metalliferous and non–metalliferous sites in southern Poland. Experiments in controlled conditions (tolerance and accumulation tests) aimed to check if relationships between tolerance and accumulation of heavy metals abilities exist in studied species.

Variation of soil properties and population behavior within M type of sites was higher than within NM type. Moreover, this study showed that M and NM sites differed significantly not only in terms of heavy metal concentration but also other physic–chemical soil parameters (i.e. C_O, N, P, K, Mg, Fe). Contrasting selection regimes due to different conditions in M and NM habitats might affect behavior of plants and then differentiated populations from different sites. Therefore, local M and NM *A. halleri* populations should be specifically adapted to the local soil metal composition as well as to other sites' properties.

Investigation of neutral nuclear markers showed that populations of *A. halleri* in southern Poland were clustered into two groups, mainly corresponding to geographic location. The results suggest that M populations from Silesia and Olkusz region have diverged independently, however from common ancestor (*i.e.* Tatra Mts. populations). Moreover, two types of NM populations were identified: (1) ancestral "ancients" NM populations in non–contaminated natural species habitats in Tatra Mts., (2) evolved recently, as a result of two successive episode of colonization: first one on M sites and second one on NM sites beyond the mountains (nNM populations in Niepołomice Forest). In this study, for the first time it was shown that foundation of NM populations by M is also possible and it was proposed to name these recently evolved NM populations as "new–NM" (= nNM) populations. The estimations of time of divergence shown that *A. halleri* populations in Silesia region were founded probably in 14th century M, in Olkusz region in 17th century, while recently, in 18th century M populations from Olkusz region have been split into NM populations in Niepołomice Forest.

In the field, populations from different edaphic types accumulated Zn at very different level, while in the pot experiment, under uniform controlled conditions, most of populations accumulated Zn at similar level. It was observed that in the field some of populations showed the capacity to mobilize and concentrate metal in shoots (Niepołomice Forest's and Tatra Mts.' populations), while the others have been limited the uptake of metal (Silesia and Olkusz regions' populations). Finally, it was shown that accumulation is genetically determined, so that variance observed between populations in the field was both genetics and environment–dependent. Accumulation is then the result of interaction between genotype and environment.

New approach for phenotyping hydroponic experiment has been worked out in this study. The main advantages of proposed method are the assessment of genetic and environmental variance components on tolerance abilities, as well as the investigation of the same genotypes in several independent experiments. Moreover, it was shown that the use of this test at the within–species level is characterized by relatively high discriminating capacities.

In controlled conditions, NM populations of *A. halleri* were significantly less tolerant and accumulated Zn to significantly higher concentration as compared to M populations. This research allowed also to identify the most tolerant genotypes (particularly interesting in terms of phytostabilisation) as well as the most hyperaccumulating populations (useful in research on phytoextraction). Further detailed studies of these selected genotypes could be useful in identification of genes (or genome regions) involved in tolerance and accumulation of heavy metals. Reciprocal transplantation experiments *in situ* are also needed to measure the "cost of tolerance" and convincingly demonstrate local adaptation of Polish *A. halleri* populations.

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CONTENTS

I. INTRODUCTION

1. Heavy metals in environment

Areas with naturally elevated content of heavy metals in soil are known from many different places on Earth. In such places soil can vary in composition and concentration of metals. While in places rich in nickel, chromium and cobalt serpentine soils evolved (Peeper & Norwood 2001), different soils are also present where copper ore deposits are abundant or where zinc and lead are predominant metals is substrate composition (Ernst 1972, 1990). As in 19th and 20th century in areas rich in ores and other mineral resources mining industry began to develop, geographic range of occurrence of metalliferous sites broadens substantially. Intense development of industry and increase in industry–related pollution (ore mining, polluted dust from furnaces, galvanisation, car fumes, fungicide and pesticide usage) gave rise to formation of metal–containing soils even outside sites with natural occurrence of heavy metals (Antonovics *et al.* 1971; Macnair 1993; Saxena *et al.* 1999). Heavy metal pollution impacts the whole natural environment. Being inorganic compounds, heavy metals do not undergo the process of biodegradation and they can persist in soil for tens, hundreds or even thousands of years (Kabata–Pendias & Pendias 1999).

In soils under impact of industry heavy metals can be presented in exceedingly high concentrations and thus causing toxic effect on the most of plant species as well as for soil fauna (Ernst 1990). Metals here as stress factors there. Among negative effects of soil polluted by heavy metals, decrease in microorganisms number, deterioration of plant growth and in harvest yields (Mc Grath 1994), substantial disequilibrium in ecosystems through drastic reduction of biodiversity (Banásová *et al.* 2006) as well as serious health hazard to the local human population (Jarup 2003) could be enumerated. As far as plants are concerned the level of toxicity depends on the type of soil and its physic–chemical characteristic, on activity of microorganisms, on the plant species, genotype and on many other factors by which organism's response to the toxic dose of metals can be modified (Siedlecka *et al.* 2001). Numerous studies proved that there exist plant's defense mechanisms against noxious impact of heavy metals. These processes enable plants to persist in harsh environmental conditions.

2. Tolerance to heavy metals and their accumulation by plants

2.1. Metallophytes, pseudometallophytes – the classification of plants from metalliferous habitats

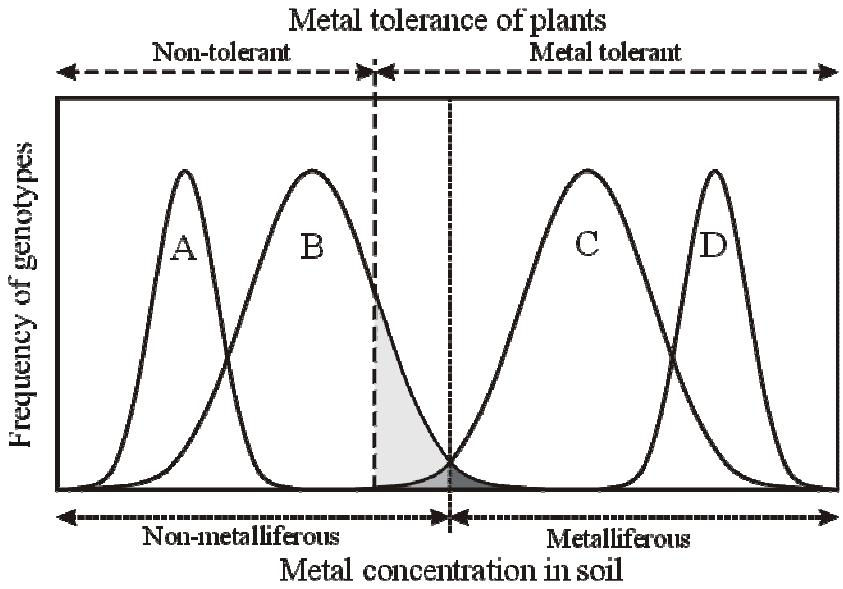
Tolerance to heavy metals is defined as "plant's ability to grow and reproduce on soils polluted with heavy metals, where most of plant species can not survive by reason of the toxicity of environment" (Antonovics *et al.* 1971; Macnair & Baker 1994; Macnair *et al.* 2000). The tolerance evolved as a response to strong selective pressure caused by high concentration of heavy metals in soil. It occurs only in limited number of species usually members of *Fabaceae* and *Poaceae* (Antonovics *et al.* 1971; Baker 1987). Tolerance to heavy metals was described for the first time by Pratt in 1934, who observed this phenomenon in *Silvestre Melandrium* (= *Silene vulgaris*) (Willems 2006). Pratt noticed that, under uniform conditions and in soil artificially contaminated with copper plants, from seeds gathered in vicinity of copper mine were developing better than plants from seeds harvested in non–polluted sites.

Plants, which are able to grow and reproduce only on soils with high concentrations of heavy metals are called metallophytes (Antonovics *et al.* 1971; Lambinon & Auquier 1964). Lambinon and Auquier (1964) divided metallophytes into: (1) absolute metallophytes – able to grow only on soils rich in metals (*e.g. Viola calaminaria, Thlaspi calaminare, Minuartia verna ssp. hercynica*) and (2) local metallophytes – able to grow on metal contaminated soils only within a particular region while outside the area colonizing non–polluted sites (*e.g. Armeria maritima*). The pseudometallophytes – taxa able to grow on metalliferous as well as on non–metalliferous soils within one area – form the second group in classification proposed by Lambinon and Auquier (1964). The authors divided pseudometallophytes into three groups: (1) elective pseudometallophytes – presenting particular vitality and abundance on metalliferous soils (*e.g. Agrostis tenuis, Campanula rotundifolia, Polygala vulgaris, Thymus pulegioides, Rumex acetosa*), (2) indifferent pseudometallophytes – growing on metalliferous soils but showing neither abundance nor particular vitality (*e.g. Plantago lanceolata, Avena pubescens, Genista tincoria, Linum catharticum*), (3) accidental metallophytes – common ruderal species occurring sporadically on metalliferous soils and presenting decreased vitality. In current literature pseudometallophytes populations on metalliferous soils are called metallicolous while these on non–metalliferous soils – non–metallicolous. In this work, metalliferous sites

as well as metallicolous populations will be marked with the letter M while non–metalliferous sites and non–metallicolous populations are marked with NM (Pauwels *et al.* 2008). There is multitude of different metallophytes classifications (Duvigneaud & Denaeyer de Smet 1963; Lambinon & Auquier 1965; Antonovics *et al.* 1971; García–González & Clark 1989; Baker & Proctor 1990). Their authors divided the group in different ways giving often new names to groups of plants previously recognized by other authors under different names. Because of that, one group of plants within metallophytes could have been known by several different names depending on the author. A review on metallophytes was presented by Pollard *et al.* (2002). They proposed a schematic division of plant species regarding their tolerance abilities and their geographical range on metallicolous and non–metallicolous sites (Fig.1). The authors divided plants into strict (= obligate) nonmetallophytes – having only NM populations – and metallophytes. Among metallophytes two groups were recognized: (1) facultative metallophytes (pseudometallophytes) – species with both M and NM populations and (2) absolute (= strict = obligate) metallophytes (eumetallophytes) – species with M populations only, endemic to metalliferous habitats. It was pointed out, that predominant majority of plants belong to nonmetallophytes (species A in Fig. 1). These plants are sensitive to toxic impact of heavy metals and their range is strictly limited to soils with only trace content of metals. There are no tolerant genotypes within populations of nonmetalophytes. Another group, the most numerous and the most interesting (especially in genetic context), is formed by plants able to tolerate heavy metals – pseudometallophytes. Pollard *et al.* (2002) recognized two categories of pseudometallophytes: species with predominance of populations occurring on non–metalliferous soils (species B) and species with predominance of populations occurring mainly on metalliferous soils (species C). Among the group of pseudometallophytes with predominance of NM populations, the majority of plants is sensitive to toxic impact of heavy metals. However, there are some tolerant genotypes within their populations (light shading in Fig. 1). These genotypes are favored (by natural selection) during colonization of metalliferous areas (heavy shading in Fig. 1). The following species belong to this group: *Agrostis capillaris, Plantago lanceolata, Mimulus guttatus, Silene vulgaris* (Pollard *et al.* 2002). The phenomenon of metal tolerance is therefore not restricted only to plants from M populations. As far as the second group is concerned (with predominance of M populations) all the genotypes are tolerant (also genotypes

from NM populations) (intermediate shading in Fig. 1). *Thlaspi caerulescens, Thlaspi goesingense, Thlaspi montanum* and *Arabidopsis halleri* belong to this group. The last group according to Polard's classification is called eumetallophytes (species D). These plants, endemic to metalliferous areas, tolerate high concentrations of heavy metals within the whole species. Numerous accumulators of nickel such as different species from *Alyssum* genus are in that group (Brooks *et al.* 1979).

According to law regulations in Poland (Monitor Polski No 23, 1986) in non–metalliferous soils total content of zinc, lead and cadmium should not be higher than 300 $mg \cdot kg^{-1}$, 100 $mg \cdot kg^{-1}$, 3 $mg \cdot kg^{-1}$, respectively. In opposite, when above mentioned norms are exceeded, these soils can be named metalliferous.



A – nonmetallophytes, M = 0; C – pseudometallophytes, M > NM
B – pseudometallophytes, NM > M D – eumetallophytes, NM = 0

Fig. 1. Schematic division of theoretical plant species: A, B, C, D. Frequency of metallicolous (M) and non–metallicolous (NM) populations as well as frequency of tolerant and non–tolerant genotypes was presented. Zones of light, intermediate and heavy shading illustrate the minority of genotypes within the species, as discussed in the text (according to Pollard *et al.* 2002).

2.2. Genetic basis of metal tolerance

Measuring tolerance

During last twenty years numerous researches has been conducted on tolerant and hyperaccumulating (having high concentrations of metals in above–ground parts)

species. That is due to their potential use in recultivation of degraded areas (Salt *et al.* 1998; Krämer 2005). Developing conception of purification and stabilization of post–industrial areas requires, however, knowledge on genetic as well as physiological basis of tolerance and hyperaccumulation (Brooks 1998; Macnair *et al.* 2000; Macnair 2003). During many years, scientists were unable to meet the challenge of describing these phenomena in genetic terms because of the lack of appropriate research tools. Initially studies on tolerance were based on observations of plants growth on contaminated soils under both controlled and natural conditions. From the results of these experiments it was possible to draw some deductions on absence or presence of tolerance (Bradshaw 1970; Antonovics *et al.* 1971). First surveys took into account tolerance to zinc in *Agrostis capillaris* (= *A. tenuis*) (Bradshaw 1952). Experiments proved that the rate of root growth was a sensitive indicator of the presence of metal ions. Different root tests of tolerance, commonly employed in contemporary research, based upon this conclusion (Wilkins 1978; Macnair 1983; De Koe *et al.* 1992; Schat & Ten Bookum 1992a). The method of root test enables not only to indicate the presence of tolerance, but also to assess its degree. Seedlings are placed in hydroponic culture in solutions of different concentrations of metals. On the basis of degree of inhibition of root growth in different solutions the value of tolerance index can be determined. Tolerance index (TI) mirrors various plant responses to ion stress. However, the validity of the tolerance index was vigorously challenged because this tolerance measurement was believed to exhibit an inherently high level of statistical noise (Macnair 1983; Schat & Ten Bookum 1992b; Macnair 1993). Indeed, the tolerance index was a quotient of two variables with presumably differently skewed probability distributions and additionally, affected by innate variation of root growth, and thus by genes other than those that were supposed to govern tolerance (Macnair 1983; Schat & Ten Bookum 1992b). In order to provide more accurate estimates of tolerance Macnair (1983) introduced the single concentration test, in which tolerance was evaluated by the ability of cuttings to produce roots at a certain fixed metal concentration. To overcome the limitations inherent to the single concentration test, the multiple concentration test was adopted. In that one, clones were exposed to different metal concentrations and tolerance corresponded to the concentration, at which growth was reduced to 50% of control root growth (Macnair 1993). However, this tolerance test was relatively time–consuming, because of series of metal concentrations,

at which tolerance had to be estimated, as well as the generation time necessary for the production of clonal replicates (Schat & Ten Bookum 1992a; Macnair 1993). In order to avoid the cloning of individual plants an alternative type of the multiple concentration test, *i.e.* the sequential exposure test (Schat & Ten Bookum 1992a) was established, in which each individual plant was exposed to a test solution in which the metal concentration was increased in time in a stepwise manner. The tolerance level corresponded to the lowest concentration at which root growth was completely inhibited (Schat & Ten Bookum 1992a). Multiple concentration tests might be considered to be the most suitable for addressing the genetic determinism of heavy metal tolerance (Bert *et al.* 2000; Assunçao *et al.* 2003b; Bert *et al.* 2003). In this kind of research crosses between plants presenting extreme phenotypes are often employed (Macnair *et al.* 1999; Assunçao *et al.* 2003a; Frerot *et al.* 2005; Willems *et al.* 2007, Courbot *et al.* 2008). Observations of differences between tested individuals became possible due to the progress of techniques of plant cultivation in uniform and controlled conditions in order to eliminate environmental variation.

Tolerance to several metals

As in metalliferous soils elevated concentration of single metal is rather rare, while situation when at least several metals are abundant is quite frequent, metallophytes usually are able to tolerate not only one but also several metals. Two hypotheses were proposed to explain this phenomenon: (1) tolerance to several metals can be determined by different, independent mechanisms (one mechanism – one metal) and (2) tolerance is a pleiotropic phenomenon (one mechanism determines tolerance to different metals). In the first case, the phenomenon should be called multiple tolerance, while in the second one co–tolerance (Schat & Vooijs 1997; Tilstone & Macnair 1997; Schat *et al.* 2000).

On one hand, according to several authors (Antonovics 1971; Ernst 1982; Karataglis 1982) plants are able to tolerate several metals, but only under conditions that they grow on soil where all the metals have high, toxic concentrations. On the other hand, according to other authors (Turner 1969; Allen & Sheppard 1971; Hogan & Rauser 1979; Cox & Hutchinson 1979, 1981; Symeonidis *et al.* 1985; Verkleij & Bast–Kramer 1985; Verkleij & Prast 1989; Schat & Ten Bookum 1992b) plants are able to tolerate several metals also when they grow on soil with relatively small,

non toxic concentrations of these metals. Recent developments in research techniques have allowed testing tolerance to different metals. Modern methods enable to differentiate between multiple and co–tolerance (Schat & Vooijs 1997; Tilstone & Macnair 1997; Bert *et al.* 2003). To achieve that, tolerance tests on progeny from crosses of tolerant and non–tolerant individuals (from the same or closely related species) or progeny from crosses between individuals with different levels of tolerance (within the same species) are performed. Research on metallicolous populations of *Silene vulgaris* employing crosses between individuals has proved that tolerance to zinc and copper is under the control of different genes (multiple tolerance). This phenomenon is also accompanied by tolerance to zinc, nickel and cobalt (Schat *et al.* 1996; Schat & Vooijs 1997). Similarly, co–tolerance to zinc and cadmium was discovered in *Arabidopsis halleri* (Bert *et al.* 2003). Studies on genetic basis of tolerance to copper in *Mimulus guttatus* (Tilstone & Macnair 1997) as well as tolerance to zinc, cadmium and nickel in *Thlaspi caerulescens* (Assunção *et al.* 2003b) have proved that these plants possess highly specific mechanism of tolerance to each tested metal.

Results of first studies on genetic bases of tolerance suggest that, in plants, tolerance is a polygenic trait (determined by numerous genes) (Antonovics *et al.* 1971). However, recent studies show that tolerance is determined by one or two major genes (Macnair 1990, 1993). Studies carried out on crosses support the idea of one or two major genes responsible for determination of tolerance to heavy metals. It was found, that tolerance to copper in *Mimulus guttatus* is determined by one gene (Macnair 1983). Tolerance in *Silene vulgaris* was found to be determined by one or two major genes (depending on population) (Schat & Ten Bookum 1992a; Schat *et al.* 1993). Among grasses tolerance to arsenic in *Holcus lanatus* is determined by one gene (Macnair 1993) as well as it is in *Agrostis capillaris* (Watkins & Macnair 1991). In case of tolerance to zinc in *Silene vulgaris* involvement of two major genes was proved (Schat *et al.* 1996). All above mentioned studies have proved that tolerance is a dominant trait coded by one or two major genes and usually by one or few modifier genes (Watkins & Macnair 1991; Schat *et al.*1996; Macnair *et al.* 2000).

(Hyper) accumulation as a strategy of tolerance to heavy metals in plants

Two major strategies evolved among plants from areas with high concentration of heavy metals: exclusion and accumulation (Fig. 2) (Baker 1981). A strategy of excluding metals from above–ground parts (shoots, leaves *etc*.) is the most widespread (Willems 2006). The strategy involves metal uptake and accumulation in roots by limitation of metal transport to above–ground parts (Baker 1981). Constant and low concentration of metals in shoots is maintained in this way until a critical dose of metal in soil is exceeded. The second strategy – accumulation – is present only in limited number of plant species. This strategy involves metal accumulation in above–ground parts in concentrations higher than in roots (Baker 1981, 1987), Hyperaccumulation is a special example of the strategy of accumulation (Brooks *et al.* 1977). There is also another group of species (indicator species), in which metal content in above–ground parts is proportional to metal concentration in soil.

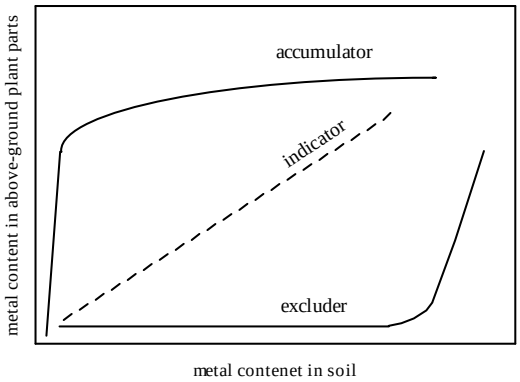


Fig. 2. Tolerant plants' reactions to concentrations of heavy metals in soil (according to Baker 1981).

Phenomenon of hyperaccumulation was first described in 1865 during research on *Thlaspi calaminare* (= *T. caerulescens*) – a species able to grow on soils rich in zinc (Sachs 1865). The term "hyperaccumulator" was first used by Brooks *et al.* (1977) to describe plants accumulating more than 1000 $mg \cdot kg^{-1}$ of dry weight (d.wt) of nickel. Further research made possible to define threshold concentrations for other metals. It is widely accepted that they equals 10 $mg \cdot kg^{-1}$ d.wt for mercury (Hg), 100 $mg \cdot kg^{-1}$ d.wt for cadmium (Cd), 1000 $mg \cdot kg^{-1}$ d.wt for lead (Pb), cobalt (Co), copper (Cu), arsenic (As), selenium (Se), nickel (Ni) and 10000 $mg \cdot kg^{-1}$ d.wt for manganese

(Mn) and zinc (Zn) (Brooks *et al.* 1977; Baker & Brooks 1989; Baker *et al.* 2000; Reeves & Baker 2000).

There are also other criteria for evaluating hyperaccumulation: a ratio of metal concentration in above–ground parts to concentration in roots (S/R ratio) (Salt & Krämer 2000 and literature cited therein) and a ratio of metal concentration in above–ground parts to metal concentration in soil (BF – bioaccumulation factor) (Baker *et al.* 1994). Plant can be perceived as hyperaccumulating when value of these ratios stands above one.

However, these two definitions of hyperaccumulation can generate contradictory conclusions according to the metal concentration in the soil. Indeed, the same plant individual, grown in a less polluted soil (*e.g.* contained 300 $mg \cdot kg^{-1}$ d.wt of Zn) and contained high amount of metal in above–ground parts (*e.g.* 4000 $mg \cdot kg^{-1}$ d.wt of Zn), could be considered as non–hyperaccumulator according to definition *sensu* Brooks *et al.* (1977), either hyperaccumulator *sensu* Baker *et al.* (1994). The opposite situation is also possible, when the same plant grows in highly polluted soil (*e.g.* 20000 $mg \cdot kg^{-1}$ d.wt of Zn) and accumulate very high amount of metal (*e.g.* 12000 $mg \cdot kg^{-1}$ d.wt of Zn) in its above–ground parts. Therefore, the definition of hyperaccumulation should stress the hyperaccumulator's behavior which is characterized by a capability of plant either to concentrate the metal in its aerial parts at high amounts when plant grows on less polluted soils, or to control and maintain metal accumulation on plateau much higher than those observed for non–hyperaccumulating plants in the same conditions when plant grown on highly polluted soils (Roosens *et al.* 2008).

In spite of numerous studies in hyperaccumulation, only little is known about its mechanisms. Six hypotheses proposed so far to describe mechanisms and reasons for which plants accumulate high amounts of heavy metals were reviewed by Boyd and Martens (1992) and Boyd (1998). First hypothesis assumes that hyperaccumulation prevents heavy metals from being absorbed inside a plant cell. Even if metals are absorbed, they are transported to places where they are sequestrated and can not disturb metabolic processes (cell wall, vacuole) (Antonovics *et al.* 1971; Baker 1981, 1987; Kruckeberg & Reeves 1995). According to another hypothesis, metals are eliminated from root cells by transporting them to the oldest leaves. When leaves desiccate accumulated metals can be removed from plant (Ernst 1972; Wild 1978; Boyd & Martens 1992a). Heavy metals can be also eliminated via leaf epiderma

and glandular hairs (Farago & Cole 1988; Clemens 2001). The other hypothesis assumes a relationship between changes in water relations in plant cells and hyperaccumulation (Severne 1974; Baker & Walker 1990; Robertson 1992). In plants adapted to drought cell water potential is much lower than in other species. Low water potential is due to high concentration of ions and other substances in cell sap. According to Baker and Walker (1990), heavy metals (such as nickel for example) may be perceived as water–potential–decreasing agents. For that reason hyperaccumulation may be beneficial to plants growing under drought conditions. There is also another hypothesis according to which high concentrations of metals in above–ground parts is a side effect of a physiological processes different than tolerance (Baker & Walker 1990). Here accumulation is seen as a non–desired consequence of chemical affinity of heavy metals to other elements that are taken up by plants (Boyd & Martens 1992b). In terms of reasons of hyperaccumulation it is hypothesized that hyperaccumulator influence other plants by enriching soil with metals and thus creating "toxic environment" in which other plant species can not develop (Baker & Brooks 1989; Wilson & Agnew 1992; Boyd & Jaffré 2001). Among other reasons of hyperaccumulation we can consider defense against herbivores and damages caused by pathogens. High tissue concentration of metals can have toxic effect on both herbivores and pathogens (Ernst *et al.* 1990; Boyd 1998; Sägner *et al.* 1998). The last hypothesis is currently the most tested (Boyd & Martens 1992b; Boyd & Martens 2002; Noret *et al.* 2005).

Hyperaccumulators are usually able to accumulate one or several metal elements. Currently 418 species of hyperaccumulating plants are known including accumulators of Ni, Zn, Cd, Pb, Cu, Co and Mn (Reeves & Baker 2000). Nickel accumulators constitute predominant majority in this group (317 species; Baker *et al.* 2000). Today we know 36 species of Co hyperaccumulators, 24 species accumulating Cu, 18 accumulating Zn, 8 accumulating Mn, 5 accumulating Pb, 3 accumulating Cd (Baker *et al.* 1992; Brooks 1994; Prasad & de Oliveira Freitas 2003). *Brassicaceae* family houses the highest number of hyperaccumulating species. Within 11 genera 87 species of hyperaccumulators are known from *Brassicaceae* (Prasad & de Oliveira Freitas 2003). Here belong *Arabidopsis halleri* and *Thlaspi caerulescens, which* were proposed as model species in research on tolerance to zinc and cadmium (Assunção *et al.* 2003a; Krämer 2003).

2.3. Adaptations to metalliferous habitats

It is accepted that heavy metal tolerance in plants is one of the best examples of adaptation and microevolution (Antonovics *et al.* 1971). Local adaptations in populations from metalliferous areas evolve due to toxic concentrations of heavy metals in soil what maintain high selection pressure (Turner 1969; Antonovics *et al.* 1971; Macnair 1987). As soil pollution caused by humans is a relatively recent phenomenon it became possible to track dynamic of local adaptations almost *in statu nascendi*. Research on evolution of zinc tolerance in NM (= non–metallicolous) populations of *Agrostis tenuis* remains one of the best examples of the process. In close vicinity of natural populations of *Agrostis tenuis* a zinc smelter was sited, causing soil pollution (Al–Hiyaly *et al.* 1988). After few years, *Agrostis tenuis* populations from the area were found to possess toleration abilities to zinc comparable with populations from much older metalliferous sites located in post–mining area. In research on *Agrostis stolonifera* after four–year exposure investigated populations showed higher tolerance than NM populations (Wu *et al.* 1975). It seems that tolerance could evolve rapidly. Different studies have proved that level of tolerance depends on the time of metal exposure: the longer time of exposure was, the more tolerant plants were (Wu *et al.* 1975, Al–Hiyaly *et al.* 1988). An hypothesis was proposed that initially selection in major gene or genes plays a key role (Macnair 1993). The selection enables plants to colonize metalliferous areas. Differences in the level of tolerance between M (= metallicolous) populations evolve subsequently as a result of selection on hypostatic modifier genes (Macnair 1997). The pace of evolution of tolerance to heavy metals depends on the strength of selection pressure and gene flow rate between M and NM populations (Macnair 1993). Colonization of metalliferous soils is possible due to strong directional selective pressure on dominant gene coding the trait of tolerance. Above mentioned mechanisms allow evolution of tolerance and thus establishment of M populations even when gene flow from neighbouring NM populations is not restricted (Lefèbvre & Vernet 1990).

Exclusion is the most common strategy of tolerance to heavy metals in plants (see chapter 2.2 and Fig. 2). Among pseudometallophytes showing this strategy, inter–populational differences in metal tolerance come from extremely different concentrations of the metal in soil from M and NM sites. Exclusion strategy evolves in M populations and it is strictly connected with selection pressure caused by toxic

concentrations of metals (Matthews *et al.* 2005). Tolerance in plant populations after exposure to high doses of metals in soil is called adaptive tolerance (Antosiewicz 1995). In contrary to exclusion the impact of differences of metal concentration on accumulation ability is less obvious. The vast majority of hyperaccumulators is endemic to metalliferous sites (Kruckeberg & Rabinowitz 1985; Kruckeberg 1986) as in case of nickel tolerant species on serpentine. They are absolute metallophytes, with natural range strictly limited to sites with high concentration of metals in soil (see chapter 2.1 and Fig. 1). There are also hyperaccumulating species, known as pseudometallophytes, which occur in both M and NM sites. Here, among others, belong *Thlaspi caerulescens* (Lloyd – Thomas 1995; Meerts & van Isacker 1997; Reeves *et al.* 2001), *T. goesingense* (Reeves & Baker 1984), *T. montanum* (Boyd & Martens 1998b), *Arabidopsis halleri* (Bert *et al.* 2000). *Thlaspi caerulescens* and *Arabidopsis halleri* are among the most investigated species. Recently, it was shown that heavy–metal tolerance in both species is constitutive (Meerts & van Isacker 1997; Bert *et al.* 2000), *i.e.* concern all genotypes, whatever their edaphic origin.

Thorough research on genetic basis of hyperaccumulation in plants may have practical applications as, for example, improvement in phytoremediation. Predominant number of known hyperaccumulators produces only low biomass. Furthermore, the most of taxa show hyperaccumulation of one metal while areas polluted with heavy metals usually contains at least several metal elements in toxic concentrations. Currently, numerous researches are carried out in order to increase growth rate, biomass production and tolerance in several plant species. Investigation aims also at making plants able to take up more than one metal (Mejáre & Bülow 2001). Discovery of genes underlying hyperaccumulation might result in production of transgenic plants having all the essential features of ideal phytoremediators (Chaney *et al.* 2000; Lasat 2002; McGrath *et al.* 2002).

2.4. Recultivation of post–industrial areas

Heavy metals disturb biological equilibrium of soil. Therefore, a need exists to remove them from polluted soils or change them into forms which are unavailable for living organisms (Pilon–Smits 2005). It can be done with usage of bacteria, fungi or plants and this method is called bioremediation. When plants are employed in order to remove noxious metals from soil the method can be called phytoremediation

(Salt *et al.* 1998). Phytoremediation can be divided into phytoextraction, phytostabilisation and phytovolatilisation (Raskin *et al.* 1997; Salt *et al.* 1998). Phytoextraction uses plant's ability to accumulate heavy metals in above–ground parts (Galiulin *et al.* 1998; McGrath & Zhao 2003). After a period of growth mature plants are removed from polluted soil and thus reduction in soil metal concentration is gained. Among features of ideal plant suitable for phytoextraction we can enumerate: high growth rate, high biomass production and tolerance to harsh environments of post–industrial wastelands (Krämer 2005; McGrath & Zhao 2003; Raskin *et al.* 1997; Salt *et al.* 1998). Plants seem to be the most suitable candidates for phytoextraction because of their natural abilities to accumulate high amounts of heavy metals in above–ground parts. However, low rate of growth and low biomass yields strongly limit their broad use (Clemens 2001). To date only few species have been used in phytoextraction. *Thlaspi caerulescens* have been used in recultivation of areas polluted with zinc and cadmium. *Thlaspi rotundifolium* have been found suitable in lead removal while species from *Alyssum* have served in recultivation of nickel–polluted sites (Siwek 2007 & literature cited therein). Another technique of phytoremediation – phytostabilisation – bases upon decreasing bioavailability of metals and thus minimizing the risk of their accumulation in food chains. In this technique tolerant plant species are also employed. Immobilization of metals entails their sedimentation or reduction by chemicals released by roots. *Festuca rubra, Sesbania rostrata* and *Typha latifolia* are sometimes mentioned as suitable phytostabilisers (Siwek 2007). A process in which some non–organic compounds are taken up from soil, reduced in plant tissues and then released to the atmosphere is called phytovolatilisation. The method was found useful in purifying soils polluted with selenium (*e.g.* using *Brassica juncea*), mercury (*e.g.* using transgenic *Arabidopsis thaliana*) and arsenic (*Pteris vittata*) (Raskin *et al.* 1997; Salt *et al.* 1998).

Low costs and lack of negative impact on soil environment are the most important advantages of phytoremediation (Krämer 2005). Employing plants in process of heavy metal removal limits considerably their possible spread to the atmosphere and water as roots penetration in soil causes its stabilization and thus decrease the risk of erosion.

3. *Arabidopsis halleri* – biological characteristics

3.1. Taxonomic position, geographic distribution and habitat

The name *Arabidopsis* was first used by de Candolle in 1821 for one section from *Sisymbrium* genus (de Candolle 1821). Twenty years later Heynhold considered *Arabidopsis* as a distinct genus comprising one species: *Arabidopsis thaliana* (Holl & Heynhold 1842). During 19th and 20th century, many different species were included to *Arabidopsis*, thus making it a very heterogeneous taxon. On the basis of morphological and molecular investigation O'Kane and Al–Shehbaz (1997, 2003) recognized only nine species within the genus (Fig. 3). *Arabidopsis halleri* is one of them. Within *Arabidopsis halleri* three subspecies were recognized: *A. halleri* subsp. *halleri*, *A. halleri* subsp. *ovirensis* (Wulfen) O'Kane & Al–Shehbaz as well as *A. halleri* subsp. *gemmifera* (Matsumura) O'Kane & Al–Shehbaz (Al–Shehbaz & O'Kane 2002). Kolnik and Marhold (2006) based on studies on morphology, kariology and molecular biology recognized two additional subspecies: *A. halleri* subsp. *tatrica* (Pawł.) Kolnik and *A. halleri* subsp. *dacica* (Heuff.) Kolnik. The authors stated that four subspecies occur in Europe: *A. halleri* subsp. *halleri*, *A. halleri* subsp. *tatrica*, *A. halleri* subsp. *dacica* and *A. halleri* subsp. *ovirensis*, they stressed, however, that taxonomic status of *A. halleri* subsp. *ovirensis* is not clear and requires special attention in future research.

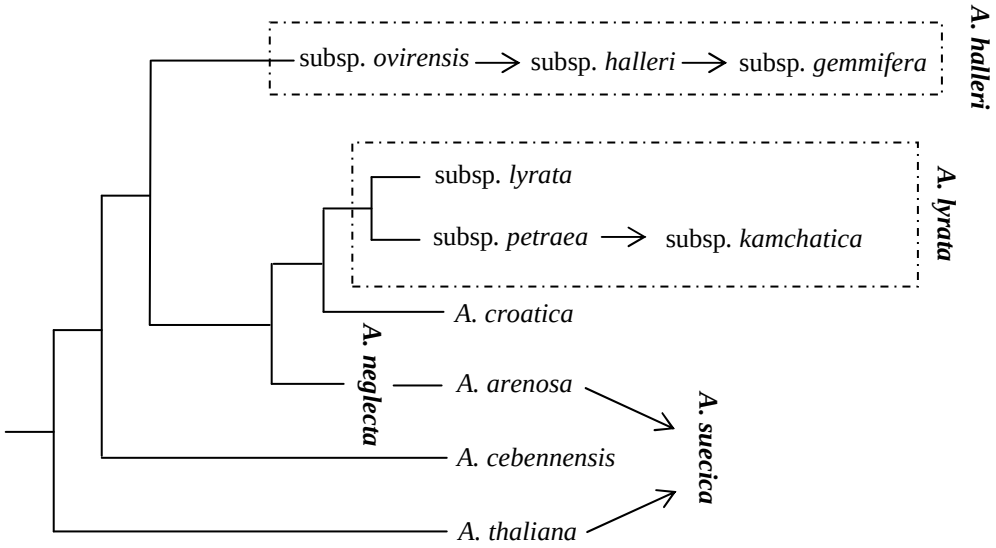


Fig. 3. Phylogenetic tree of the *Arabidopsis* genus (Al–Shehbaz & O'Kane 2002).

Arabidopsis halleri subsp. *halleri* is widespread in Europe while occurrence of other subspecies is restricted only to mountain areas. *Arabidopsis halleri* subsp. *tatrica* occurs in western part of Carpathians (to which it is endemic), subsp. *dacica* occurs in eastern and southern Carpathians while subsp. *ovirensis* is said to occur in both Alps and Carpathians (Kolnik & Marhold 2006) (Fig. 4).

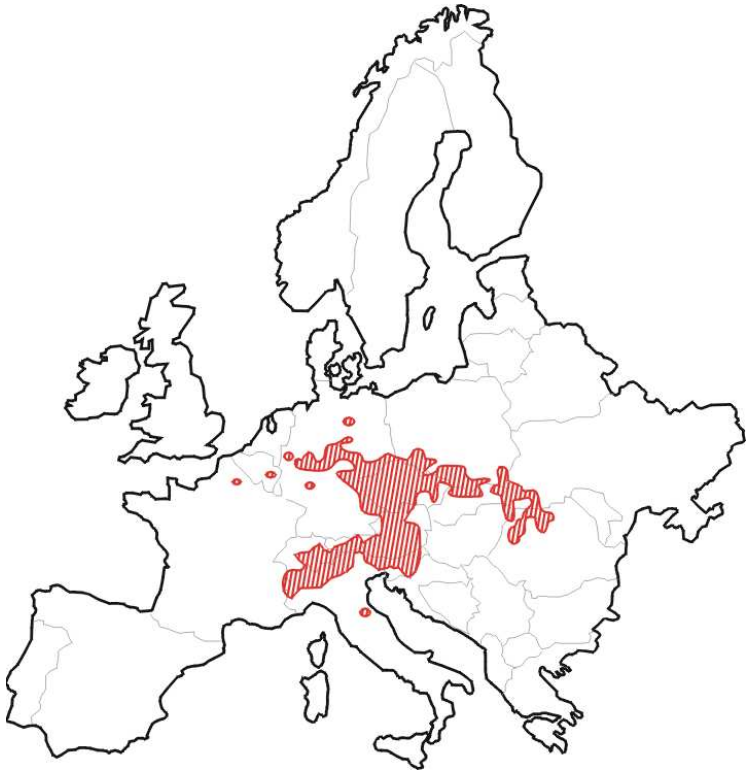


Fig. 4. Distribution map of *Arabidopsis halleri* subsp. *halleri* in Europe (according to *Atlas Florae Europeae* software by Botanical Museum, Finnish Museum of Natural History; modified); http://www.fmnh.helsinki.fi/english/botany/afe/publishing/database.htm.

In Poland three subspecies occur: *A. halleri* subsp. *halleri*, *A. halleri* subsp. *tatrica* and *A. halleri* subsp. *ovirensis.* Their natural range is restricted only to southern part of the country (Fig. 5) (Kolnik & Marhold 2006). *Arabidopsis halleri* subsp. *halleri* has the widest distribution occurring in Carpathians, Sudetes, Śląsko–Krakowska Upland as well as in some lowland areas (Niepołomice Forest, Sandomierska Lowland) (Zając & Zając 2001). *Arabidopsis halleri* subsp. *tatrica* grows in Tatra Mts., Beskid Żywiecki Mts., Pieniny Mts. and Orawa – Nowy Targ Basin (Kolnik & Marhold 2006; Zając & Zając 2001). Distribution of *A. halleri* subsp. *ovirensis* is the matter of controversy. All three subspecies grow on acidic as well as on alkaline soils

both calcareous and dolomites which are frequently naturally rich in heavy metals (Bert *et al.* 2002). They occur mostly in shady areas in grasslands, meadows, shrubs, forest margins and forests.

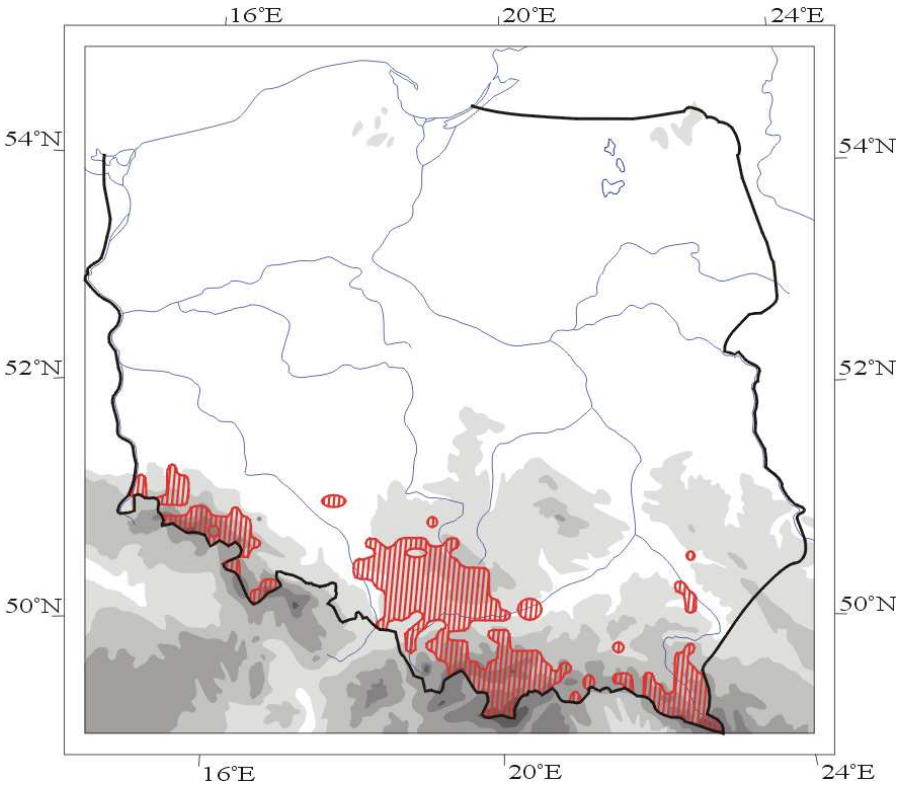


Fig. 5. Distribution map of *Arabidopsis halleri* subsp. *halleri* in Poland (according to Zając & Zając 2001; modified).

3.2. Species characteristic

Plant material used in present study represents subspecies *Arabidopsis halleri* subsp. *halleri*. Below its detailed characteristic is given.

Perennial herb with stolons. Stems (7) 10 – 53 (70) cm tall, branched above, with simple or 1–forked trichomes, rarely glabrous. Basal leaves rosulate with long petioles, pinnatifid to lyrate–pinnatifid, with terminal lobe much larger than (1) 2 – 6 (7) leteral lobes. Stem leaves shortly petiolate, coarsely toothed, gradually reduced in size upward. Lower and central stem leaves oval to oblong, distantly toothed. Leaves covered with simple of 1–forked trichoms, upper leaves often glabrous. Flowers abundant (6) 8 – 33 (37), petals white or lilac (4.1) 4.5 – 7.5 (8.1) mm long and (1.9) 2.2 – 3.8 (4.1) mm wide. Sepals green to green–purplish, glabrous or with few simple trichoms apically. Siliques linear (1.2) 1.4 – 2.6 (3) cm long

on pedicells (0.5) 0.6 – 1.2 (1.3) cm long. Flowering and fruiting May – August (Al–Shehbaz & O'Kane 2002; Kolnik & Marhold 2006).

Fig. 6. *Arabidopsis halleri* on metalliferous site in southern Poland. Vicinity of Bolesław Mine and Metallurgical Plant.

Arabidopsis halleri is an enthomophilic species (Bert *et al.* 2000; Van Rossum *et al.* 2004; Castric & Vekemans 2004). The impact of differences in petal color on reproduction success in not known (Maria & Koch 2006). The species can reproduce also vegetatively by stolons (Al–Shehbaz & O'Kane 2002) no longer than 1 m (Van Rossum *et al.* 2004). *Arabidopsis halleri* is a diploid species having basic chromosome number 2n = 16 (Al–Shehbaz & O'Kane 2002; Kolnik & Marhold 2006). DNA coding regions show high homology (90 – 95 %) to *Arabidopsis thaliana* – a model species in molecular biology (Koch *et al.* 2001; Weber *et al.* 2004). Close relationship between both species made it possible to use knowledge from *A. thaliana* studies in research on *A. halleri* (Mitchell–Olds 2001; Feder & Mitchell–Olds 2003).

Arabidopsis halleri occurs on both metalliferous (rich in Zn, Cd and Pb) and non–metalliferous soils and can be therefore classified as pseudometallophyte (Bert *et al.* 2000). Although all the populations of the species studied to date (metallicolous and non–metallicolous) show tolerance to high concentrations of heavy metals in soil (constitutional tolerance; Pauwels *et al.* 2005) they differ significantly in terms of the level of the tolerance (Macnair 2002; Pauwels *et al.* 2008). *Arabidopsis halleri* is one of two known Cd hyperaccumulators (Brooks 1998; Küpper *et al.* 2000) and one of more than ten known Zn hyperaccumulators (Macnair 2002). To date research carried out on crosses between tolerant *A. halleri* and non–tolerant *A. lyrata* individuals have shown that hyperaccumulation and tolerance

of Zn and Cd in *A. halleri* are encoded by several genes (Macnair *et al.* 1999; Bert *et al.* 2003, Willems *et al.* 2008, Courbot *et al.* 2008). Numerous researches are currently conducted to identify genes underlying tolerance and accumulation. The species was accepted as a model in studies in heavy metal accumulation and tolerance in plants (Mitchell–Olds 2001; Weber et al 2004; Lexer & Fay 2005, Pauwels *et al.* 2008, Roosens 2008 *et al.* 2008).

4. Research aims and hypotheses

Why to study A. halleri populations from southern Poland? Hitherto knowledge

Studies conducted to date in *A. halleri* have shown that although Zn tolerance is assumed to be constitutive in the species, populations differ significantly in terms quantitative values of tolerance to zinc (Pauwels *et al.* 2005). It should be stressed that the observed quantitative polymorphism is a prerequisite for allowing diversifying selection to adapt populations to local conditions (Latta 1998). However, the evolution of local adaptation is under influence of gene flow, population history and the reproductive system. Therefore, to get to know inferences about the role of local adaptation in shaping phenotypic differentiation, patterns of genetic variation for quantitative selected traits should be compared with that for neutral molecular markers.

Both selected phenotypic and neutral genetic differentiation between M and NM *A. halleri* populations has been already studied (Pauwels *et al.* 2005, 2006). Genetic approach, using molecular markers, was performed at a large geographic scale in which investigated populations were isolated by several hundred kilometers. Genetic gaps could have occurred among the sampled regions and therefore, the causal effect for the phenotypic differentiation observed among M and NM populations are steel poorly known: phenotypic differentiation could be related either to evolutionary relationships between the two types of populations or to existence of specific local adaptation of populations to metal contamination. However, the phylogeographic study using cpDNA (Pauwels *et al.* 2005) showed that *A. halleri* population structure was related to geographic isolation rather than to Zn exposure in northern Europe. On this basis it was supposed that M sites in Europe were colonized by *A. halleri* due to plant migration from the nearest NM sites, and those distant M populations

have evolved independently. Consequently, because enhanced tolerance was confirmed to be a general feature of M populations, it was hypothesized that natural selection towards enhanced tolerance could have acted during the recent colonization of polluted areas. Authors also suggested that Zn tolerance in *A. halleri* is still submitted to selection. Moreover, by cultivating progenies in growth chambers, under uniform environmental conditions, Pauwels *et al.* (2006) showed that the most tolerant populations were those located on the most polluted soils, which might suggest that the strength of selection is related to the local degree of heavy metal contamination of the soil. However, these experiments have been performed with plants from very distant sites (several hundred kilometers), among which genetic exchange was unlikely. Therefore, it is difficult to precise if phenotypical differentiation observed between *A. halleri* populations have to be related to the geographical distances which strongly/or completely isolate the populations, or to the selection which generates processes for local adaptation. It is, therefore, essential to study gene flow between M and NM populations, that can physically exchange genes through pollen and/or seed flow, to verify above mentioned hypothesis. As distances between previously studied populations were too big, this kind of study was impossible.

The respective role of gene flow, drift and selection in shaping life history differences between ecotypes can be addressed on M and NM populations located in close proximity by contrasting patterns of variation for selected quantitative traits and neutral molecular markers in response to soil Zn concentration. It should be also stressed that the selective forces promoting genetic differentiation of traits likely to be involved in local adaptation can be efficiently analysed by reciprocal transplants and common garden experiments (Kawecki & Ebert 2004).

The occurrence of quantitative polymorphism for metal tolerance separating NM and M populations in response to natural selection has recently been sustained in *Thlaspi caerulescens* (Jiménez *et al.* 2007; Dechamps *et al.* 2007, 2008). Data from field observations, common garden experiment manipulating soil Zn concentration and light availability, and microsatellite molecular variation were contrasted for a set of neighbouring metallicolous and non–metallicolous populations (Jiménez *et al.* 2007). It was shown that M and NM plants differed in response to soil Zn concentration. A predominant role in shaping life history differences between ecotypes has had related to soil toxicity divergent selection.

Interestingly, despite geographical proximity gene flow between populations was weak and then local adaptation was opposed also weakly. However, authors suggested that to take into account the impact of all environmental components on plant fitness, and then to verify if populations between and within ecotypes are locally adapted to their own sites, the field reciprocal transplantations of M and NM populations are needed. Such experiment was recently performed (Dechamps *et al.* 2008). It was shown that the local adaptations of metallicolous and non–metallicolous ecotypes of *T. caerulescens* to their own environments are not equal. Selection found in the metalliferous environment was strong and specific, and then the colonization by a foreign genotype was there extremely difficult. Therefore, colonization of the non–metalliferous environment by the metallicolous ecotype was considered as more probable than the colonization of the metalliferous environment by the non–metallicolous ecotype. However, the metallicolous ecotype showed decreased fitness in the non–metalliferous environment, which may arguably represent a cost of adaptation to the metalliferous environment.

Aims of this work

The present study attempted to provide a better description and understanding of the origin and the evolutionary dynamics of Zn tolerance and accumulation ability in *A. halleri* populations from a single geographic region of southern Poland, and clear up the history and colonization patterns of this species in investigated area. The research on relationship between phenotypic and genetic structure in metallicolous and non–metallicolous populations was undertook. It was expected that in geographical low–scale study differences in discussed traits will be mirrored by genetic differentiation, because of supposed gene flow. The research was carried out on plant material from 15 *A. halleri* populations from southern Poland. As the area is highly differentiated regarding habitats, it was assumed that it will be the most appropriate and representative for this study. Here, in southern Poland, metalliferous and non–metalliferous sites occur within relatively short distances. This fact makes from this part of Europe the only known area where gene flow between two different edaphic types of *A. halleri* populations can be studied. In terms of research on adaptation to extreme environments and colonization of metalliferous sites southern Poland is a unique and particularly interesting area.

Research aimed to gain a better insight into process of plants adaptation to extreme environments. Experiments were performed on plant material collected directly from field as well as on plant material from seeds collected from fields and grown under controlled greenhouse conditions. Physic–chemical analyses of field soil and plant samples were performed in order to characterize *A. halleri* habitats and plants' behavior in southern Poland. Then, genetic analyses using neutral molecular markers (performed on nuclear and chloroplast DNA) aimed to resolve genetic structure and to reconstruct phylogeography of *A. halleri* populations from investigated area. It was expected that this research should allow to reconstruct migration routes and processes which enabled *A. halleri* to colonize metalliferous and non–metalliferous sites in southern Poland. Then, experiments in controlled conditions (tolerance and accumulation tests) aimed to check if relationships between tolerance and accumulation of heavy metals abilities exist in studied species. Both, genetic and phenotypic analyses were performed on the same populations from particular region of southern Poland. It was expected that the research will enable to characterize adaptive abilities of each studied population as well as to determine the level of phenotypic plasticity.

Opportunity to find genotypes suitable for experiments on genetically modified plants for phytoremediation was additional aspect of the research. It was expected that genetic study would allow to identify the most tolerant genotypes (particularly interesting in terms of phytostabilisation) as well as the most hyperaccumulating genotypes (useful in research on phytoextraction). Further detailed analysis of these selected genotypes could be useful in identification of genes (or genome regions) involved in tolerance and accumulation of heavy metals.

The thesis comprises of 5 main parts:

– introduction in the topic and state the problems (chapter I),
– studies on *Arabidopsis halleri* populations in the field – chemical analysis of plants and their habitats (chapter II),
– studies on neutral genetic diversity using molecular markers in examined populations (chapter III),
– studies on selected genetic diversity using variation in accumulation abilities in examined populations (chapter IV),

– studies on selected genetic diversity using variation in tolerance abilities in examined populations (chapter V).

The following questions were addressed:

1. Are metallicolous and non–metallicolous *Arabidopsis halleri* populations occurring in southern Poland differentiated by plants micro– and macro–elements, and heavy metal content?
2. Which was the migration route during colonisation of investigated area of southern Poland? Are non–metallicolous *A. halleri* populations from Tatra Mts. ancestral in investigated area?
3. Is there any detectable relationship between growth, accumulation and tolerance abilities in plants' of studied populations?
4. Is there genetic variation between *Arabidopsis halleri* individuals for zinc accumulation and/or tolerance?
5. Do analyses of variation for selected quantitative traits (tolerance and accumulation) and neutral molecular markers allow inferences about the putative role of gene flow and selection in shaping those differences?

II. STUDIES ON *ARABIDOPSIS HALLERI* POPULATIONS IN THE FIELD – CHEMICAL CHARACTERISTIC OF SOIL AND PLANTS

One of the basic aims of this study was to characterize the sites in southern Poland in which investigated species occurs and then to characterize the plants' behavior in relation to sites' conditions. On the other hand, this knowledge was also essential to infer, more precisely than in the hitherto studies, about adaptation to heavy metal contaminated sites. Indeed, data concerning accumulation abilities in *A. halleri* populations in the field, which were an objective of presented above chapter, were then compared to experimental observations of shoot zinc concentrations in the controlled conditions (see chapter IV of this study). Both, field and experimental observations, in terms of phenotypic variation, could contribute to the achievement of a better knowledge of adaptive value of zinc (Zn) accumulation and its phenotypic variation.

1. Materials and methods

1.1. Studied sites

As much as 15 sites with occurrence of *Arabidopsis halleri* were included to the present study. They were all located in southern Poland (Tab. 1; Fig. 7). Numbers of investigated sites (32, 33, 12, 13, 14, 22, 24, 27, 28, 29, 15, 17, 19, 30, 21) were given following the method of coding of the European populations of *A. halleri* used in GEPV laboratory in Lille.

Table 1. Studied sites in southern Poland.

Site number	Location	Geographic coordinates		Altitude
		N	E	a.s.l. (m)
32	Western Tatra Mts.	49°16'26.94"	19°52'41.76"	970
33	Western Tatra Mts.	49°17'32.52"	19°55'36.54"	876
12	Niepołomice Forest	50°06'24.36"	20°21'55.98"	226
13	Niepołomice Forest	50°06'35.64"	20°21'40.26"	206
14	Niepołomice Forest	50°06'31.80"	20°22'02.88"	188
22	Bukowno	50°16'58.08"	19°28'43.38"	339
24	Bolesław	50°17'00.18"	19°29'05.64"	334
27	Galman	50°11'36.78"	19°32'15.12"	447
28	Galman	50°11'54.12"	19°32'19.74"	471
29	Galman	50°11'54.06"	19°32'30.96"	496
15	Wełnowiec	50°17'12.96"	19°01'32.04"	302
17	Wełnowiec	50°16'57.12"	19°01'46.98"	297
19	Miasteczko Śląskie	50°30'12.84"	18°56'08.34"	308
30	Miasteczko Śląskie	50°30'10.03"	18°56'20.02"	325
21	Bibiela	50°29'45.66"	18°59'00.12"	300

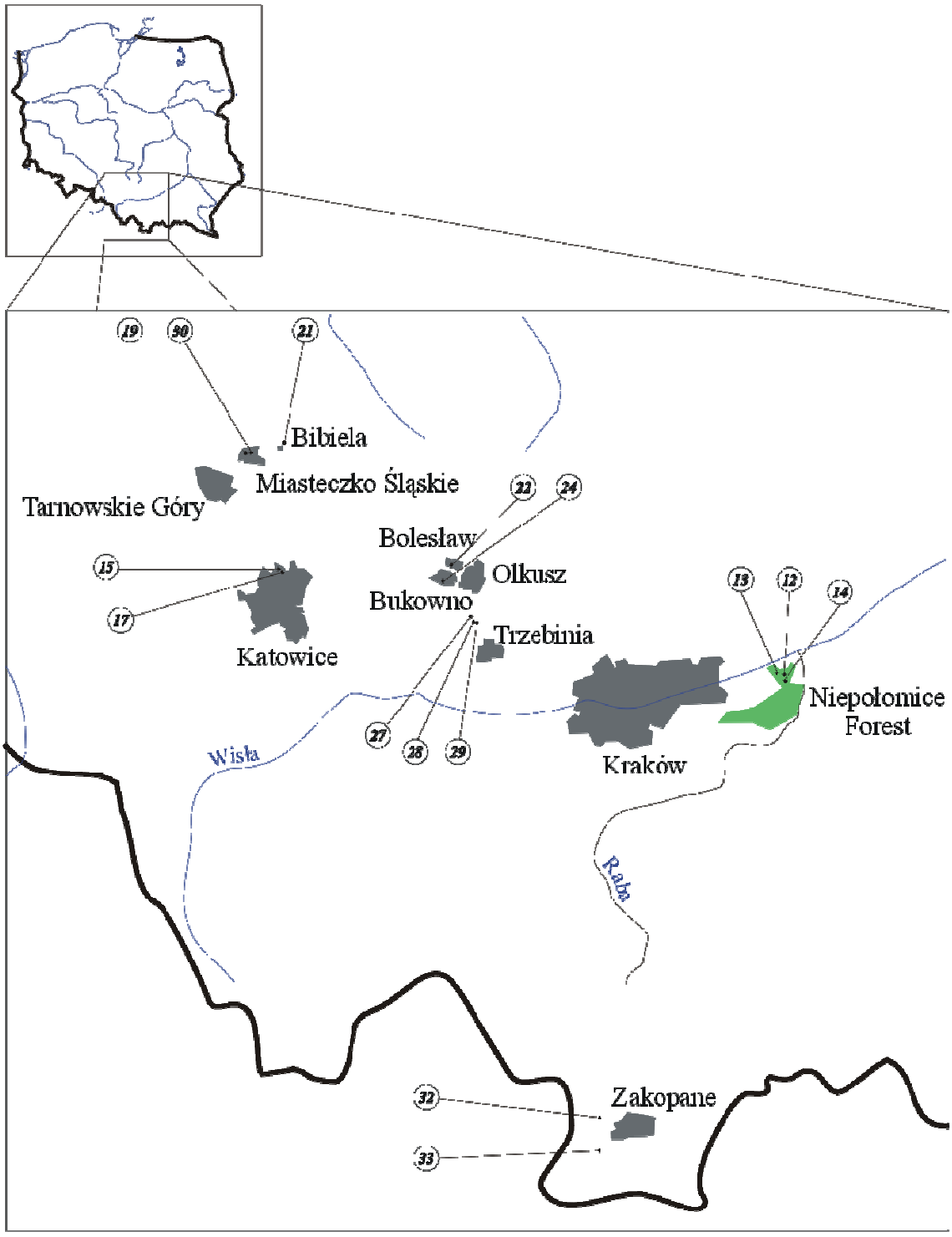


Fig. 7. Studied sites and their location in southern Poland.

Sites from non–industrial areas

Sites 32 and 33 (Fig. 8) are located in western part of Tatra Mts. in spruce (*Picea abies*) forest near "Droga pod Reglami" road and Kościeliska valley.

Fig. 8. Sites 32 and 33 in western part of Tatra Mts.

Sites 12, 13 and 14 (Fig. 9) are located in Niepołomice Forest about 20 km eastwards from Kraków between two rivers: Wisła and Raba. The area is as big as 10000 ha. All the investigated sites are located in northern part of Niepołomice Forest in "Grobla" range in oak–hornbeam forest.

Fig. 9. Sites 12, 13 and 14 in Niepołomice Forest.

Sites from industrial areas

Sites 22, 24, 27, 28 and 29 (Figs 10–12) are located near Olkusz – the area housing the richest zinc and lead ore deposits in Poland. According to historians, ore mining started here as early as in 13th century in vicinity of Bukowno and Bolesław (Molenda 1963). Initially ores were extracted by open–pit mining, then (following development in mining techniques) shaft mining became a predominant method

of extraction. Mining area is surrounded by different factories connected with metallurgical industry. Among them Bolesław Mine and Metallurgical Plant is the biggest. All the mining activities caused a huge damage to natural environment of this area. Here, in the nearest vicinity of ore deposits near Bukowno and Bolesław sites 22 and 24 are located. Site 22 (Fig. 10) is situated about 100 m from the metallurgical plant in the edge of young (about 20 years–old) anthropogenic forest. Site 24 (Fig. 11) is located about 500 m from the metallurgical plant on meadow. Both sites are under impact of dust contaminated with heavy metals and originating from the Bolesław Mine and Metallurgical Plant.

Fig. 10. Site 22 in Bolesław.

Fig. 11. Site 24 in Bukowno.

Sites 27, 28 and 29 (Fig. 12) are located between Olkusz and Trzebinia in "Galman" forest (about 70 years–old anthropogenic forest). In 19th century, mining was carried out here in open–pit mine "Katarzyna". Mining activities ceased

in 1912. Even nowadays sings of mining can be seen everywhere around. Within the area of 8 km^2 more than 400 pits can be found. Each pit is surrounded by small waste heap. This pattern of pits and surrounding heaps is called in Polish "warpie".

Fig. 12. Sites 27, 28 and 29 in „Galman" forest.

Sites 15 and 17 (Figs 13, 14) are located in Wełnowiec – a quarter of Katowice. Here a large waste heap from zinc smelter started to exist in the first half of 19th century. The heap has never been recultivated and all the plant colonizing this place can be considered as spontaneous flora. Site 15 (Fig. 13) is located at the top of the heap in dry and sunny place. A year ago, this site was destroyed as the most of the deposits from waste heap was used as a material in road construction activities. Site 17 (Fig. 14) is located in lower part of the heap, in partly shadowed and moist place.

Fig. 13. Site 15 in Wełnowiec.

Fig. 14. Site 17 in Wełnowiec.

Sites 19 and 30 (Figs 15, 16) are located in Miasteczko Śląskie, 30 km north westwards to Katowice in Tarnowskie Góry region. The area is clearly one of the most polluted in whole Silesia (Siebielec *et al.* 2004). Since middle ages, the area was a centre of zinc and lead mining. Miasteczko Śląskie foundry is the biggest industrial plant in this region. The foundry was founded in 1966. Site number 19 (Fig. 15) is located just 50 m from the foundry fence. The place is inhospitable to living organisms as here pollution with a dust containing heavy metals and originating from the foundry is the highest. The area around the foundry was being recultivated by planting shrubs and trees. As pollution in the nearest neighborhood of the foundry is extremely high only several plants was able to survive. Conditions good enough for growth and development of plants occur at least 300 m from chimneys. Here, about 500 m from Miasteczko Śląskie foundry, site 30 is located (Fig. 16).

Fig. 15. Site 19 in Miasteczko Śląskie.

Fig. 16. Site 30 in Miasteczko Śląskie.

Site 21 (Fig. 17) is located in the edge of 70 years–old forest, in Bibiela village, about 7 km from Miasteczko Śląskie foundry. As distance from foundry is relatively small, this site is probably under the influence of industrial emissions of Miasteczko Śląskie. In 1880's huge deposits of iron and silver ores were discovered in close vicinity of Bibiela village and between 1889 and 1917 an ore mine was working here (Nowak 1927). After a catastrophic flood, which destroyed the mine in 1917, mining has never been started again.

Fig. 17. Site 21 in Bibiela.

1.2. Soil and plant sampling

Soil and plants were sampled from 15 sites (see Tab. 1) during flowering period of *Arabidopsis halleri* in May 2005. In each site a 60 m long transect was set out. The transect was sampled each 12 m. From each site, 5 individuals

of *A. halleri* plants (5 samples of roots and 5 samples of above–ground parts) were obtained. Rhizospheric soil (5 samples per site) was sampled using cylinder of the diameter of 7 cm to a depth of 10 cm.

In order to precisely characterize the studied sites a lot of physic–chemical soil parameters have been investigated. Firstly, the soil pH was determined as a parameter which influence on metals' and nutrients' biodisponibility. Then, the micro– and macroelements as well as heavy metal content were appointed. Both, nutrient and heavy metal content might be limiting factors for plants, and so are essential to know in study of the plants' adaptation to growth in metalliferous soils. At last, to verify if populations from different sites differ in nutrient demands and their behavior, the same elements which determined in soil, have been appointed in plant material.

1.3. Analysis of soil samples

Samples were dried in a room temperature, passed through a 2 mm sieve and ground with an agate mortar and pestle. Then material was dried at 70^0C in order to keep the weight of the sample stable.

Soil pH was measured in distilled water by means of potentiometric method using pH–meter Mettler–Toledo MP 125. Samples of air–dried soil (2.5 g) were mixed with 10 ml of deionised water and left for 24 h. pH was determined in solution from above sediment at 22^0C using standard solutions with known pH of 7.2, 4.01 and 9.21 (Wąchalewski 1999).

1 g samples of ground soil were mixed with perchloric acid $HClO_4$ (10 ml) and left for 24 h. Then each sample was mineralized during 48 h at temperature gradually increasing up to 270^0C. After mineralization, sample volume was set at 100 ml with deionised water. In this solution total concentration of zinc (Zn), lead (Pb), cadmium (Cd), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg) and iron (Fe) was determined using atomic absorption spectrometry (AAS, Varian 220FS).

0.5 g samples of ground soil was mixed with sulphuric acid (H_2SO_4) (12 ml) and copper sulphate ($CuSO_4$) (2 tablets Kjeltabs). Mineralization was carried out in 440^0C during 1 h. In so–prepared samples nitrogen (N) concentration was determined following the Kjeldahl's method (Kjeltec 2003 analyzer).

Phosphorus (P) concentration was determined by vanadium–molibdenium method. 0.05 g samples of ground soil were dissolved in 100 ml of 1 % solution of hydrochloric acid (HCl). 10 ml of each sample was mixed with water (30 ml) and vanadiummolibdenium mixture (10 ml). After 10 min, absorbance of the tested sample was measured at 415 nm against a sample of reference.

0.1 g samples of ground soil were burnt in over at 1000^0C. Then sulphur (S) concentration was determined using coulometric method (CS30 analyzer).

Organic carbon (C_O) concentration was determined by Turin's method. Samples of 0.1 – 0.5 g of sieved soil were mixed with 10 ml of potassium dichromate ($K_2Cr_2O_7$) and boiled during 5 min with mercury sulphate ($HgSO_4$) as a catalyst. Excess of $K_2Cr_2O_7$ was titrated with Mohr's salt ($FeSO_4 \cdot (NH_4)_2 \cdot (SO_4)_2 \cdot 6H_2O$).

Available forms of zinc (Zn_A), lead (Pb_A) and cadmium (Cd_A) were determined by vortexing soil solution with EDTA (Escarre *et al.* 2000). Soil samples (5 g from metallicolous sites and 20 g samples from non–metallicolous sites) were mixed with buffer solution (0.5 M CH_3COONH_4 + 0.5 M CH_3COOH + 0.02 M EDTA) in volume of 50 and 100 ml respectively. Each sample was vortexed during 30 min. The solution was then filtered (Schleider & Schull filter paper no. 595 ½) and metal concentration was determined by atomic absorption spectrometry (AAS, Varian 220FS).

1.4. Analysis of plant samples

Samples of plant material were divided in two parts: roots and above–ground part and subsequently washed with deionised water. Roots were additionally washed in ultrasound bath. Each sample was dried at room temperature and then at 70^0C in order to keep the weight of the sample stable. 0.2 g samples of ground tissue were mixed with 5 ml of nitric acid (HNO_3) and perchloric acid ($HClO_4$) (both acids were mixed in proportion 4:1 respectively) and left for 24 h. Then each sample was mineralized during 48 h at temperature gradually increasing up to 270^0C. Mineralized samples were dissolved up to volume of 100 ml with deionised water. In each sample total concentration of Zn, Pb and Cd was determined by atomic absorption spectrometry (AAS, Varian 220 FS). Furthermore, in shoots total concentration of calcium (Ca), magnesium (Mg) and iron (Fe) was determined following the same method.

0.5 g samples of ground tissue were mixed with 12 ml of sulphuric acid (H_2SO_4) and two Kjeltabs tablets ($CuSO_4$) and mineralized during 1 h in 440^0C. In so–prepared samples N concentration was determined following the Kjeldahl's method (Kjeltec 2003).

Phosphorus concentration was determined by vanadium–molybdenum method. 0.2 g samples of ground tissue were dissolved in 100 ml of 1% solution of hydrochloric acid (HCl). 10 ml of each sample was mixed with vanadiummolybdenum mixture (10 ml) and water (30 ml). After 10 min, absorbance of the tested sample was measured at 415 nm against a sample of reference.

0.1 g samples of ground soil were burnt in oven at 1000^0C. Then sulphur (S) concentration was determined using coulometric method (CS30 analyzer).

1.5. Data analysis

Abilities of *A. halleri* to accumulate Zn and Cd was determined on the basis of S/R parameter. It is a ratio of the metal concentration in shoots to the concentration in roots. The parameter is commonly used in studies on metal accumulation in plants. It is accepted that plants with the ratio above 1 are hyperaccumulators (Krämer *et al.* 1996; Lasat *et al.* 1996; Shen *et al.* 1997; Lasat *et al.* 1998; Schat *et al.* 2000). Bioaccumulation factor (BF; Baker *et al.* 1994) was also determined for all tested plants. It is a ratio of the metal concentration in shoots to the total concentration of the metal in soil.

1.6. Statistical analyses

Assessment of soil and plant characteristics: nonparametric tests and PCA analyses

The differences of total and extractable concentrations of metals in soil between sites were assessed. Because of small sample sizes nonparametric Kruskal–Wallis test ($p < 0.05$) have been performed using Statistica 8.0 integrated package.

To reduce the number of variables explaining the variance between sites and populations, and to appoint combination of parameters explaining differentiation between tested sites, a Pearson correlation matrix and PCA (Principal Component Analysis) analyses were computed for all tested parameters and all investigated sites as well as populations. Graphic presentations of results were achieved in order to verify distribution and separation of samples originated from different soil types. In case of soil analysis, first component PC 1 clearly separated metal–contaminated

and non–contaminated soils and then the parameters correlated with PC 1 (with factor loadings higher than 0.50) were considered as resolving to the sites separation. Then, on the basis of such chosen parameters two successive PCA analyses have been performed on M and NM types of soil independently. This allowed to thoroughly characterize the properties of both (M and NM) types of soil. Analyses were carried out using Statistica 8.0 integrated package.

For parameters selected by first PCA analysis, the coefficient of variation (CV) was computed. This statistics was used to compare standard deviations of investigated elements between contaminated and non–contaminated sites. In this analysis, data from different types of sites were treated independently.

Comparison of different data sets: linear regressions

The comparison of mean metal concentrations in above–ground plant parts to that in their roots was achieved for each population, by linear regression.

Then, the influence of total as well as extractable metal content in soil on metal concentration in above–ground plants' parts was achieved for each site and their occurred population. Analysis was performed in order to verify the shape of the curve of metal plant/soil relation.

Similar analysis, based on bioaccumulation factor (BF) and total metal concentration in soil, was performed in order to compare the accumulation abilities of populations in investigated sites. The BF, defined as a ratio of Zn concentration in shoots to soil Zn concentration measured in the rhizospheric soil (in this study: the total Zn concentration in soil), is indeed usually used in order to estimate quantitative expression of accumulation (Deram *et al.* 2006).

All regressions were carried out using Statistica 8.0 integrated package.

2. Results

2.1. Physic–chemical analysis of soils – characterization of sites

Soil pH and heavy metal content

Values of pH, concentrations (total and available) of Zn, Pb and Cd in samples from 15 investigated sites as well as percent share of available forms of metals in their total content, were presented in Table 2. Soil pH varied from 5.33 to 6.95. The lowest pH value was measured in soil samples from Niepołomice Forest (sites 13, 14) while the highest in Bukowno (site 22) and Bolesław (site 24).

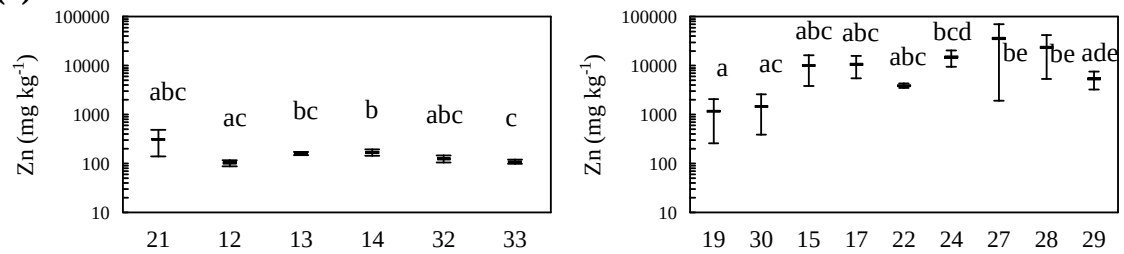
Table 2. Soil pH, total and available concentrations ($mg \cdot kg^{-1}$ d.wt) of metals (Zn, Pb, Cd) ($\overline{X} \pm SD$) and the percent (%) of available forms of metals in their total content (Avail / Tot) in soil from studied sites. A – available form; M – metalliferous sites; NM – non–metalliferous sites – see chapter II 3.1).

Site	Type	pH	Total			Extractable			Avail / Tot		
			Zn	Pb	Cd	Zn_A	Pb_A	Cd_A	Zn	Pb	Cd
32	NM	6.04 ± 0.13	125 ± 19	49 ± 16	1.4 ± 0.5	6 ± 2	5 ± 1	0.9 ± 0.1	5	10	64
33	NM	5.89 ± 0.08	109 ± 8	56 ± 16	0.8 ± 0.1	6 ± 1	5 ± 1	0.6 ± 0.1	6	9	75
12	NM	6.12 ± 0.57	103 ± 12	9 ± 2	0.2 ± 0.1	8 ± 2	6 ± 2	0.2 ± 0.0	8	67	100
13	NM	5.43 ± 0.17	160 ± 10	25 ± 2	0.5 ± 0.1	18 ± 2	11 ± 1	0.4 ± 0.1	11	44	80
14	NM	5.33 ± 0.17	169 ± 20	28 ± 3	0.5 ± 0.2	18 ± 4	11 ± 2	0.4 ± 0.2	11	39	80
22	M	6.95 ± 0.33	3911 ± 340	1045 ± 115	27.7 ± 3.1	103 ± 13	67 ± 5	16.9 ± 2.2	3	6	61
24	M	6.71 ± 0.11	14964 ± 4395	3315 ± 840	91.0 ± 27.0	348 ± 203	123 ± 53	51.9 ± 33.3	2	4	57
27	M	6.26 ± 0.37	35942 ± 27393	10367 ± 3956	182.2 ± 112.0	3631 ± 824	3446 ± 761	49.3 ± 5.8	10	33	27
28	M	6.09 ± 0.34	23664 ± 14762	3323 ± 1935	158.0 ± 103.0	3974 ± 1555	1617 ± 673	74.7 ± 32.5	17	49	47
29	M	5.67 ± 0.37	5390 ± 1743	1804 ± 955	51.9 ± 18.6	749 ± 496	668 ± 388	36.2 ± 11.9	14	37	70
15	M	6.25 ± 0.13	10163 ± 5085	3109 ± 1667	68.7 ± 44.5	311 ± 200	504 ± 331	39.1 ± 21.3	3	16	57
17	M	6.46 ± 0.09	10642 ± 4146	6396 ± 1553	37.9 ± 13.4	184 ± 65	4613 ± 747	21.0 ± 5.5	2	72	55
19	M	6.22 ± 0.43	1167 ± 732	925 ± 574	20.4 ± 10.7	47 ± 32	84 ± 53	15.8 ± 9.1	4	9	77
30	M	6.04 ± 0.13	1481 ± 882	1398 ± 1088	21.6 ± 10.6	47 ± 23	97 ± 72	15.0 ± 6.1	3	7	69
21	NM	5.76 ± 0.39	327 ± 139	219 ± 99	5.1 ± 2.3	7 ± 3	6 ± 1	1.2 ± 0.4	2	3	24

Total concentrations of heavy metals varied broadly for Zn from 103 to 35942 mg·kg^{-1} d.wt, for Pb from 9 to 10367 mg·kg^{-1} d.wt, and for Cd from 0.2 to 182.2 mg·kg^{-1} d.wt. The lowest concentrations were measured in Niepołomice Forest (sites 12, 13, 14) and in Tatra Mts. (sites 32, 33), while the highest concentrations of heavy metals were found in soil from Galman (sites 27, 28, 29). In Bibiela (site 21) intermediate concentrations of investigated metals were found: several times higher than in soils from Niepołomice Forest and Tatra, but much lower than in samples from Miasteczko Śląskie, Wełnowiec, Bukowno, Bolesław and Galman. In soils from industrial areas average concentrations of Zn was 70 times higher and concentration of Pb was 50 times higher than in non–contaminated soils. Content of available forms of heavy metals varied broadly from 6 to 3974 mg·kg^{-1} d.wt in case of Zn, from 5 to 4613 mg·kg^{-1} d.wt in case of Pb and from 0.2 to 74.7 mg·kg^{-1} d.wt in case of Cd. In soil samples from Tatra, Bibiela and Niepołomice Forest (sites 32, 33, 21, 12, 13, 14) the lowest concentrations of available forms were found, while the highest values were determined for Galman (sites 27, 28). Percent of available forms of Zn varied from 2 to 17 %. Zinc was the least available in Wełnowiec (site 17), Bibiela (site 21) and Bolesław (site 24) while being the most available in Galman (site 28). Available forms of Pb comprised from 3 to 72 % of total Pb content. In Bibiela (site 21) and Bolesław (site 24) Pb was the least available, while in Wełnowiec (site 17) was the most available. In case of Cd, percent share of available forms varied from 24 to 100. In soil samples from Bibiela (site 21) Cd was the least available while in samples from Niepołomice Forest (sites 12, 13, 14) the most available.

Analysis carried out on data from non–contaminated and contaminated sites revealed that this two major groups of sites differed in term of homogeneity / heterogeneity of heavy metal content in soil samples concerning each site. In general, variability within sites from non–contaminated areas was much less than those within contaminated sites (Fig. 18).

(a)



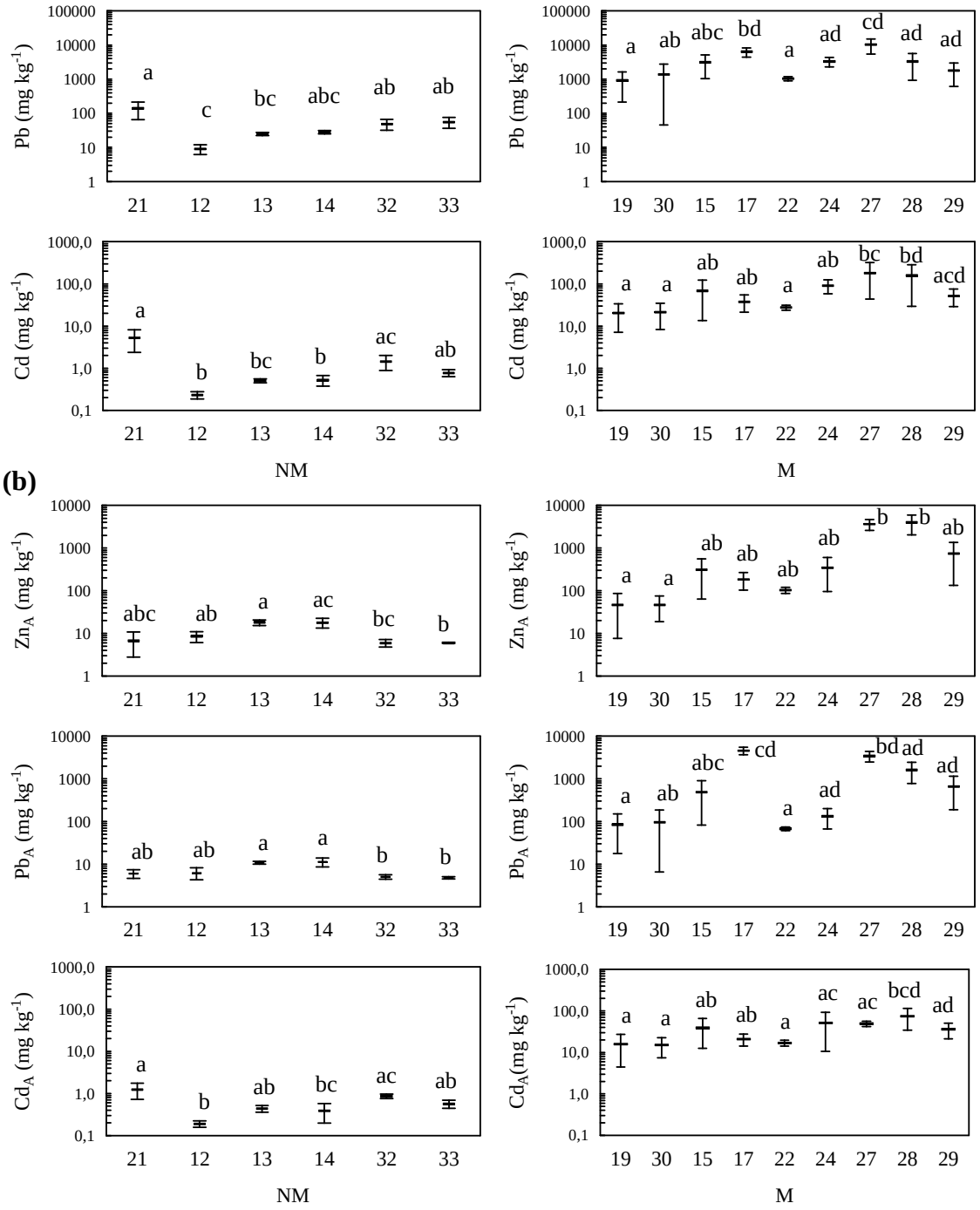


Fig. 18. **(a)** Total concentrations (mg·kg^{-1} d.wt) of metals (Zn, Pb, Cd) in soil from investigated sites (X ± SD). **(b)** Available concentrations (mg·kg^{-1} d.wt) of metals (Zn, Pb, Cd) in soil from investigated sites (X ± SD). Different letters indicate a significant difference at 5 % level (Kruskal–Wallis test). M – metalliferous sites; NM – non–metalliferous sites – see chapter II 3.1).

Macroelements content

In Table 3 concentration of organic forms of C as well as total concentrations of N, K, P, S, Ca, Mg and Fe in soil samples were presented.

Table 3. Total concentrations (% d.wt) of macroelements in soil from investigated sites ($\overline{X} \pm$ SD). M – metalliferous sites; NM – non–metalliferous sites – see chapter II 3.1); O – organic form.

Site	Type	C_O	N	P	K	S	Ca	Mg	Fe
32	NM	2.92 ± 0.29	0.37 ± 0.09	0.09 ± 0.01	0.65 ± 0.04	0.032 ± 0.005	0.66 ± 0.28	0.75 ± 0.10	2.72 ± 0.39
33	NM	2.50 ± 0.25	0.36 ± 0.04	0.12 ± 0.01	0.60 ± 0.01	0.027 ± 0.005	0.24 ± 0.04	0.65 ± 0.03	2.28 ± 0.07
12	NM	1.70 ± 0.47	0.18 ± 0.02	0.04 ± 0.01	0.41 ± 0.13	0.013 ± 0.003	0.22 ± 0.10	0.43 ± 0.13	1.07 ± 0.27
13	NM	2.03 ± 0.24	0.27 ± 0.01	0.06 ± 0.01	0.78 ± 0.09	0.012 ± 0.002	0.27 ± 0.08	0.68 ± 0.09	1.52 ± 0.14
14	NM	3.00 ± 0.69	0.30 ± 0.02	0.07 ± 0.01	0.88 ± 0.12	0.028 ± 0.003	0.57 ± 0.34	0.79 ± 0.14	1.70 ± 0.17
22	M	3.22 ± 1.34	0.19 ± 0.06	0.08 ± 0.01	0.16 ± 0.08	0.042 ± 0.025	0.82 ± 0.22	0.54 ± 0.16	1.22 ± 0.10
24	M	8.14 ± 3.16	0.41 ± 0.21	0.17 ± 0.03	0.16 ± 0.02	0.082 ± 0.035	4.35 ± 0.69	1.92 ± 0.46	2.76 ± 0.71
27	M	18.20 ± 7.28	0.68 ± 0.26	0.13 ± 0.01	0.12 ± 0.03	0.042 ± 0.014	6.07 ± 2.14	3.59 ± 1.53	1.93 ± 0.36
28	M	20.01 ± 8.80	0.75 ± 0.33	0.13 ± 0.04	0.14 ± 0.04	0.049 ± 0.028	5.44 ± 2.92	3.06 ± 1.79	1.42 ± 0.29
29	M	26.73 ± 6.87	0.86 ± 0.06	0.13 ± 0.06	0.18 ± 0.04	0.104 ± 0.045	4.53 ± 2.39	2.73 ± 1.83	1.59 ± 0.19
15	M	9.63 ± 5.34	0.32 ± 0.10	0.06 ± 0.02	0.30 ± 0.10	0.070 ± 0.052	0.48 ± 0.27	0.29 ± 0.13	1.99 ± 0.82
17	M	11.50 ± 2.62	0.39 ± 0.10	0.08 ± 0.01	0.37 ± 0.21	0.074 ± 0.012	0.70 ± 0.24	0.54 ± 0.14	2.10 ± 0.40
19	M	1.73 ± 0.86	0.14 ± 0.07	0.05 ± 0.01	0.04 ± 0.04	0.021 ± 0.009	0.10 ± 0.03	0.07 ± 0.01	0.35 ± 0.06
30	M	1.74 ± 0.49	0.08 ± 0.03	0.03 ± 0.01	0.05 ± 0.01	0.021 ± 0.011	0.29 ± 0.17	0.08 ± 0.03	0.29 ± 0.08
21	NM	2.74 ± 0.57	0.14 ± 0.02	0.06 ± 0.02	0.03 ± 0.01	0.084 ± 0.025	0.30 ± 0.23	0.06 ± 0.03	0.26 ± 0.04

Content of C_O varied from 1.70 to 26.73 % d.wt. The lowest concentration was found in Niepołomice Forest (site 12) and Miasteczko Śląskie (sites 19, 30) while the highest in samples from Galman (27, 28, 29). Total content of N varied from 0.08 to 0.86 % d.wt. The lowest contents of N were found in samples from Miasteczko Śląskie (sites 19, 30) and Bibiela (site 21), the highest in soil from Galman (sites 27, 28, 29). Total content of P varied from 0.03 to 0.17 % d.wt. The lowest concentration was found in samples from Miasteczko Śląskie (site 30) and Niepołomice Forest (site 12), while the highest in soil from Bolesław (site 24).

Total content of K varied from 0.03 to 0.88 % d.wt with the lowest values found in soil samples from Bibiela (site 21) and Miasteczko Śląskie (sites 19, 30) and the highest values in soil from Niepołomice Forest (sites 13, 14) and Tatra (sites 32, 33). For S content range was from 0.012 to 0.104 % d.wt. The lowest values were found in Niepołomice Forest (sites 12, 13) while the highest in samples from Galman (site 29). Total content of Ca varied from 0.10 to 6.07 % d.wt while content of Mg from 0.07 to 3.59 % d.wt. The lowest concentrations of both elements were found in Miasteczko Śląskie (site 19) while the highest in samples from Galman (sites 27, 28, 29) as well as from Bolesław (site 24). Total content of Fe varied from 0.26 to 2.76 % d.wt. The poorest in Fe was soil from Bibiela (site 21) and Miasteczko Śląskie (sites 19, 30) while the richest were samples from Bolesław (site 24) and Tatra Mts. (sites 32, 33).

Soil classification – contaminated and non–contaminated type of soil

Presented above chemical analyses shown that sites differ significantly in heavy metal content and then indicate the occurrence of 2 main subgroups of investigated sites: heavy metal contaminated and uncontaminated. In order to test if the subgroups differ also in term of all determined physic–chemical parameters and then they possess different properties, a principal component analysis (PCA) was performed on a data matrix covering information about all investigated factors (pH, C_O, N, P, K, S, Ca, Mg, Fe, Zn, Pb, Cd, Zn_A, Pb_A, Cd_A) in soil samples from all scrutinized sites (Tab. 4). The two first principal components (PC) explained 77.07 % of the total variance (56.59 % and 20.48 % explained by PC 1 and PC 2, respectively; Fig. 19). The first principal component separates sites towards to pH, C_O, K, S, Ca, Zn, Pb, Cd, Zn_A, Pb_A, Cd_A. The heavy metals (total as well as available) content is the major source of variation along this axis. The PC 2 was related to the C_O, N, P, K, Mg, Fe and Zn.

Table 4. PCA loadings of all investigated physic–chemical soils' parameters. The analysis was carried out for all 15 studied sites. PC 1, PC 2 – two first Principal Components; O – organic form; A – available form. Factor loadings higher than 0.50 were given in bold.

Parameter	PC 1	PC 2
pH	**0.53**	–0.22
C_O	**0.71**	**0.57**
N	0.33	**0.82**
P	0.38	**0.76**
K	**–0.63**	**0.59**
S	**0.59**	0.36
Ca	**0.60**	0.67
Mg	0.45	**0.70**
Fe	0.05	**0.85**
Zn	**0.96**	**0.12**
Pb	**0.97**	0.00
Cd	**0.98**	–0.04
Zn_A	**0.91**	0.20
Pb_A	**0.89**	0.18
Cd_A	**0.97**	0.04
Explained variation (%)	56.59	20.48

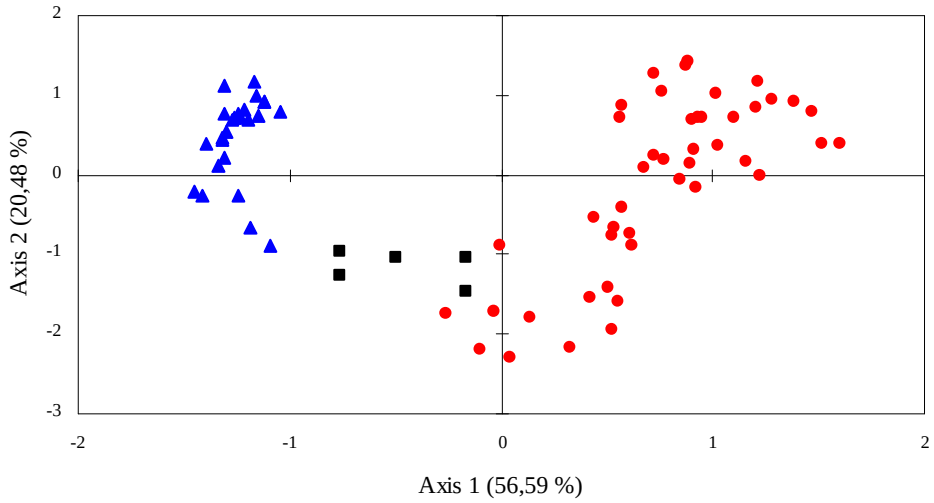


Fig. 19. Principal component analysis of all investigated sites and all investigated elements in soils. Axes 1 and 2 represent 56.59 % and 20.48 % of total variance, respectively. Blue triangles represent samples from non–industrial sites (12, 13, 14, 32, 33), red circles – samples from industrial sites (19, 30, 21, 15, 17, 22, 24, 27, 28, 29), black squares – samples from site 21 (industrial area of Silesia region).

Figure 19 shows that the separation between metal–contaminated and non–contaminated soils can be clearly seen in the two–dimensional space delimited by PC 1 and PC 2.

However, the second component is not resolving to the sites separation. And then, all the soil samples were divided into two main groups across the PC 1. First one, represented by blue triangles, contained samples from non–contaminated sites (12, 13, 14, 32, 33), second one, represented by red circles, contained samples from contaminated sites (19, 30, 21, 15, 17, 22, 24, 27, 28, 29). Largest differences in reaction norms explained by PC 1 were found between contaminated sites. In particular, some M individuals displayed quite low negative values on PC 1 and a majority of positive values on this axis, whereas NM individuals showed high negative values. Some samples, represented by black squares, displayed quite low negative values on PC 1 and then showed intermediate characteristics. These intermediate properties were found in site 21, from industrial area of Silesia region.

Because of not resolving properties of the second component of presented above PCA analysis, the parameters in which PC 2 was related could be then considered as inutile in present study of sites separation. Therefore, to characterize two identified type of sites (from contaminated and non–contaminated areas) two other PCA analyses were performed on the parameters to which the PC 1 was related (*i.e.* pH, C_O, K, S, Ca, Zn, Pb, Cd, Zn_A, Pb_A, Cd_A). It should be also stressed, that data from different type of sites (contaminated *vs* non–contaminated) were analysed independently. Because of intermediate properties of site 21 (Bibiela), these samples were excluded from the present analysis.

In Table 5 factor loadings and total variance explained by each principal component were given.

Table 5. PCA loadings of chosen investigated physic–chemical soils' parameters. The analysis was carried out on contaminated and non–contaminated sites separately. PC 1, PC 2 – two first Principal Components; O – organic form; A – available form. Factor loadings higher than 0.50 were given in bold.

	Non–contaminated sites		**Contaminated sites**	
	PC 1	PC 2	PC 1	PC 2
pH	-0.01	**-0.85**	0.00	**-0.96**
C_O	**0.85**	0.25	**0.82**	0.47
K	0.46	**0.78**	**0.54**	-0.19
S	**0.82**	-0.15	**0.70**	0.18
Ca	**0.74**	0.29	**0.84**	0.00
Zn	0.22	**0.92**	**0.91**	-0.31
Pb	**0.86**	0.00	**0.86**	-0.17
Cd	**0.91**	-0.18	**0.91**	-0.10
Zn_A	-0.31	**0.90**	**0.91**	0.15
Pb_A	-0.28	**0.91**	**0.77**	0.16
Cd_A	**0.88**	-0.15	**0.86**	0.01
Explained variation (%)	42.98	36.76	61.09	12.69

During data analysis for non–contaminated sites it was found that first principal component explained 42.98 % and second 36.76 % of total variance. The following parameters were highly correlated with first component: C_O, S, Ca, Pb, Cd, Cd_A, while pH, K, Zn, Zn_A and Pb_A were highly correlated with second component. In case of sites from metal–contaminated areas, the two first principal components explained 61.09 % and 12.69 % of total variance, respectively. The following parameters were highly correlated with the first PC 1: C_O, K, S, Ca, Zn, Pb, Cd, Zn_A, Pb_A, Cd_A. Soil pH was highly correlated with second principal component.

For both data sets (contaminated and non–contaminated), the coefficient of variation (CV) was also computed. This statistic was used to compare standard deviations of investigated elements (C_O, N, P, K, S, Ca, Mg, Fe) between contaminated and non–contaminated sites. Data from different type of sites were analysed independently. Because of intermediate properties of site 21 (Bibiela), samples from the site were excluded from analysis. Highest values of CV were obtained between samples from contaminated sites (average CV = 0.78) than between samples from non–contaminated areas (average CV = 0.32) (Tab. 6).

Table 6. Coefficient of variation computed from distributions of investigated soil data from contaminated and non–contaminated areas. O – organic form.

Investigated elements	**Coefficient of variation**	
	samples from contaminated sites	samples from non–contaminated sites
C_O	0.84	0.03
N	0.71	0.27
P	0.52	0.39
K	0.76	0.28
S	0.67	0.41
Ca	1.10	0.66
Mg	1.10	0.24
Fe	0.56	0.34
Average	0.78	0.32

2.2. Chemical analysis of plants: content of investigated elements in *A. halleri* field samples

Metal content in shoots and roots

Total content of Zn, Pb and Cd in shoots as well as in roots is presented in Table 7. These concentrations varied broadly between investigated sites.

It was found that total content of Zn varied from 3449 to 21752 $mg \cdot kg^{-1}$ d.wt in shoots and from 1199 to 13340 $mg \cdot kg^{-1}$ d.wt in roots. Concentration of Pb in shoots varied from 0.2 to 583.0 $mg \cdot kg^{-1}$ d.wt and from 0.3 to 995.7 $mg \cdot kg^{-1}$ d.wt in roots. In green parts total concentration of Cd varied from 31 to 493, while in roots from 20 to 277 $mg \cdot kg^{-1}$ d.wt. The highest concentration of Zn and Pb were found in plants from Miasteczko Śląskie (sites 19, 30), while the highest concentration of Cd was found in samples from Galman (site 28) and Miasteczko Śląskie (sites 19, 30). The lowest concentrations of both above mentioned elements were found in plants from Tatra Mts. (sites 32, 33).

Table 7. Total content ($mg \cdot kg^{-1}$ d.wt) of metals (Zn. Pb. Cd) in shoots and roots of *A. halleri* field samples ($\bar{X} \pm$ SD). M – metallicolous populations; NM – non–metallicolous populations; NMp – non–metallicolous populations in industrial area – see chapter II 3.2).

Site	Type	Shoot content			Root content		
		Zn	Pb	Cd	Zn	Pb	Cd
32	NM	4247 ± 1048	0.2 ± 0.1	55 ± 19	2386 ± 523	0.6 ± 0.4	20 ± 8
33	NM	3449 ± 65	0.2 ± 0.1	31 ± 5	1199 ± 66	0.3 ± 0.1	20 ± 1
12	NM	8383 ± 1660	0.6 ± 0.3	53 ± 25	3917 ± 1976	43.1 ± 20.2	64 ± 36
13	NM	9724 ± 1733	0.5 ± 0.2	88 ± 28	2863 ± 1304	16.7 ± 14.7	49 ± 14
14	NM	14341 ± 4707	0.3 ± 0.2	123 ± 65	4731 ± 1571	10.2 ± 4.1	42 ± 17
22	M	13720 ± 2887	35.1 ± 20.5	321 ± 65	5924 ± 1799	74.8 ± 75.8	197 ± 24
24	M	10494 ± 1818	50.4 ± 19.8	186 ± 59	3930 ± 873	191.3 ± 48.5	118 ± 65
27	M	6751 ± 3999	33.5 ± 12.1	121 ± 61	3669 ± 1306	184.4 ± 124.5	84 ± 31
28	M	14773 ± 5109	47.1 ± 34.1	493 ± 361	5222 ± 2093	636.0 ± 357.5	252 ± 184
29	M	10078 ± 693	14.1 ± 8.1	118 ± 71	5551 ± 1226	209.7 ± 128.8	79 ± 57
15	M	11810 ± 2933	20.0 ± 6.0	116 ± 78	6780 ± 2142	342.8 ± 217.4	96 ± 70
17	M	13964 ± 3204	22.9 ± 10.2	67 ± 21	7396 ± 5076	311.7 ± 300.1	52 ± 17
19	M	21752 ± 5605	540.0 ± 385.0	486 ± 111	13340 ± 2783	985.4 ± 661.8	272 ± 55
30	M	19372 ± 7225	583.0 ± 359.0	421 ± 61	12581 ± 1773	995.7 ± 346.9	277 ± 59
21	NMp	13193 ± 4475	70.0 ± 46.0	152 ± 39	7329 ± 2608	59.3 ± 19.6	90 ± 39

In Figure 20 linear dependencies between metal (Zn, Pb, Cd) concentrations in shoots and roots in *A. halleri* plants were presented. In case of Zn the dependency is described by equation $y = 0.61x–1427$. High R^2 value ($R^2 = 0.82$; $p < 0.001$) indicates that this function fits closely to experimental data set. For Cd dependency is given by equation $y = 0.55x+10.34$ ($R^2 = 0.95$; $p < 0.001$). In case of Pb no linear correlation was found even after removal of extreme values from experimental data set ($R^2 = 0.36$).

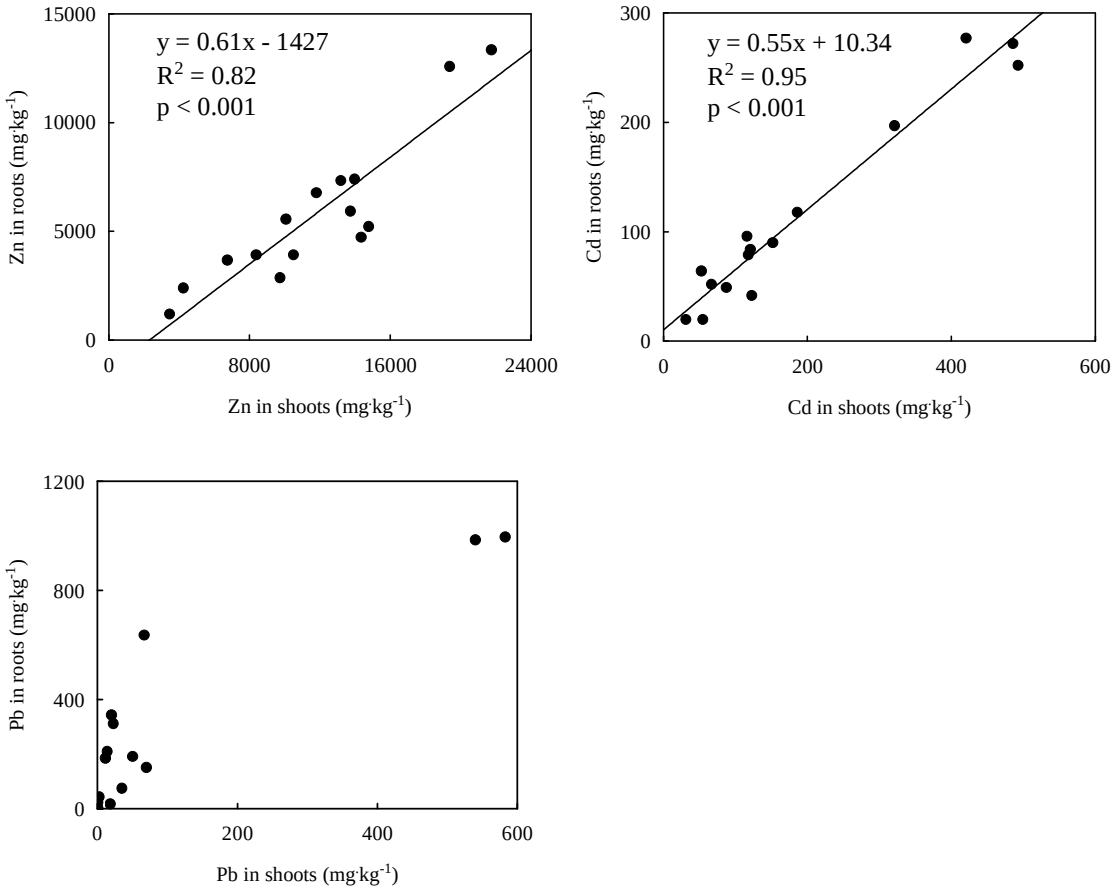


Fig. 20. Regression lines presenting relationship between concentrations of Zn, Pb and Cd in shoots and roots of *A. halleri* plant samples from investigated sites.

Macroelements content in shoots

Concentrations of other tested elements (N, P, S, Ca, Mg, Fe) in shoots of plants from 15 investigated sites were presented in Table 8. These concentrations did not varied broadly. Total content of N varied from 2.20 to 3.95 % d.wt. The lowest N content showed plants from Niepołomice Forest (site 12) and Bukowno (site 22), while the highest showed plants from Galman (site 29). Concentration of P varied from 0.20 to 0.62 % d.wt with the lowest values for samples from Wełnowiec (sites 15, 17) and the highest in samples from Tatra Mts. (site 33). Total content of S varied from 0.19 to 0.61 % d.wt having the lowest values in samples from Bukowno (site 22) and the highest values in samples from Galman (site 29). Content of Ca varied from 1.05 to 4.09 % d.wt. The lowest content of this element was found in plants from Galman (site 27) while the highest in samples from Bukowno (site 22). The lowest concentration of Mg was found in samples from Wełnowiec (site

17) while the highest in samples from Bukowno (site 22). Total content of Fe in plants varied from 0.01 to 0.23 % d.wt having the lowest values in plants from Wełnowiec (sites 15, 17) as well as from Galman (sites 27, 28) while in plants from Niepołomice Forest (sites 12, 13, 14) having the highest values.

Table 8. Total content (% d.wt) of macroelements in shoots of *A. halleri* field samples ($\bar{X} \pm$ SD). M – metallicolous populations; NM – non–metallicolous populations ; NMp – non–metallicolous populations in industrial area – see chapter II 3.2).

Site	Type	N	P	S	Ca	Mg	Fe
32	NM	3.11 ±0.09	0.35 ±0.04	0.30 ±0.02	1.64± 0.27	0.44 ± 0.10	0.04 ± 0.03
33	NM	3.47 ±0.22	0.62 ±0.06	0.30 ±0.02	2.20± 0.05	0.62 ± 0.03	0.03 ± 0.01
12	NM	2.39 ±0.36	0.32 ±0.09	0.48 ±0.06	1.56± 0.47	0.43 ± 0.08	0.21 ± 0.18
13	NM	2.69 ±0.09	0.31 ±0.06	0.52 ±0.09	1.29± 0.14	0.47 ± 0.09	0.23 ± 0.12
14	NM	2.20 ±0.51	0.37 ±0.11	0.42 ±0.05	1.57± 0.38	0.50 ± 0.04	0.17 ± 0.14
22	M	2.22 ±0.15	0.44 ±0.06	0.19 ±0.01	4.09± 1.29	0.95 ± 0.51	0.03 ± 0.02
24	M	2.54 ±0.23	0.26 ±0.07	0.21 ±0.02	2.35± 0.22	0.52 ± 0.11	0.02 ± 0.01
27	M	3.76 ±0.13	0.30 ±0.13	0.41 ±0.02	1.05± 0.38	0.49 ± 0.13	0.01 ± 0.01
28	M	3.46 ±0.40	0.30 ±0.05	0.34 ±0.08	1.61± 0.19	0.81 ± 0.32	0.02 ± 0.01
29	M	3.95 ±0.42	0.47 ±0.07	0.61 ±0.02	1.89± 0.57	0.64 ± 0.07	0.03 ± 0.01
15	M	3.50 ±0.18	0.23 ±0.08	0.46 ±0.04	1.86± 0.63	0.53 ± 0.17	0.02 ± 0.01
17	M	2.99 ±0.16	0.20 ±0.11	0.36 ±0.05	1.62± 0.19	0.36 ± 0.07	0.01 ± 0.01
19	M	3.65 ±0.32	0.45 ±0.05	0.32 ±0.03	2.64± 1.28	0.65 ± 0.22	0.07 ± 0.05
30	M	3.64 ±0.30	0.44 ±0.03	0.30 ±0.01	2.10± 0.69	0.69 ± 0.10	0.08 ± 0.01
21	NMp	3.55 ±0.60	0.29 ±0.10	0.23 ±0.02	2.13± 1.13	0.89 ± 0.27	0.04 ± 0.03

2.3. Heavy metal accumulation by *A. halleri* populations in the field and relationship with physic–chemical properties of soils

In Figure 21 dependencies between metal (Zn, Pb, Cd) concentrations in soils and *A. halleri* shoots were presented. For each investigated metal, the trends of relations were similar for both total and available soils' metal content. In case of Zn, in non–contaminated sites plants accumulated Zn at much diversified level (from relatively low to extremely high), while in contaminated sites plants accumulated less and at more homogenous level. For Pb, independently on soil metal content most plants accumulated low amount of them, however some plants accumulated Pb at relatively high level when grown in non–contaminated sites. In case of Cd the trends in relationships were similar to those described for Zn.

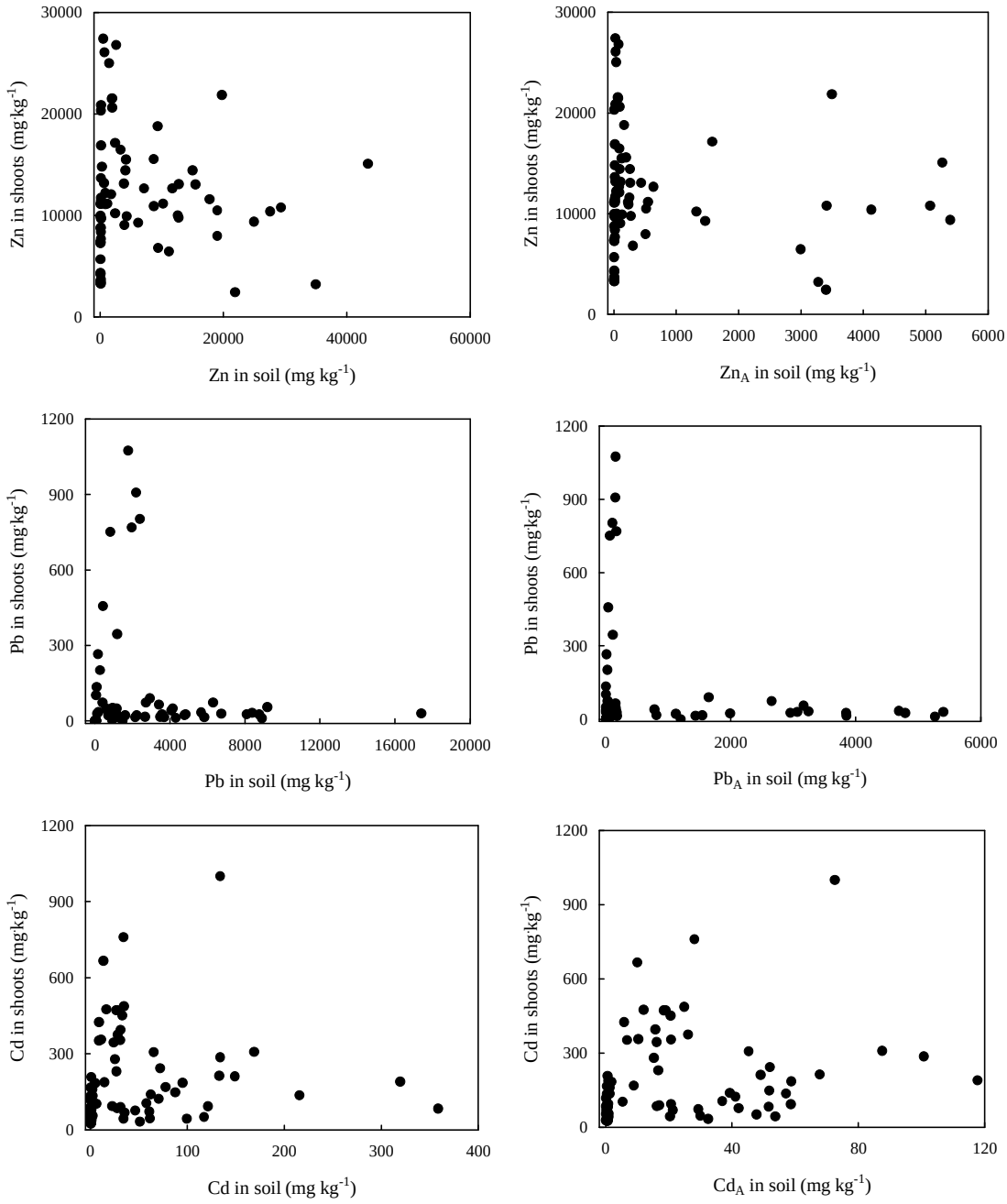


Fig. 21. Relationship between total (Zn, Pb, Cd) and available (Zn_A, Pb_A, Cd_A) content of metal in soil and their total content in *A. halleri* shoots.

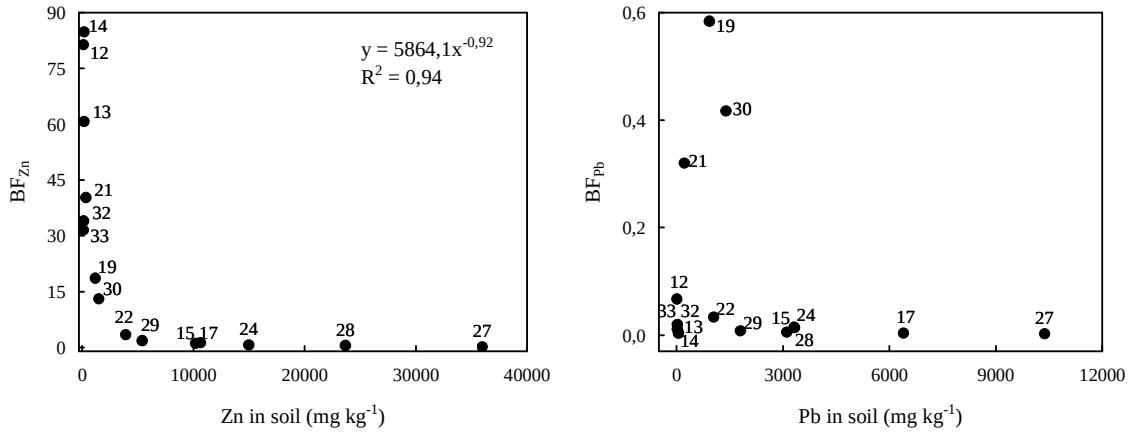
A ratio of Zn, Pb and Cd content in shoots to their content in roots (S/R) as well as a ratio of metal concentration in shoots to concentration in soil (bioaccumulation factor – BF) were presented in Table 9. Both Zn and Cd presented S/R ratio above one in all tested populations (with the exception of population 12). For lead S/R ratio was lower than 1 in all tested populations. In case of Pb also BF stood constantly below 1. For Zn and Cd BF values were diverse and in majority of cases higher than 1. The lowest BF values were found in population from Galman

(site 27: BF_{Zn} = 0.2; BF_{Cd} = 0.7), the highest in populations from Niepołomice Forest (site 12: BF_{Zn} = 81.4; BF_{Cd} = 265.0; site 14: BF_{Zn} = 84.9; BF_{Cd} = 249.0).

Table 9. A ratio of metal (Zn, Pb, Cd) content in *A. halleri* shoots to their content in roots (S/R) and ratio of metal concentration in shoots to total concentration in soil (bioaccumulation factor – BF) (X ± SD). M – metallicolous populations; NM – non–metallicolous populations ; NMp – non–metallicolous populations in industrial area – see chapter II 3.2).

Site	Type	S/R			BF		
		Zn	Pb	Cd	Zn	Pb	Cd
32	NM	1.8 ± 0.8	0.33 ± 0.25	2.8 ± 0.6	34.0 ± 10.7	0.004 ± 0.001	39.3 ± 18.0
33	NM	2.9 ± 0.2	0.67 ± 0.32	1.6 ± 0.4	31.6 ± 8.1	0.004 ± 0.002	38.8 ± 5.0
12	NM	2.1 ± 1.4	0.01 ± 0.01	0.9 ± 0.2	81.4 ± 29.4	0.067 ± 0.015	265.0 ± 104.3
13	NM	3.4 ± 1.5	0.03 ± 0.01	1.8 ± 0.2	60.8 ± 17.3	0.020 ± 0.010	176.0 ± 80.0
14	NM	3.0 ± 0.7	0.01 ± 0.00	2.9 ± 2.0	84.9 ± 10.9	0.011 ± 0.007	246.0 ± 75.2
22	M	2.3 ± 0.5	0.47 ± 0.27	1.6 ± 0.4	3.5 ± 0.9	0.034 ± 0.016	11.6 ± 9.3
24	M	2.7 ± 0.3	0.26 ± 0.11	1.6 ± 0.6	0.7 ± 0.4	0.015 ± 0.002	2.0 ± 0.6
27	M	1.8 ± 1.0	0.18 ± 0.10	1.4 ± 0.8	0.2 ± 0.1	0.003 ± 0.003	0.7 ± 0.5
28	M	2.8 ± 0.8	0.07 ± 0.04	2.0 ± 0.5	0.6 ± 0.3	0.014 ± 0.009	3.1 ± 2.5
29	M	1.8 ± 0.6	0.07 ± 0.06	1.5 ± 0.8	1.9 ± 0.4	0.008 ± 0.002	2.3 ± 1.8
15	M	1.7 ± 0.9	0.06 ± 0.03	1.2 ± 0.5	1.2 ± 0.6	0.006 ± 0.004	1.7 ± 1.1
17	M	1.9 ± 1.3	0.07 ± 0.03	1.3 ± 0.2	1.3 ± 0.8	0.004 ± 0.001	1.8 ± 0.7
19	M	1.6 ± 0.7	0.55 ± 0.28	1.8 ± 0.4	18.6 ± 7.7	0.584 ± 0.400	23.8 ± 10.4
30	M	1.5 ± 0.4	0.59 ± 0.30	1.5 ± 0.1	13.1 ± 8.2	0.417 ± 0.330	19.5 ± 5.8
21	NMp	1.8 ± 1.2	0.46 ± 0.30	1.7 ± 1.1	40.3 ± 32.2	0.320 ± 0.120	29.8 ± 17.0

Total content of Zn and Cd in soil and BF values were inversely proportional with R^2 values 0.94 and 0.87, respectively (Fig. 22). There was no relationship between soil content and BF value for Pb (R^2 = 0.01). Having in mind BF_{Zn} and BF_{Cd} values investigated populations were ordered according to decreasing ability in Zn uptake: 27 < 24 < 17 < 28 < 15 < 22 = 29 < 30 < 19 < 33 < 32 < 13 < 21 < 14 = 12. Similarly, investigated populations were ordered for Cd: 27 < 24 = 17 < 29 < 15 < 28 < 22 < 30 < 19 < 33 < 32 < 21 < 13 < 12 < 14.



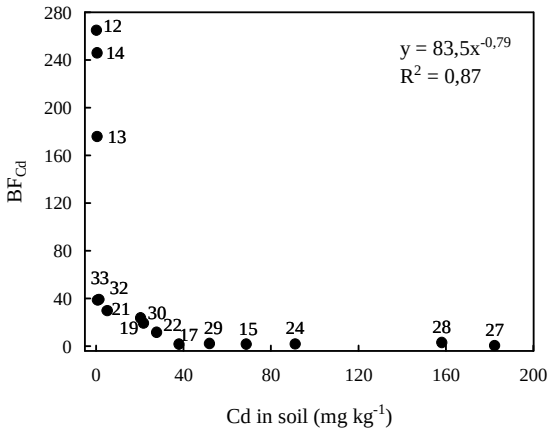


Fig. 22. Relationship between total content of metal (Zn, Pb, Cd) in soil and bioaccumulation factor **(**BF) value. The numbers of populations are shown next to the symbols.

Population classification and relationship with physic–chemical analysis of soils

In order to test if investigated populations from industrial areas differed from those from non–contaminated areas, a principal component analysis (PCA) was performed on a data matrix covering information about all investigated elements (N, P, S, Ca, Mg, Fe, Zn_S, Pb_S, Cd_S, Zn_R, Pb_R, Cd_R, S/R_{Zn}, S/R_{Cd}, BF_{Zn}, BF_{Cd}) in soil samples from all scrutinized sites (Tab. 10). All parameters were highly correlated with two principal components (PC 1, PC 2). Figure 23 shows that populations were differentiated by the axes 1 and 2, which represented 34.58 % and 15.25 % of total variance respectively. All the soil samples were grouped into two main groups. First one, represented by blue triangles, contained samples from non–contaminated sites (12, 13, 14, 32, 33), second one, represented by red circles, contained samples from industrial sites (19, 30, 21, 15, 17, 22, 24, 27, 28, 29). Samples from population 21 (Bibiela), represented by black squares, were classified within the second group.

Table 10. Results of principal component analysis (PCA). The analysis was carried out for *A. halleri* plants from non–contaminated and contaminated sites separately. S – metal content in shoots; R – metal content in roots; S/R – ratio of metal content in shoots to roots; BF – bioaccumulation factor (ratio of metal concentration in shoots to total concentration in soil). Factor loadings higher than 0.50 were given in bold.

	PC 1	**PC 2**
N	0.11	**–0.65**
P	0.04	0.12
S	–0.37	0.08
Ca	0.39	0.05
Mg	**0.53**	–0.05
Fe	–0.01	**0.74**
Zn_S	**0.84**	0.26
Pb_S	**0.85**	–0.37
Cd_S	**0.91**	0.03
Zn_R	**0.82**	–0.07
Pb_R	**0.76**	–0.32
Cd_R	**0.90**	–0.11
S/R_{Zn}	–0.24	0.38
S/R_{Cd}	–0.22	0.27
BF_{Zn}	–0.21	**0.80**
BF_{Cd}	–0.23	**0.84**
Explained variation (%)	34.58	15.25

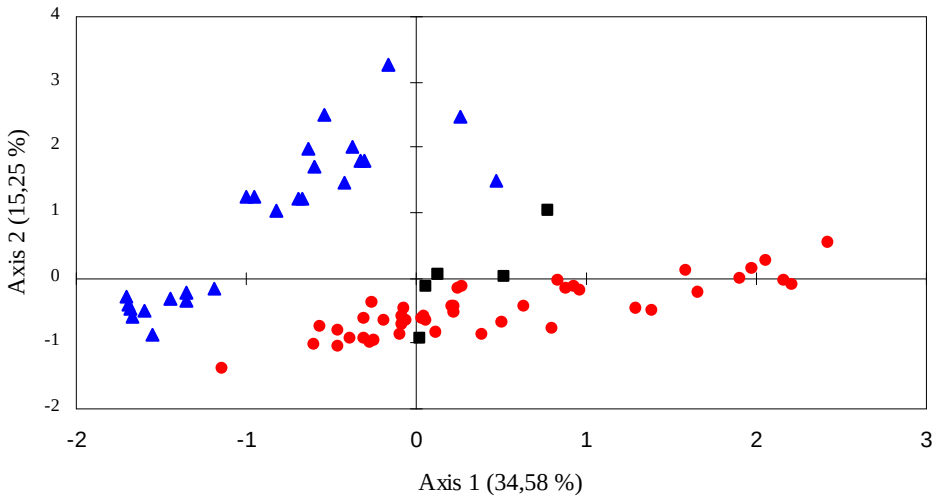


Fig. 23. Principal component analysis carried out on data from all investigated *A. halleri* populations. Axes 1 and 2 represent 34.58 % and 15.25 % of total variance respectively. Blue triangles represent samples from non–contaminated sites (12, 13, 14, 32, 33), red circles – samples from industrial sites (19, 30, 21, 15, 17, 22, 24, 27, 28, 29), black squares – samples from site 21 (industrial area of Silesia region).

3. Discussion

During extensive research on *Arabidopsis halleri* in Europe (study was carried out on plant material from Austria, Belgium, Croatia, Germany, Poland, Romania, Slovakia and Italy) all of NM populations were scarred in the mountain ranges, at middle to high altitudes (mean 702 m) whereas all of M populations were sampled outside the mountain ranges, at low to moderate altitudes, in heavy metal contaminated areas (Pauwels *et al.* 2006). The majority of hitherto investigated European populations of *A. halleri* consist in M populations (Pollard *et al.* 2002). There are two main metalliferous (calamine) areas in Europe with occurrence of *A. halleri*: Harz Mts. in Germany and upland regions of southern Poland (Berton 1946, Ernst 1990). In upland regions of southern Poland, heaps containing post–mining wastes are common due to mining of Zn–Pb ores. There are also numerous places with abandoned arable grounds. Among characteristic features of the area low content of nutrient in soil as well as high content of heavy metals are the most important from the point of view of the present study (Godzik 1985, 1993; Grodzińska *et al.* 2000; Szarek–Łukaszewska & Niklińska 2002). On these contaminated areas occurs a vegetation composed of metal tolerant species – for example *A. halleri*. Interestingly, in upland regions of southern Poland *A. halleri* occurs not only in sites with high content of heavy metals but also where heavy metals are absent and sometime in close vicinity of mining areas. The aim of this study was to characterize properties of these closely related sites as well as the behaviors of the *A. halleri* plants and populations occurring on these sites.

3.1. Classification of metalliferous and non–metalliferous sites with *A. halleri* in southern Poland

Sites characteristic on the base of heavy metals' content

Results presented above have proved that heavy metal content in soil samples from investigated sites varies broadly, and allow to divided investigated sites into two distinct groups: heavy metal contaminated and non–contaminated. Previous research carried out on unpolluted natural areas of Poland, described values of heavy metal content which are considered as typical to Polish soils (Kabata–Pendias 1995). According to this data, natural content in superficial soil layer varies between

50 and 100 mg·kg^{-1} in case of Zn, 20 to 60 mg·kg^{-1} of Pb and 0.3 and 1.0 mg·kg^{-1} of Cd (Kabata–Pendias 1995, Greinert & Greinert 1999). In present study, content of Zn in samples from Tatra Mts. and Niepołomice Forest was slightly higher than in natural non–metalliferous soils, while in case of Pb these samples were included within the range of normal content. Samples from Bibiela have shown concentrations of both, Zn and Pb about 3 times higher than in natural non–contaminated soils concentration, while in samples from the rest of investigated sites both concentration was considerably higher. In case of Cd content, samples from Niepołomice Forest and Tatra Mts. (excluding site 32) showed more or less the same low pattern, samples from Bibiela contained 5 times higher amounts than upper limit of the natural content range whereas in remaining samples even several hundred times higher. All sites which presented content of Zn, Pb as well as Cd considerably higher than those found by Kabata–Pendias (1995) in typical natural soil samples from Poland, were then considered as metallicolous while sites for which those concentrations were included within the range of normal content, as non–metalliferous. Therefore, sites from Tatra Mts. (sites 32, 33) and Niepołomice Forest (sites 12, 13, 14) were classified as non–metalliferous (NM), whereas sites from Bukowno (site 22), Bolesław (site 24), Galman (sites 27, 28, 29), Wełnowiec (sites 15, 17), Miasteczko Śląskie (sites 19, 30) were classified as metalliferous (M). Bibiela (site 21) is a site located within industrial area, with intermediate concentrations of investigated metals were found (relatively low when compared with other metalliferous sites from southern Poland). This site was classified together with non–metalliferous because the comparison between Bibiela and other M and NM sites in southern Poland showed that in terms of available metals contents and physic–chemical properties of soil (*i.e.* pH, micro– and macro–elements' content) Bibiela site is more similar to other NM sites.

Heavy metal concentrations in samples from investigated M and NM sites in present study are also comparable with concentrations obtained by other scientists during their research on M and/or NM sites in Europe (Brown 2001; Bert *et al.* 2002; Pauwels 2007). In these studies, sites have been classified as M and NM in reference to the French agricultural approved NFU 44 041 norm. According to this norm soil will be considered as a contaminated one if it contains more than 300 mg·kg^{-1} of Zn or 100 mg·kg^{-1} of Pb or 2 mg·kg^{-1} of Cd (Bert *et al.* 2002).

Similarity of this study as well as presented above results' have proved that included to the present study sites, have similar amounts of heavy metals to other previously investigated European sites in which occurred *A. halleri* and then classification of investigated sites as M and NM was supported by both Polish as well as European norms.

Different sources of heavy metals in studied sites

Furthermore, results have shown that in southern Poland *A. halleri* is able to grow in mountain areas, on soils with low metal content, as well as in lowlands on heavy–metal–contaminated soils. It should be stressed, that all investigated sites represent very heterogeneous group. Interestingly, in NM sites from Tatra Mts. and Niepołomice Forest the Zn content was found as slightly higher than in natural non–metalliferous soils. In case of Tatra Mts. this may be consequence of natural geological soil and rock properties, while in Niepołomice Forest of industrial pollution from closely located Kraków. On the other hand, also high amounts of metals in investigated metalliferous soils originated from different sources and were respectively a consequence of mining, a fall of contaminated industrial dust as well as storage of waste material from smelting. In particular, sites in Bolesław, Bukowno and Galman (sites 22, 24, 27, 28, 29) were located in post–mining area. However they differ in age of mining activity. In Galman mining was carried out in 19th century and was ceased in 1912, while in vicinity of Bolesław and Bukowno mining started as early as in 13th century and was continue till 90. of 20th century. These two last sites (sites 22 and 24) occurred additionally in close vicinity of Zn smelter pollution, and then Zn contamination proceeded from soil and atmospheric origin. Dust contaminated with heavy metals can fall over land surface and interact with soil solution and thus making metals highly available for plants (Szarek–Łukaszewska & Niklińska 2002). This pattern of pollution is a predominant one also in case of sites located in vicinity of zinc smelter in Miasteczko Śląskie (sites 19, 30). Interesting research on Zn, Pb and Cd pollution of superficial layers of soil were carried out in proximity of Zn smelter Miasteczko Śląskie (Karweta 1988). This smelter was build in 1966, and Karweta's (1988) study was conducted during first 15 years of their activity. During this period of time heavy metal content in soil from place 900 m far from the smelter increased from 17 to 1650 $mg \cdot kg^{-1}$ d.wt in case of Zn, from 39 to 1250 $mg \cdot kg^{-1}$ d.wt in case of Pb

and from 0.1 to 11.0 $mg \cdot kg^{-1}$ d.wt in case of Cd. Karweta (1988) noticed also a tendency to pH increase from 3.3 to 5.7. Although pollution from Miasteczko Śląskie zinc smelter was significantly reduced by 1990's, soils in close vicinity remain heavily polluted. This site is particular because of recent and specific source of contamination of Zn smelter and any natural soil enrichment in heavy metals. The last one type of M site of different kind, investigated in this study, was situated in an area of heavy metal smelter waste heap in Wełnowiec (sites 15 and 17). In case of Wełnowiec, heavy metals are there really abundant (Jędrzejczyk *et al.* 2003) and this location seems to be very heterogeneous in term of heavy metal content.

Then, it has been proved that *A. halleri* habitats in a single geographic region of southern Poland are very heterogeneous group in case of heavy metal content, and the distance between M and NM sites is relatively small. This heterogeneity as well as proximity of M and NM sites is very important in case of present study, because in such heterogeneous environments the local adaptation in *A. halleri* populations could evolve. This region is thus particular and model to study of plant adaptation. In the next chapters of this study (III & IV) it will be verify if heavy metal content might lead to strong divergent selection on *A. halleri* in investigated sites.

Sites characteristic on the base of pH and nutrients' content

In terms of soil pH investigated soil samples presented pH values optimal for development of flora and soil fauna. The optimal pH values for vast majority of plants lays between weakly acidic and weakly alkaline (Uggla 1971). Soil pH influences its' biological activity. In weakly acidic soils bioavailability of nutrient elements is increased (Kabata–Pendias 2004). With low soil pH values bioavailability of heavy metals is also increased and therefore in metalliferous soils with low pH heavy metal ions can have toxic effect on plants. Herms and Brummer (1984) have pointed out that pH play a key role in heavy metals mobility and their uptake by plants. According to Kabata–Pendias and Pendias (1979) the mobility of Zn in acidic soils can be even ten times higher than in soils of pH > 6.4. Boekhold and van der Zee (1992) have found that pH is the most important factor influencing Cd mobility in soil. Christensen (1984) have showed that decrease in pH by 2 units had caused increase in Cd solubility by 75 %. As Zn and Cd only weakly interact with soil colloids their bioavailability is therefore high (Alloway & Ayres 1998). In case of Pb strong interaction with organic matter

significantly decreases its availability for plants (Alloway & Ayres 1998). Values of available forms of heavy metals in investigated soils differed broadly between authors (Karweta 1988, Godzik 1993, Szarek–Łukaszewska & Niklińska 2002). It can be due to the fact that soil in post–mining areas can be highly differentiated in terms of physic–chemical properties as well as due to different methods employed by above mentioned authors. However, determined in this study soil pH did not vary broadly, and then probably did not strongly affect on heavy metals biodisponibility in different sites.

A ratio of carbon to nitrogen content (C/N) can serve as a criterion useful in assessing the degree of soil degradation (Siuta 1995). In case of mineral soils C/N ratio varies between from 8 to 10 in natural, non polluted soils, from 10 to 17 in low–degraded soils, from 17 to 30 in moderate–degraded soils and from 30 to 45 in highly degraded soils (Siuta 1995). Having these values in mind soil from investigated NM sites can be perceived as non–degraded (with exception of site 21) whereas all the M sites contain more or less highly degraded soils. This division is congruent with two groups of investigated sites: M and NM.

Interestingly, separation of sites into NM and M was also clear when, apart the heavy metals, other physic–chemical soils' parameters have been taking into account. However, it was shown that not all determined parameters had influence on sites assembling but only pH, C_O, K, S, Ca, Zn, Pb, Cd, Zn_A, Pb_A, Cd_A (except N, P, Mg, Fe). Then, successive analyses enabled to learn more about properties of soil in NM and M sites. In particular, on data obtained from NM sites, parameters represent organic matter (C_O, S, Pb, Cd, Cd_A) were highly positively correlated. Small amounts of heavy metals found in soil samples from NM sites come probably from deposition of airborne dust pollutants. Moreover, in non–contaminated soils organic matter is a main source of S (Johnson 1984) and the quantity of S in soil increase with increase in organic matter content (Terelak *et al.* 1998). Maynard *et al.* (1985) and Haynes and Williams (1992) have found a similar dependency between S and organic matter during their research and mineralization rate of sulphur organic compounds in arable and abandoned fields. The ratio of carbon and sulphur content is also an important parameter in characterization of soil properties. According to numerous publications when ratio C/S < 200 mineralization prevails; whereas, when C/S > 400, immobilization of sulphur organic compounds prevails over mineralization (Freney & Stevenson 1966; Ghami *et al.* 1992).

In all analysed samples C/S ratio showed that mineralization of sulphur organic compounds prevails over immobilization. In case of NM sites the correlation between total and available forms of heavy metals in soil was found only for Cd and Zn. The fact that total and available forms of Pb are not correlated is also confirmed by Bert *et al.* (2002), for soils with trace concentration of heavy metals. Solubility as well as mobility of heavy metals in soil depends on different factors and among others on their total content. The lower the content of metal is, the lower impact on physic–chemical properties of soil it has (Elston *et al.* 1976). Soil pH as well as available forms of Zn and Pb were negatively correlated, which suggest that availability of this metals in NM sites depends on pH value.

As far as analysis carried out for M sites heavy metals as well as nutrient elements were highly positively correlated. It might be due to origins of elements in soil. In investigated soils heavy metals as well as S come from remains of metal ores in mining wastes or from dust emitted by Pb and Zn smelters. Soils developing on mining waste material are rich in calcium and magnesium as Zn and Pb ores are abundant in Tertiary, Jurassic and Triassic rocks rich in dolomite and calcite. Sulphides of Zn and Pb are also among main components of ores (Rose *et al.* 1979). Sphalerite and galena being Zn and Pb sulphides are main ores. Moreover Ca is also frequently found in both Zn and Pb ores. For that reason contamination with Zn is usually accompanied with pollution with Pb and Cd. According to Cabała and Sutkowska (2006) in Zn– and–Pb–rich mining wastes Zn and Pb carbonates and different iron oxides are predominant compounds. They have also found that Cd is often present in aggregates of carbonates and sulphides of Pb. In contaminated soils ZnS, PbS and $CuFeS_2$ undergo the process of oxidation and ions of Zn, Pb, Cu and Fe are released into soil solution. The fact that Ca and Cd were highly correlated may be also explained by a tendency to co–sedimentation with other minerals presented by heavy metals. This phenomenon is an important mechanism of heavy metal adsorption in soils of unstable humidity. Cadmium shows tendency to co–sedimentation *e.g.* with calcium carbonates (Sposito 1983). The phenomenon of co–sedimentation of metals and carbonates (mainly CaCO3) is particularly important in semi–dry soils developed on calcareous substrate. Organic matter is a source of essential nutrients as well as binds heavy metals and S. Potassium and S are the components which represent mineral fraction in soil contaminated with mining waste material and therefore were correlated with heavy metals. Potassium

alum is a frequent mineral in places where rocks containing sulphides of Zn and Pb undergo the process of weathering. The mineral is common especially in southern Poland (Cabała & Sutkowska 2006).

General properties of NM and M sites in which A. halleri occurs

It has been proved that there is a clear difference between M and NM sites in southern Poland, in which *A. halleri* occur. It should be stressed that these sites are separate by different level of lot of physic–chemical soils' parameters (*i.e.* pH, $C_{O,}$ K, S, Ca) and not only heavy metals. It was shown that M sites from southern Poland from very heterogeneous group in terms of both heavy metal content as well as the content of micro– and macroelements, while NM sites were characterized by considerable homogeneity of physic–chemical properties.

3.2. Classification of metallicolous and non–metallicolous populations of *A. halleri* and their relationship with physic–chemical properties of soils

Status of metallicolous, non–metallicolous and "non–metallicolous in a polluted region" populations in studied area of southern Poland

On metalliferous soils grow populations known as metallicolous, while populations growing on non–metalliferous soils are called non–metallicolous (Pauwels *et al.* 2008). Detailed chemical analyses of plant material from investigated sites enabled to establish classification of different *A. halleri* populations occurring in metalliferous and non–metalliferous sites in southern Poland. In this study, populations of *A. halleri* from NM sites differed from those from heavy metal contaminated areas, therefore classification of population's type could be supported by both, soils' as well as plants' data set. In particular, populations from Tatra Mts. (32, 33) and Niepołomice Forest (12, 13, 14) were classified together and considered as NM, on the contrary populations from Bukowno (22), Bolesław (24), Galman (27, 28, 29), Wełnowiec (15, 17) and Miasteczko Śląskie (19, 30) were classified as M. In terms of metal content, site and population in Bibiela (21) seems to be particularly interesting. This site is located in industrial area of Silesia region, but on the basis of soil analysis the site was described as slightly polluted and considered to be NM. Nevertheless, a detailed study of heavy metals and macroelements concentration in *A. halleri* plants from this site, revealed chemical

characteristic similar to other tested M populations. As it has been already noticed by Pauwels *et al.* (2006), in industrial areas, "non–M" populations may differed from true NM ones by their abilities to exchange genes with geographically proximate M populations. On the other hand, during colonization of low polluted habitats selection pressure towards enhanced tolerance probably was strongly reduced (such as in NM populations) (Bert *et al.* 2002; Pauwels *et al.* 2005). For this reason, population from Bibiela (21) was considered to be in particular category and qualified as NMp (for "non–metallicolous in a polluted region"). Category of NMp population was firstly proposed by Pauwels *et al.* (2006).

Summing up, in the relatively small region of southern Poland, three edaphic types of *A. halleri* populations occurred: NM, M and NMp. All of them were studied in this work.

Heavy metal accumulation and hyperaccumulation by A. halleri populations in the field

Arabidopsis halleri as hyperaccumulator sensu Brooks (1998)
Zinc

Regardless the type, all scrutinized populations showed high accumulation of Zn. In shoots of NM as well as M sampled plants' the values of Zn exceed greatly (30–140 and 6–20 times more, respectively) typical values for non–accumulating plant species (Brooks 1998). Therefore, all investigated *A. halleri* populations, NM as well as M, hyperaccumulated Zn in the field. Additionally, as concentrations of Zn found in investigated plants are widely accepted as toxic (Marschner 1995, Steinborn & Breen 1999) and as *A. halleri* plants showed no signs of damage, it can be concluded that all the investigated plants can be considered as tolerant. These results confirm previous work in which hyperaccumulation of Zn in *A. halleri* field samples have been reported. On the base of the same criterion, other research on European populations of *A. halleri* have showed that typical for hyperaccumulators concentration of Zn (*i.e.* 10000 $mg \cdot kg^{-1}$ d.wt, Brooks 1998,) was observed only in metallicolous populations (Bert *et al.* 2002).

Cadmium

In case of Cd, similarly to Zn, typical concentration usually found in non–accumulating plants (Brooks 1998) was exceeded in all tested populations (30–150 and 7–50 times more in NM and M, respectively). According

to Dahmani–Muller *et al.* (2001) all the *A. halleri* plants from sites with elevated Cd content possess ability to hyperaccumulate this metal. High concentrations of Cd in *A. halleri* from field collected both M and NM populations have been already reported by Bert *et al.* (2002). However, only 2 out of 33 tested populations were classified as Cd hyperaccumulators (according to Brooks' (1988) criterion of hyperaccumulation).

Lead

Unlike Zn and Cd *A. halleri* can not hyperaccumulate Pb. Total concentration of Pb in soil was, in every case, higher than concentration in shoots. This result is entirely consisted with previous research of Dahmani–Muller *et al.* (2000) who have stated that Pb concentration in shoots of *A. halleri* do not exceeds total concentration in soil. As Pb show low mobility and consequently is relatively poorly available for plants its accumulation is rather rare. According to Baker and Brooks (1989) Pb accumulator must contain no less than 1000 $mg \cdot kg^{-1}$ of Pb in shoots and there are only five species meeting this criterion. During the present study no plants presented as high level of Pb were observed, which have confirmed that *A. halleri* can not hyperaccumulate Pb. Similar results were showed by Bert *et al.* (2002).

Metal accumulation in shoots in relation to metal content in soil

There was no relation between metal accumulating ability in *A. halleri* populations in the field with the degree of contamination of investigated sites. The metals' concentrations in plants did not depend directly on metal concentration in soil and then obtained results, when presented graphically, differed from those drown (and recognized as typical for accumulators) by Baker (1981) (see chapter I 2.2). Because this relation was not observed neither for total nor available forms of metals, and distribution of both curves (based on total and available metal content) was much the same (even if the analysis of these forms of metals were performed separately and using different methodology) the data were considered as correctly performed. Thus no significant effect of measurements was expected to contribute observed heterogeneity. However, it should be stressed that in this study metal concentrations were determined on field–collected samples, while presented by Baker (1981) typical curve of relation of soil / above–ground metal content have been performed under standard laboratory conditions. In increasing number

of hyperaccumulating species it is clear that variation in metal uptake within and between populations is large, even under standard conditions (for review see Macnair 2003). In case of *A. halleri,* Macnair (2002) has studied 17 populations under standard conditions, and showed that the final concentration of Zn in leaves of *A. halleri* is highly variable and there was no relationship between these content and Zn contamination of soils from which plants occurred, which was in agreement with this field study. Part of this variation could be ascribed to genetic variation between individuals. However, there are clearly other environmental factors which determine the final above–ground concentration of metal, therefore to known metal accumulation ability of Polish *A. halleri* populations the experiment in controlled conditions have been performed (see chapter IV).

Shoot to root ratio criterion

In present study, it was noticed that growth in heavy metal concentration in roots causes growth in metal concentration in shoots. Transport of metal elements from roots to shoots (Baker 1981) and its storage in shoots at concentrations higher than in roots is involves by hyperaccumulation. Therefore, a ratio of metal content in shoots to metal content in roots was frequent considered as a criterion of hyperaccumulation (= S/R ratio stands above 1) (Krämer *et al.* 1996, Lasat *et al.* 1996, Shen *et al.* 1997, Lasat *et al.* 1998, Schat *et al.* 2000). In this study, according to S/R criterion all the tested plants strategy of Zn and Cd distribution was typical of hyperaccumulators (with exception of population 12 from Niepołomice Forest where S/R ratio was found to be slightly lower than 1: S/R = 0,9). In case of Pb its concentration on shoots was lower than in roots and this result confirms the fact, already reported by other hyperaccumulation criterion, that *A. halleri* do not hyperaccumulate Pb.

Metal uptake capabilities – bioaccumulation factor

Metal accumulation capability of investigated *A. halleri* plants in natural conditions was estimated by bioaccumulation factor (BF > 1 in hyperaccumulating plants; Baker 1981). Taking this criterion into account it can be stated that majority of tested plants hyperaccumulated Zn and Cd, while in case of Pb it was confirmed that this element can not be hyperaccumulated by *A. halleri*. Non–metallicolous populations were characterized by considerably higher efficiency in Zn and Cd uptake than M populations. Efficiency in metal uptake was as higher as lower total content

of metal in soil was. Higher efficiency in Zn and Cd uptake by NM than M populations, have been already shown by Zhao *et al.* (2000) during research on *A. halleri* cultivated in artificially contaminated soil and by Knight *et al.* (1997) and Robinson *et al.* (1998) in field research on *Thlaspi caerulescens*. In this study, exceptionally low BF values were obtained for M population 27 from Galman. Interestingly, this site had also extremely high total content of tested metals in soil and then as it has been reported by Backer (1981) extreme concentrations of metals in soil can cause a decrease in BF value (BF≤1).

Moreover, this study suggests, as it was already proposed by Bert *et al.* (2002), that with increasing total Zn soil content, the Zn content of the aerial parts of the plants reaches a plateau. This plateau response could be due to plant physiology and be explained by blocking of the translocation of metals from the roots to the shoots and /or saturation of the metal uptake mechanism at the root surface (Hamon *et al.* 1999). Thus, plateau response could be a safety mechanism that limits plant metal uptake and prevents phytotoxicity at high metal soil concentration. Results of this study suggest that, in NM *A. halleri* populations, this safety mechanism did not occur (neither for Zn nor Cd). Interestingly, in case of Zn this threshold seems to be about 5000 $mg \cdot kg^{-1}$ of total Zn content in soil, while for Cd about 40 $mg \cdot kg^{-1}$ of total Cd content in soil. Below these values plants showed an exponential increase in the capacity to mobilize and concentrate metal in their above–grounds parts, while above these thresholds an exponential increase of resistance and limit plant metal uptake was observed. Despite extremely high metal content in soil, their uptake by plants have never been stopped, and plants from highly contaminated sites showed BF at relatively small but homogeneous level. In case of this study, the capacity to mobilize and concentrate metal in above–grounds parts were thus observed in populations from Niepołomice Forest (12, 13, 14), Tatra Mts. (32, 33) and Bibiela (21), while metal uptake was mostly limited in populations from Galman (27, 28), Bolesław (24) and Wełnowiec (15, 17). Populations from Bukowno (22) and Miasteczko Śląskie (19, 30) were intermediates.

It was established during previous researches that metal uptake in plants can be influenced by other metal ions present in soil (Marschner 1993, Sajwan & Lindsay 1986). Numerous researches have showed that cations uptake can be limited in presence of other cations in high concentrations (Alva & Edwards 1993, Filipek & Badora 1993, Badora & Filipek 1994).

Also in this study the best fit to the observed data, *i.e.* the best regression model showed on which parameters depended Zn and Cd concentration in plants. However, on this data sets, characterized by strong heterogeneity within as well as between sites and populations estimation of the model parameters proved to be not confident. Therefore, to perform similar analysis in this heterogonous investigated area the multiplying of sample size is needed.

Behavior of M and *NM populations of A. halleri*

We can learn from literature that highly tolerant plants, when compared with those with low tolerance, show lower nutrient demands and their mineral balance is well adapted to presence of heavy metals in soil (Antosiewicz 1995). In present study investigated M populations occurs in habitats highly differentiated in terms of micro– and macroelements content. It can be therefore hypothesized that high metal content plays a key role during the process of colonisation of M soils whereas content of nutrient is not a limiting factor. In contrary, NM populations occurred in habitats which did not differed substantially in micro– and macroelement content in soil. These results suggest that genotypes from M populations of *A. halleri* are able to colonise heterogeneous industrial environments, while on the other hand, it is possible that investigated in this study NM genotypes, posses probably only small adaptational abilities and they colonise NM sites where environmental conditions meet their needs.

In present study content of nutrient elements in plants was normal and did not differ between individuals from M and NM populations. According to Krupa *et al.* (2002) increased amount of Cd in environment leads to decrease in Ca content in plant tissues. This fact was, however, not confirmed in the present study. Calcium concentrations in tissues in plants sampled from NM populations were comparable with concentrations obtained for samples from M populations. Similarly, Krupa *et al.* (2002) have stated that heavy metals may lead towards decrease in cell content of P and consequently disturb photosynthesis. In tested samples P content was normal also and did not differ significantly between M and NM sites.

Heavy metal content in plant samples from investigated populations did not differ significantly within NM type, nor within M type (except Miasteczko Śląskie and Galman, which is probably due to the fact that first site is under impact

of airborne pollutants from Zn smelter while the second site is located on post–mining area). These might suggest that in natural environment (in both M and NM sites) ecological differences do not influence accumulation ability in *A. halleri*.

However, data concerning Zn and Cd hyperaccumulation abilities in *A. halleri* populations in the filed presented in this study should be additionally verified in controlled experimental conditions. In natural habitat plant reactions to heavy metals can be modified by numerous environmental variables as well as by physic–chemical properties of soil. Because of that only experiment carried out in uniform and controlled conditions would enabled to characterize investigated populations in terms of their hyperaccumulation abilities. To meet this chalange accumulation of Zn by populations from investigated sites was tested during the pot experiment, presented in chapter IV of this study.

III. GENETIC DIVERSITY AND STRUCTURE OF *ARABIDOPSIS HALLERI* POPULATIONS IN SOUTHERN POLAND

1. Materials and methods

1.1. Plant material and DNA extraction

In summer 2005 fifteen populations of *Arabidopsis halleri* were sampled in southern Poland (see chapter II 1.1). Fourteen of them were included to the present study (except population 19 from Miasteczko Śląskie which was closely located – 450 m – to population 30; Tab. 11).

Table 11. Studied sites in southern Poland. M – metallicolous population; NM – non–metallicolous population; n_i – number of genotyped individuals per population.

Site	Origin	Location	Geographic coordinates		n_i
			N	E	
32	NM	Western Tatra Mts.	49°16'26.94"	19°52'41.76"	27
33	NM	Western Tatra Mts.	49°17'32.52"	19°55'36.54"	21
12	NM	Niepołomice Forest	50°06'24.36"	20°21'55.98"	28
13	NM	Niepołomice Forest	50°06'35.64"	20°21'40.26"	28
14	NM	Niepołomice Forest	50°06'31.80"	20°22'02.88"	27
22	M	Bukowno	50°16'58.08"	19°28'43.38"	25
24	M	Bolesław	50°17'00.18"	19°29'05.64"	22
27	M	Galman	50°11'36.78"	19°32'15.12"	25
28	M	Galman	50°11'54.12"	19°32'19.74"	18
29	M	Galman	50°11'54.06"	19°32'30.96"	18
15	M	Wełnowiec	50°17'12.96"	19°01'32.04"	22
17	M	Wełnowiec	50°16'57.12"	19°01'46.98"	28
30	M	Miasteczko Śląskie	50°30'10.03"	18°56'20.02"	19
21	NMp	Bibiela	50°29'45.66"	18°59'00.12"	22

The scale of sampling ranged from less than 1 km between populations (*e.g.* 12 and 13) to more than 150 kilometers for the most distant populations (*e.g.* 32 and 21). Mean geographic distance was 65.26 ± 43.22 km. In each study site a 60 m long transect was set out. Leaf material was sampled from 25 to 30 individual rosettes of *Arabidopsis halleri*, along a transect covering the population area. Sampling was made following two patterns: (1) 3 m minimal distance between neighbors samples, to avoid clone sampling (Van Rossum *et al.* 2004), (2) in distance of 0.5 – 3 m from transect line. Overall, 330 genotypes were collected. Leaves were dried at 55^0C for 24 h and stored in silica gel prior to molecular analysis.

DNA from each genotype was extracted from 10 to 15 mg of dry leaf material, using *NucleoSpin® 8/96 Plant* kit from MACHEREY–NAGEL. PCR amplification was performed on 1/100 dilutions.

1.2. cpDNA analyses

Data sets from the chloroplast DNA (cpDNA) were obtained from intergenic regions (trnC–trnD and psbC–trnS) and the *trnK* region containing the *matK* intron (trnK1–trnK2). Amplified fragments ranged in size from 1584 bp for psbC–trnS (fragment CS1 – 1084 bp, CS2 – 698 bp) to 2475 bp for trnC–trnD (CD1 – 1000 bp, CD2 – 887 bp, CD3 – 996 bp) and 2591 bp for trnK1–trnK2 (fragment matK1 – 1077 bp, matK2 – 1009 bp, matK3 – 995 bp). The primer pairs used for amplification of these fragments were defined by Demesure *et al.* (1995), Dumolin–Lapegue *et al.* (1997) and Pauwels *et al.* (2005).

In order to screen the chloroplastic DNA polymorphisms in analysed populations, an EcoTILLING approach was used. This method has been developed from the TILLING method (targeting induced local lesions in genomes, set up in *Arabidopsis*) for analyzing natural variability in plants. TILLING is a low–cost, high–throughput reverse genetic method that combines random chemical mutagenesis with PCR–based screening of gene regions of interest (Colbert *et al.* 2001; McCallum *et al.* 2000a, b).

The DNA of each of 330 individuals was amplified by PCR. Amplification of about 1–kbp fragments covering the three targeted regions was made with asymmetrically labelled fluorescent primers, specific for chosen fragments. Forward–strand primers were end–labelled with IRDye 700 dye and reverse–strand primers with IRDye 800 dye. Heteroduplexes between wild–type fragments and fragments harboring an induced mutation were formed by denaturing and reannealing PCR products. Nucleotide changes were identified by enzymatic digestion of heteroduplexes with the mismatch clearage endonuclease ENDO I (kindly provided by A. Bendhamane from the INRA–UMRGV France). Cleaved fluorescent products were resolved and visualized on electrophoretic gels using Li–Cor 4200 gel analyzer (LiCor–ScienceTec) (Kulinski *et al.* 2000; Oleykowski *et al.* 1998). The ends of the amplified DNA were labelled differentially with either the IRD700 dye on the left end, or IRD800 dye on the right end. Two fluorescent dyes were detected

in different channels and two images are generated for each electrophoretic run. Candidate polymorphic sites identified in the IRD700 channel were confirmed in the IRD800 channel, which showed the cleavage product labelled on the right end. Each nucleotide polymorphism was first recorded by its gel mobility (fragments were separated on 7% denaturing polyacrylamide gels of 25 cm; Long ranger, FMC), which approximates position within a few nucleotides. Given the positional information provided by ENDO I cutting, polymorphisms and haplotypes were identified. LiCor gel images were analysed with the aid of the ImageJ software (http://rsb.info.nih.gov/ij/).

Few samples representing each detected haplotype were sequenced to confirm polymorphisms. For DNA sequencing, amplifications were performed with 20 ng genomic DNA in a 50 µl final volume following the manufacturer's suggestions for the AmpliTaq® Standard (Perkin Elmer®). Samples were run on ABI 3100 capillary sequencing machines. Sequence trace analysis was performed using BioEdit software.

PCR and TILLING conditions

Amplification each of the 341 DNA samples, for each tested region, was performed using the following conditions: a total volume of 15 µl consisting of 10 ng of template DNA, 3 mM $MgCl_2$, 200 µM of each of the four dNTPs, 200 µg/ml BSA, 0.04 µM unlabelled reverse primer, 0.08 µM unlabelled forward primer, 0.16 µM IRD800 reverse primer, 0.12 µM IRD700 forward primer, 0.025 units of AmpliTaq® Standard (Perkin Elmer®) and 1x AmpliTaq® Standard buffer (Perkin Elmer®). The cycling conditions were 5 min initial denaturing at 94^0C, followed by 40 cycles of: denaturing for 30 s at 94^0C; annealing at 50 ^{0} C for 45 s and extension for 60 s at 72^0C. A final extension followed at 72^0C for 7 min. Conditions of CH2.1 wild–type DNA amplification and cycling were identical as described above.

To create heteroduplexes, PCR products of tested DNA were mixed 1:1 with CH2.1 wild–type DNA. The reaction was carried out in the temperature gradient starting at 94^0C and followed by a slow cooling step of -0.1^0C / s.

ENDO I reactions were carried out using the following conditions: a total volume of 30 µl consisting of 100 ng of heteroduplexes DNA, 3 µl ENDO I, 3 µl buffer ENDO I

10x and 19 µl water. Incubation was performed in termocycler at 37^0C for 40 min. Samples of digested heteroduplexes were passed through a Sephadex G50 (S–G50; GE Healthcare Life Sciences, Little Chalfont, UK) spin plate to remove salts and buffer components that are inhibitory to gel runs and laser detection. Purified samples were mixed with 5µl of denaturizing loading buffer (LiCor–ScienceTec). Sizes were assessed using the software Image J v. 4.1 by comparison with molecular weight marker (50–700 bp) labelled with IRDye 700 and IRDye 800 (LiCor–ScienceTec).

1.3. Microsatellite analyses

Each of the 341 DNA samples was genotyped for ten microsatellite loci. Five microsatellites (*ATH, GC16, LYR132, LYR133, LYR104*) have been previously used at the within–population level (Van Rossum *et al.* 2004). Five other (*GC22, NGA112, ICE13, MDC16, NGA361*) were transferred from *A. thaliana* and recently combined in a multiplex (Llaurens *et al.* 2008) (Tab. 12).

Table 12. Primer sequences of used microsatellite loci.

Locus	Primer sequence (5′–3′)	
ATH	F:	TCTATCAACAGAAACGCACCGAG
	R:	CCACTTGTTTCTCTCTCTAG
GC16	F:	TTTTGGAGTTAGACACGGATCTG
	R:	GTTGATCGCAGCTTGATAAGC
LYR132	F:	GCCGTGAGATTAAAGAAGACG
	R:	GCAAGAGCTGATCTCCATCC
LYR133	F:	GTTGCTGCTGCTGATGGTT
	R:	CAAGGAAGGCAGCAAAGAAA
LYR104	F:	GAGGCGAATGTAGTGGAAGG
	R:	CGACCTCCATCATCGATCTCAGCA
GC22	F:	GGTCTAATTGCCGTTGTTGC
	R:	GAATTCTGTAACATCCCATTTCC
NGA112	F:	TAATCACGTGTATGCAGCTGC
	R:	CTCTCCACCTCCTCCAGTACC
ICE13	F:	GATCCTTCACCGGGTCTTG
	R:	GTGGTGGAGACTCTTCGAGC
MDC16	F:	GAGTGGCCTCGTGTAGAGAAAG
	R:	TGTCACTCTTTTCCTCTGGTTTG
NGA361	F:	AGGGTTTTCCCAAAGAGATGA
	R:	TCTTGTCCTTCGATTTTAGACCA

PCR conditions

For loci *LYR132*, *LYR133*, LYR104, *GC16* and *ATH*, PCR reactions were carried out in a total volume of 15 µl containing 20 ng of DNA template, 1x polymerase chain reaction buffer (20 mM Tris–HCl pH 8.3, 50 mM KCl), 3.5 mM $MgCl_2$, 100 µM dNTP, 200 µg/mL BSA, 0.2 µM of each unlabelled primer and 0.15 µM of the M13 fluorescently labelled primer (either IRD–700 or IRD–800) and 0.4 units of AmpliTaq® DNA Polymerase (Applied Biosytems).

In order to obtain labelled PCR products either the forward or reverse primer containing a 5'–tail of 19 bp or 20 bp, respectively, homologous to the universal consensus M13 primer sequence followed by the locus–specific sequence were used (Oetting *et al.*1995). This made PCR products detectable on the automated genotyper Li–Cor 4200 (Li–Cor–ScienceTec). PCRs were performed on a Perkin–Elmer Gene–Amp system 9700 (94^0C for 5 min, followed by locus–specific amplification:

GC16: 94^0C for 30 s, 50^0C annealing temperature for 45 s, 72^0C for 40 s, for eight cycles, followed by M13 labelling amplification: 94^0C for 30 s, 50^0C for 20 s, 72^0C for 40 s, for 30 cycles, and a final extension 72^0C for 7 min;

LYR133: 94^0C for 30 s, 60^0C annealing temperature for 35 s, 72^0C for 30 s, for eight cycles, followed by M13 labelling amplification: 94^0C for 30 s, 56^0C for 25 s, 72^0C for 30 s, for 32 cycles, and a final extension 72^0C for 7 min;

LYR132: 94^0C for 30 s, 60^0C annealing temperature for 30 s, 72^0C for 30 s, for eight cycles, followed by M13 labelling amplification: 94^0C for 30 s, 56^0C for 15 s, 72^0C for 30 s, for 32 cycles, and a final extension 72^0C for 7 min;

ATH: 94^0C for 30 s, 55^0C annealing temperature for 35 s, 72^0C for 30 s, for eight cycles, followed by M13 labelling amplification: 94^0C for 30 s, 56^0C for 25 s, 72^0C for 30 s, for 32 cycles, and a final extension 72^0C for 7 min;

LYR104: 94^0C for 30 s, 50^0C annealing temperature for 45 s, 72^0C for 40 s, for eight cycles, followed by M13 labelling amplification: 94^0C for 30 s, 50^0C for 20 s, 72^0C for 40 s, for 30 cycles, and a final extension 72^0C for 2 min).

Amplification products were analysed on a LiCor automated DNA sequencer 4200 (Li–Cor–ScienceTec). PCR fragments were separated on 7% denaturing polyacrylamide gels (Long ranger, FMC) of 33 cm, sizes were assessed using the software Image J v. 4.1 by comparison with an appropriate labelled molecular weight marker (50–700 bp, Li–Cor–ScienceTec).

Remaining microsatellite markers: *GC22*, *NGA11*, *ICE 13*, *MDC16* and *NGA36* (Tab. 12) were amplified simultaneously by multiplex PCR using primers labelled with Applied Biosystems dyes (VIC for *MDC16* and *GC22*, 6FAM for *NGA112* and *NGA361*, NED for *ICE13*). Markers were amplified using QIAGEN® Multiplex PCR Kit. The PCR reactions of these five–plex were carried out in a total volume of 10 µl, containing 20 ng of DNA template. PCRs were performed on a Perkin–Elmer Gene–Amp system 9700. The cycling conditions were: 15 min initial denaturing at 95^0C, followed by 25 cycles of: denaturing for 30 s at 94^0C, annealing at 50^0C for 90 s and extension for 60 s at 72^0C. A final extension was followed at 60^0C for 30 min. The PCR products were applied on ABI Prism® 3100 DNA sequencer (Applied Biosystems). Capillary electrophoresis analysis was carried out in mix containing 1.5 µl of PCR product, 9.5 µl of Hi–Di Formamide and 0.5 µl of GeneScan 500 LIZ Size Standard (Applied Biosystems). Samples were analysed using GeneMapper Software version 3.7 (Applied Biosystems).

1.4. Statistical analyses

Chlorotype diversity, phylogenetic relationship among chlorotypes and populations

Genetic diversity was quantified by estimation of allelic richness and chlorotype diversity. Chlorotypic allelic richness (a_g^i) was estimated for each population (*i*) with the rarefaction method (Kalinowski 2004) using Fstat 2.9.3 (Goudet 2001). Data were standardized to a standard sample size *g* = 18 (the smallest sample of genotyped individuals per population). Chlorotype diversity (H_S) and its sampling variance (Nei 1987) were estimated within each population using ARLEQUIN 2.0 (Schneider *et al.* 2000).

Using cpDNA variation, for each individual, the data for all investigated loci were combined into chlorotypes (cpDNA haplotypes). A minimum–spanning tree (MST) of the chlorotypes was computed from a genetic distance matrix containing the number of differences between each pair of chlorotypes. The MST construction assumes that each chlorotype is linked by a single or the series of mutational events to all other chlorotypes through a unique pathway and the construction method minimizes the number of such events (Excoffier & Smouse 1994). This analysis was performed using ARLEQUIN 2.0 software (Schneider *et al.* 2000).

Phylogenetic relationships among populations were estimated using PHYLIP V3.5c (Felsenstein 1993). Cavalli–Sforza genetic distances among populations were calculated from haplotype frequencies and resulting distance matrix was used to create a neighbour–joining (NJ) tree. For visualizing NJ tree the TreeView 1.6.6 software (Page 1996) was used.

Analysis of microsatellite variation

In order to quantify the within–population genetic diversity, performed using 10 microsatellite loci, various parameters were estimated. For each population the mean allelic richness across loci (A_S) was calculated with the rarefaction method (Kalinowski 2004) using a standardized sample size (g = 18) in FSTAT 2.9.3 (Goudet 2001). Expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated using GENETIX 4.05 (Belkhir *et al.* 1996). Significance of F_{IS} values was tested using the permutation procedure (1000 permutations) of the GENETIX 4.05 program. The sequential Bonferroni correction was applied on each p–value.

To test for differences among groups of populations in allelic richness (A_S of populations 32, 33 *vs* A_S of populations 12, 13, 14, 15, 17, 30, 22, 24, 27, 28, 29; A_S of populations 12, 13, 14 *vs* A_S of populations 22, 24, 27, 28, 29) a Mann–Whitney–Wilcoxon test was performed using STATISTICA 8.

Cluster analyses

A Bayesian clustering procedure using multi–locus genotype data, implemented in STRUCTURE V2.1 (Pritchard *et al.* 2000), was used to identify the number of groups (clusters, K) corresponding to the uppermost hierarchical level of genetic partitioning between investigated populations. Data set consisted of 10 nuclear loci. All runs employed the correlated allele frequency model and the admixture ancestry model in which the fraction of ancestry from each cluster was estimated for each individual. Estimated log likelihoods were obtained for user–defined number of clusters (K) set from 2 to 14, with 10 replicates of each K. A length of the burn–in of 10^5 Markov chain Monte Carlo (MCMC) generations and run length of 10^4 MCMC generations was employed. All other parameters were default. The most probable number of clusters was chosen with ΔK – the second order rate of change

of the log likelihood [Ln(P)] function with respect to K – by the graphical method proposed by Evanno *et al.* (2005). According to Evanno *et al.* (2005) the modal value of the distribution of ΔK is an unbiased estimator of K and the height of this modal value is used as an indicator of the strength of the signal detected. To confirm population substructure identified by STRUCTURE, the Bayesian clustering method implemented in the BAPS 3.1 software (Corander *et al.* 2004) was performed.

Phenetic relationships among populations were generated using the Neighbour–Joining algorithm (Saitou & Nei 1987) in the PHYLIP V3.5c software package (Felsenstein 1993). Evolutionary distance matrix for the Neighbour–Joining method was generated based on Cavalli–Sforza and Edwards's (1967) chord distance. The robustness of the inferred tree topologies was evaluated using 1000 bootstrap resamplings of loci and the majority–rule (extended) consensus tree was calculated.

Estimation of demographic parameters of divergence

In order to estimate how and when *A. halleri* populations diverged in the investigated area of southern Poland, recently developed methods for fitting the "isolation with migration" (= IM) model have been applied (Hey 2005). A general IM model, in which a population gives rise to 2 populations, after what gene exchange between these populations can occur, was considered. This method lets estimate 6 demographic parameters in the model: divergence time, migration rates, effective population sizes of two current and the ancestral populations, using Markov chain Monte Carlo (MCMC) algorithm (Nielsen & Wakeley 2001).

In the present study, considering previous results, 3 combinations of samples in subgroups have been analysed. Three independent runs and 3 analyses have been done: first – to estimate divergence time of subgroups from Tatra Mts. and Olkusz region; second – to estimate divergence time of subgroups from Tatra Mts. and Silesia region; third – to estimate divergence time of subgroups from Olkusz region and Niepołomice Forest. The analysis was performed on genotyped data from 10 microsatellite loci. A simulation procedure has been initiated with a burn–in period of 10^5 updates and the total run length of each analysis was 10^6 updates. Parameter values were scaled by the mutation rate (mutation rate of 10^{-4} per generation and two–year generation time were assumed). The analyses have been performed using IMa software (Hey & Nielsen 2007).

2. Results

2.1. Chloroplast DNA diversity, phylogenetic relationships and geographic distribution of haplotypes

Ten chlorotypes, labelled from I to X, were identified (Tab. 13, Fig. 24). Chlorotypes were linked by 9 mutational steps (Fig. 24 a).

Table 13. Distribution of chlorotypes among 14 *A. halleri* populations. M – metallicolous population; NM – non–metallicolous population; n_i – number of genotyped individuals per population; a^i_{18} – standardized chlorotypic allelic richness; H_S – chlorotype diversity; I:X – defined chlorotypes.

Pop.	Origin	n_i	a^i_{18}	$H_S \pm SE$	Chlorotype									
					I	II	III	IV	V	VI	VII	VIII	IX	X
32	NM	27	2.89	0.416±0.095	.	20	.	.	.	.	1	.	6	.
33	NM	21	4.97	0.752±0.048	.	7	.	.	.	1	5	.	7	1
12	NM	28	2.00	0.198±0.092	.	3	.	25	.	.	.	.	.	.
13	NM	28	3.75	0.492±0.088	1	7	.	19	1	.	.	.	.	.
14	NM	27	2.00	0.484±0.054	.	10	.	17	.	.	.	.	.	.
22	M	25	1.00	0	.	25	.	.	.	.	.	.	.	.
24	M	22	1.00	0	.	22	.	.	.	.	.	.	.	.
27	M	25	1.00	0	.	25	.	.	.	.	.	.	.	.
28	M	18	3.00	0.451±0.117	.	13	1	4	.	.	.	.	.	.
29	M	18	1.00	0	.	18	.	.	.	.	.	.	.	.
15	M	22	2.97	0.437±0.105	.	16	1	5	.	.	.	.	.	.
17	M	28	2.88	0.315±0.102	.	23	.	4	.	.	.	1	.	.
30	M	19	2.00	0.105±0.092	.	18	.	1	.	.	.	.	.	.
21	NMp	22	2.00	0.247±0.108	.	19	.	3	.	.	.	.	.	.
Total					1	226	2	78	1	1	6	1	13	1
Frequencies in the overall sample [%]					*0.3*	*68.5*	*0.6*	*23.6*	*0.3*	*0.3*	*1.8*	*0.3*	*3.9*	*0.3*

In all cases chlorotypes were separated by a single mutation. Most of revealed polymorphisms were due to point mutations rather than to size polymorphism. However, deletions / insertions of more than 5 bp were also observed (Tab. 14). Chlorotypes were not equally frequent (Tab. 13). Chlorotype II and IV were the most common (68.5 % and 23.6 % respectively) and widespread (present in fourteen and eight populations, respectively). As usually expected, the most widely represented chlorotypes (II, IV) were internal. Five chlorotypes were represented only in single individuals (I, V, VI, VIII, X; frequency each of them was 0.3 %). Only two chlorotypes (II, IV) were shared between NM and M populations, the others were specific to one edaphic type. Most of them (I, V, VI, VII, IX, X) were NM specific.

Table 14. Description of the polymorphic sites identified in 14 *A. halleri* populations. Insertions (ins) and deletions (del) are presented with their estimated lengths (given in parentheses).

Mutation number	PCR fragment	Mutation	Position[1]
1	CD	T (C)	636
2	CD	A (C)	768
3	CD	A (G)	2081
4	K1K2	del (–5)	246–250
5	K1K2	G (T)	573
6	K1K2	ins (75)	1032–1106
7	K1K2	A (C)	1240
8	K1K2	T (G)	1991
9	K1K2	del (–5)	2045–2049

[1] – position of the mutation in *A. thaliana* chloroplast genome http://www.ncbi.nlm.nih.gov)

Ten out of 14 investigated populations were polymorphic. Chlorotypic allelic richness across populations varied from 1.00 (populations 22, 24, 27, 29) to 4.97 (population 33). Chlorotype diversity within populations (H_S) varied from 0 to 0.752. The chlorotype distribution presented in Figure 24 b suggests reduced genetic variation in all lowland populations (M, NMp and NM) when compared to NM populations from Tatra Mts. The highest level of chlorotype diversity found in populations from Tatra Mts. populations suggests that these NM populations could be ancestral in investigated area. Interestingly, chlorotype IV, relatively frequent in whole sampling, was, however, not found in. populations from Tatra Mts. Moreover, this chlorotype was the major one in NM populations from Niepołomice Forest samples (12, 13, 14) (Fig. 24 b). Furthermore, the NJ populations' tree further confirmed that these two groups of NM populations (32, 33 from Tatra Mts. *vs* 12, 13, 14 from Niepołomice Forest) constitute the most distant genetic groups (Fig. 25). In sharp contrast, all investigated M populations showed very similar distribution of chlorotype frequencies (Fig 24 b) and hence high genetic proximity as illustrated by their tight clustering in the NJ populations tree (Fig. 25).

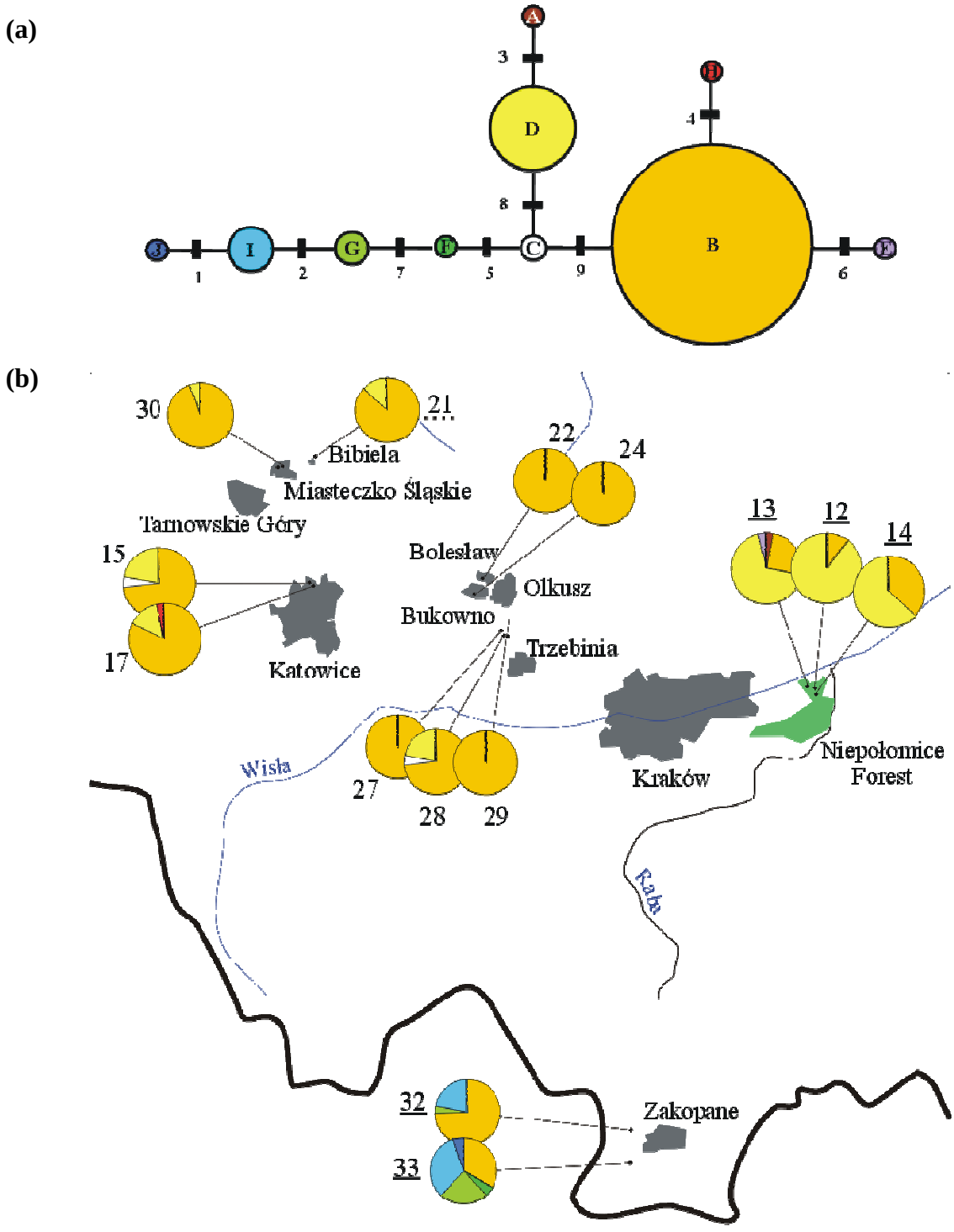


Fig. 24. **(a)** Minimum–spanning tree representing the phylogenetic relationships between 10 cpDNA haplotypes detected in investigated *A. halleri* populations. Circle: haplotypes; black rectangle: mutation event. The size of circles corresponds to the frequency of each haplotype. **(b)** Geographic distribution of cpDNA haplotypes detected in *A. halleri* populations from southern Poland. The numbers correspond to the numbers of populations. The colors correspond to the haplotypes defined in the MST and their surfaces in the circle correspond to the frequency of each haplotype in the population. The numbers underlined by solid lines correspond to NM populations, the number underlined by dashed line correspond to NMp population and the numbers non underlined correspond to M populations.

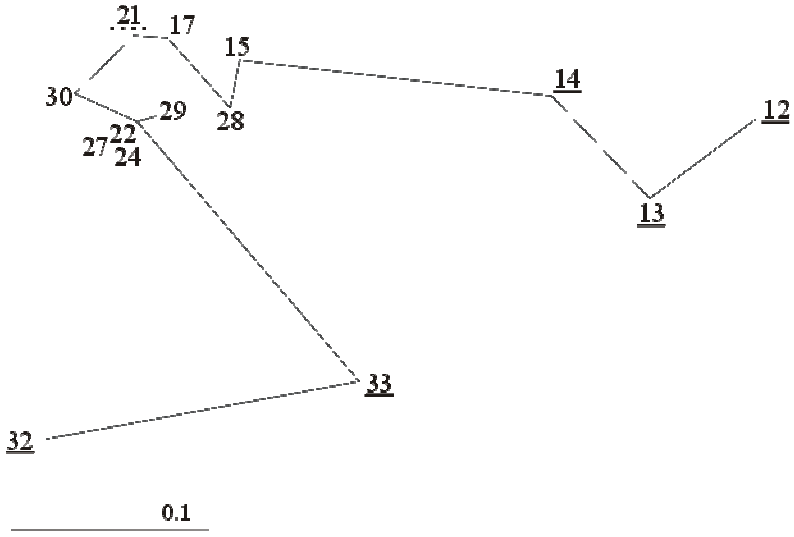


Fig. 25. Neighbour–Joining population tree for 14 *A. halleri* populations, based on cpDNA haplotypes. Genetic distances among populations were calculated from haplotype frequencies. The numbers underlined by solid lines correspond to NM populations, the number underlined by dashed line correspond to NMp population and the numbers non underlined correspond to M populations.

Taking into account ancestral status of populations from Tatra Mts., it could be supposed that all investigated M populations have been derived from NM populations from Tatra Mts., while these second did not/or not directly affected formation of populations from Niepołomice Forest. On this basis, two colonisation scenarios can be proposed and tested:

1. The ancestral populations were those from Tatra Mts. Metalliferous sites of Olkusz region (sites 22, 24, 27, 28, 29) were then first founded, followed by M populations in Silesia region (sites 15, 17 in Wełnowiec and 30 in Miasteczko Śląskie). Recently, within the industrialized Silesia region, in the low contaminated site in Bibiela, NMp population has been founded by their neighborhooding M populations. Recently also the "new" NM (= nNM) group in non–contaminated site in Niepołomice Forest was founded by M populations from geographically closest metalliferous Olkusz region.
2. Colonisation of metalliferous area as above, while an alternative scenario in case of NM group from Niepołomice Forest: no phylogenic relation with Tatra Mts. populations, but foundation of Niepołomice Forests' group from the other (yet unknown), which has not been included in the sampling of the present study.

Both scenarios should leave contrasted patterns of nuclear genetic variation. If the first scenario is correct, populations from Niepołomice Forest should clustered

with their closely related populations from Olkusz region. On the other hand, distinct and unrelated to the other population group of Niepołomice Forest's plants is expected if the second scenario is correct.

2.2. Microsatellite diversity and population structure

All 10 microsatellite loci investigated in this study were highly polymorphic. Mean allelic richness (A_S), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated for each population (Tab. 15). Values of multilocus allelic richness ranged from 29.1 to 51.7. The highest values of allelic richness were detected in populations from Tatra Mts. (32, 33) ($p < 0.05$), while the lowest in populations from Niepołomice Forest (12, 13, 14) ($p < 0.05$; Fig. 26 a). Expected heterozygosity ranged from 0.342 to 0.608. The highest and the lowest values of expected heterozygosity (H_E) were detected respectively in Tatra Mts.' (32, 33) populations ($p < 0.05$) and Niepołomice Forest's (12, 13, 14) populations ($p < 0.05$; Fig. 26b). The inbreeding coefficient (F_{IS}) values differed from 0.017 (population 33) to 0.252 (population 30). Only two populations (14 and 33) had F_{IS} values significantly greater than 0.

Table 15. Genetic variation within *A. halleri* populations. M – metallicolous population; NM – non–metallicolous population; A_S – nuclear allelic richness (multilocus); H_E – expected heterozygosity; F_{IS} – inbreeding coefficient (and associated p–value; *– significant ($p < 0.05$; significant values after Bonferroni correction given in bold); ns – not significant ($p > 0.05$).

Population	Origin	nuclear DNA		
		A_S	H_E	F_{IS}
32	NM	51.5	0.583	0.084n.s.
33	NM	51.7	0.608	0.017n.s.
12	NM	29.7	0.342	0.047n.s.
13	NM	30.6	0.435	0.054n.s.
14	NM	29.1	0.368	**0.217***
22	M	30.8	0.440	0.062n.s.
24	M	40.7	0.490	0.094n.s.
27	M	37.9	0.519	0.043n.s.
28	M	37.3	0.520	0.067n.s.
29	M	36.7	0.461	0.046n.s.
15	M	39.5	0.489	0.082n.s.
17	M	41.4	0.530	0.060n.s.
30	M	43.5	0.551	**0.252***
21	NMp	38.4	0.553	0.101n.s.

Not only considering these results, but also chlorotype composition as well as supposed scenarios of migration routes of *A. halleri* in investigated area, 4 distinct subgroups of whole sampling were defined to allow making among–subgroups comparison (Fig. 26). In particular, 2 subgroups (labelled A and B) have been chosen to verify the significance of differences between supposedly ancestral and derived population, 2 other (labelled C and D) – to verify affinity of the NM populations from Niepołomice Forest with geographically closest M populations from Olkusz region. The subgroups composition was as follow: A – Tatra Mts. populations (32, 33); B – Niepołomice Forests' (12, 13, 14) & Silesian (15, 17, 30) & Olkusz region's (22, 24, 27, 28, 29) populations; C – Niepołomice Forests' populations (12, 13, 14); D – Olkusz region's (22, 24, 27, 28, 29) populations. Among–subgroups' comparison of allelic richness (Fig. 26 a) as well as expected heterozygosity (Fig. 26 b) highlighted significant differences between Tatra Mts.' and all other investigated populations. Additionally, when looking into detailes, NM populations from Niepołomice Forest differed significantly from M populations from Olkusz region. These results support the first colonisation scenario.

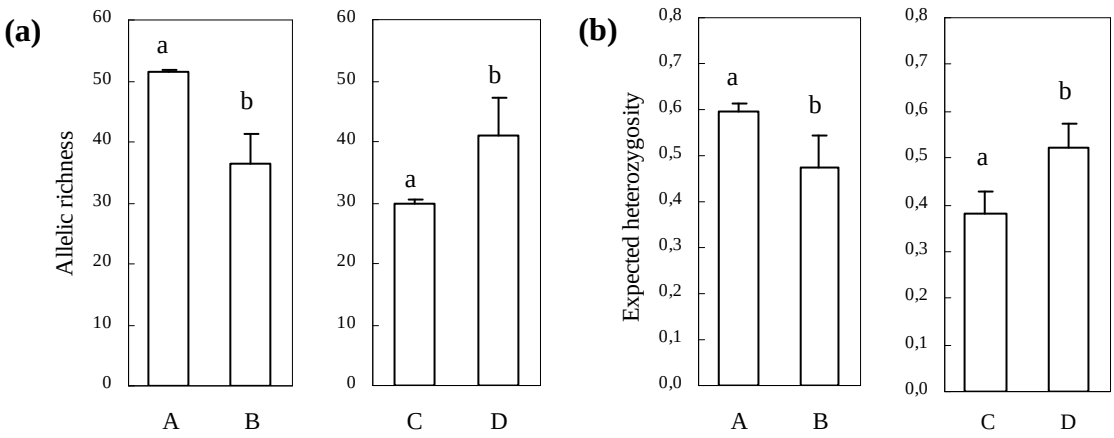


Fig. 26. (a) Multilocus allelic richness ($\bar{X}$ ± SD) of groups of investigated *Arabidopsis halleri* populations. **(b)** Expected heterozygosity ($\bar{X}$ ± SD) of groups of investigated *Arabidopsis halleri* populations. A – populations 32, 33; B – populations 12, 13, 14, 15, 17, 30, 22, 24, 27, 28, 29; C – populations 12, 13, 14; D – populations 22, 24, 27, 28, 29. Different letters above the box plots indicate a significant difference at the 5% level (Mann–Whitney–Wilcoxon test).

Cluster analysis

The microsatellite data were analysed for signs of population subdivision using the program STRUCTURE. As the uppermost hierarchical level of genetic partitioning

between investigated *A. halleri* populations, two main clusters were identified (the highest likelihood was observed for K = 2 (Fig. 27 a) and the highest modal value of ΔK was at K = 2 too (Fig. 27b).

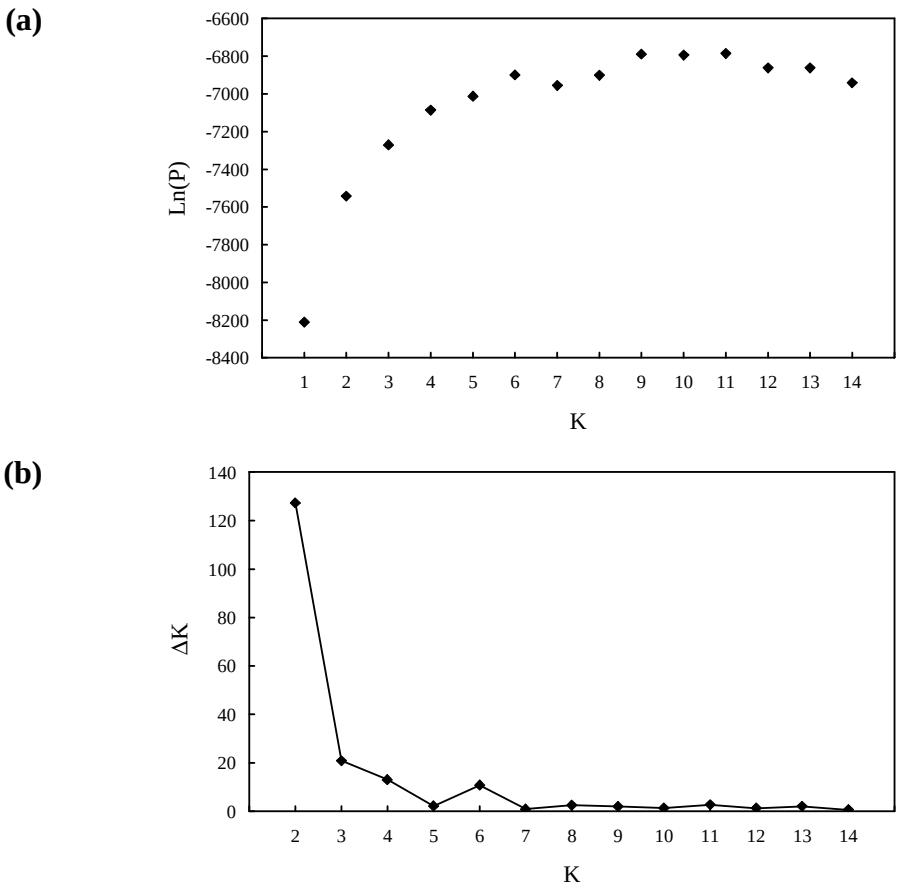


Fig. 27. (a) Mean values of log marginal likelihood of the multilocus genotypic data (P), as a function of the number of clusters (K), obtained with STRUCTURE. **(b)** Corresponding distribution of ΔK as a function of K (mean over 10 replicates).

Interestingly, K = 2 did not separate Niepołomice Forest's group of NM populations from the other, as predicted if scenario 1 is correct. Niepołomice Forest's populations (12, 13, 14) remained tightly clustered with populations from the Olkusz region (22, 24, 27, 28, 29), suggesting that they diverged from a recent common ancestor. The second cluster contained populations from Tatra Mts. (32, 33) and Silesia region (15, 17, 21, 30), what confirmed the first colonization scenario, rejecting the second one.

Magnitude of ΔK as a function of K (Fig. 27 b) showed a second level of population organization at K = 3 (the second highest modal value of ΔK

was at K = 3). Existence of this second level of populations organization (K = 3) was confirmed also by BAPS analysis (data not shown). In case of 3 clusters, the first one contained populations from Niepołomice Forest (12, 13, 14), the second populations from Olkusz region (22, 24, 27, 28, 29) and the third one from Tatra Mts. (32, 33) and Silesia region (15, 17, 21, 30). Presented results further confirmed relatively strong genetic similarity of Tatra Mts.' and Silesia's populations.

Moreover, the Neighbour–Joining tree reflected groups identified by STRUCTURE and BAPS (Fig. 28). Both levels of clustering (K = 2 and K = 3), presented by cardinal tree's branches, contained the same populations, grouped by STRUCTURE and BAPS.

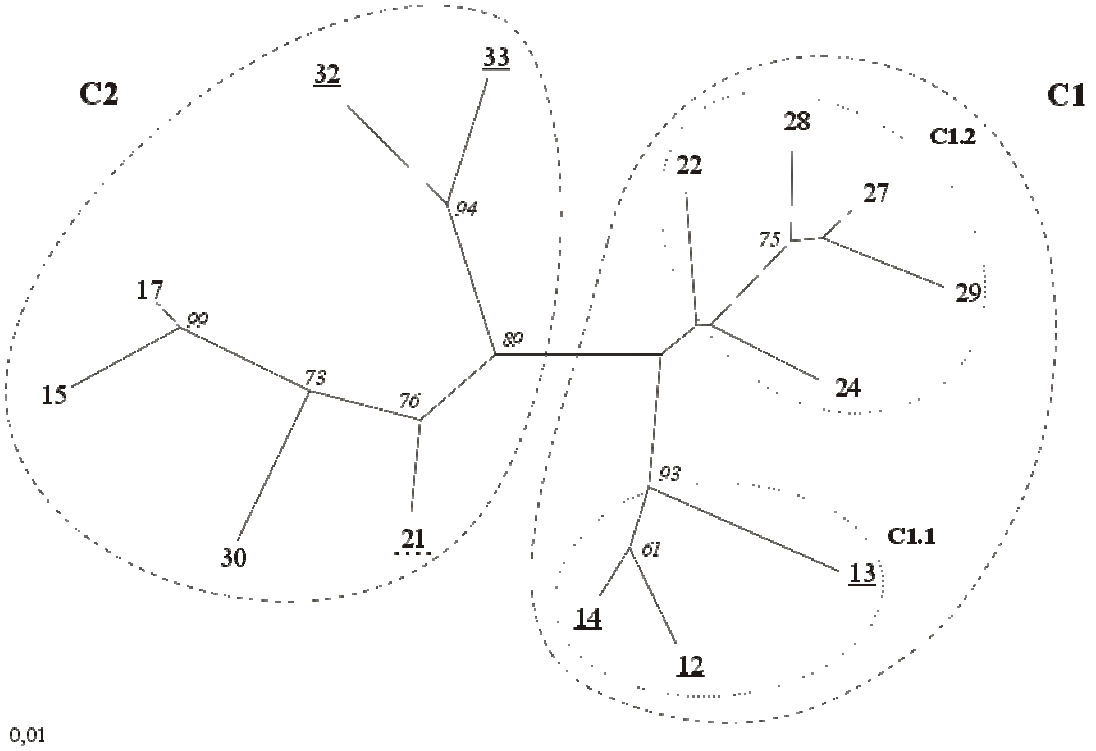


Fig. 28. Neighbour–Joining population tree using Cavalli–Sforza distance (nuclear DNA). Bootstrap values (in percent from 1000 replicates) are indicated with italics near branches (only values grater than 50 % are shown). The numbers underlined by solid lines correspond to NM populations, the number underlined by dashed line correspond to NMp population and the numbers non underlined correspond to M populations. C1 (C1.1, C1.2), C2 are the clusters of studied populations, according to their genetic distances.

The inferred tree topologies were robust, with most of bootstrap values greater than 70 %. According to the uppermost hierarchical level of genetic partitioning

(K = 2 was the best level of populations organization) populations clustered in two distinct groups (C1, C2), inside which M populations dispersed among NM populations. Populations 12, 13, 14 (NM) clustered together with populations 22, 24, 27, 28, 29 (M), whereas populations 32, 33 (NM) with populations 15, 17, 21, 30 (M). When the second level of population organization (3 clusters) was reflected at NJ tree, populations clustered inside C1 were subordinated into two groups (C1.1, C1.2). First of them (C1.1) comprised only NM (12, 13, 14), second (C1.2) only M populations (22, 24, 27, 28, 29).

These results depicted the pattern of genetic relationship among populations (and among analysed subgroups of populations) and further highlighted the evidence in favour of the first colonization scenario of *A. halleri* in investigated area.

Estimation of demographic parameters for the first scenario of divergence

The average divergence time between NM Tatra Mts. populations and two subgroups of M populations from southern Poland, examined with the IMa model, was estimated as 604 (95% HPD interval: 214 to 1504) years before present (ybp) for the Tatra Mts. – Silesia region pair and 340 (95% HPD interval: 140 to 1260) ybp for the Tatra Mts. – Olkusz region pair (Tab. 16). Divergence time between Olkusz region – Niepołomice Forest pair was estimated as 240 (95% HPD interval: 100 to 1030) ybp.

Table 16. Demographic parameter estimates for subgroups of *A. halleri* populations. N_A – effective size of the ancestral population estimates; M_1, M_2 – population migration rates estimates (M_1 pertains to genes moving from subgroup 2 to subgroup 1 and, M_2 – from 1 to 2); t_G – divergence time estimates in generations; t_Y – divergence time estimates in years. The 95% HPD interval are given in parenthesis.

Subgroups of examined populations	**N_A**	**Migration rate per generation**		**Divergence time**	
		M_1	**M_2**	**t_G**	**t_Y**
1. Tatra Mts.	226013	0.00561	0.00077	302	604
2. Silesia region	(146738–408188)	(0.00267–0.02211)	(0.00005–0.00117)	(107–752)	(214–1504)
1. Tatra Mts.	174625	0.00571	0.00069	170	340
2. Olkusz region	(113025–315975)	(0.00235–0.02461)	(0.00012–0.00282)	(70–630)	(140–1260)
1. Olkusz region	192375	0.03506	0.00487	120	240
2. Niepołomice Forest	(121125–358875)	(0.01218–0.08943)	(0.00352–0.09667)	(59–279)	(118–558)

3. Discussion

In the present study, both chloroplast DNA (cpDNA) and nuclear microsatellite markers have been investigated to estimate the genetic diversity, population structure as well as demographic history of *A. halleri* in southern Poland. The cpDNA is maternally inherited and so genetic structure at cpDNA markers mostly depend on seed–mediated gene flow (Petit *et al.* 1993). Because colonization of new habitats occurs through seeds, cpDNA markers provide information on past changes in species distribution that is unaffected by subsequent pollen movements (Petit & Vendramin 2004). On the other hand, nuclear DNA markers offer biparental view of genetic change and therefore can provide higher resolution of the relationships between samples (while both parents are strongly affected by concerted evolution) (Buckler *et al.* 1997). Hence, genetic structure at nuclear markers depends on both seed– and pollen–mediated gene flow (Petit *et al.* 1993).

3.1. Colonisation of metalliferous and non– metalliferous areas by *A. halleri* in southern Poland – phylogenetic reconstructions

This study provided evidence for genetic diversity among *A. halleri* populations in southern Poland. However, no relation between genetic and geographic structure was found (data not shown). The lack of geographically characterized genetic structure and relatively small number of chlorotypes out of the Tatra region indicate that investigated sites might have been colonized from a refuge in the Carpathian Mts. The results suggested also that observed level of population differentiation could have been due to the relatively recent colonization of industrially polluted sites. Based on the distribution of determined cpDNA haplotypes, the probably colonization routes from refuge were determined. A reasonable hypothesis for this survey is that in region of southern Poland Tatra Mts. populations are ancestral, while the others are derived. The haplotype composition may have been shaped by genetic drift due to the founder effect during colonization of industrially polluted areas. These results are in agreement with many studies of pseudometallophytes, which show reduced genetic variation in M populations as compared to NM ones (for a review see Vekemans & Lefèbvre 1997; Mengoni *et al.* 2001). Interestingly, in NM Tatra Mts. subgroup, despite the highest genetic diversity, haplotype which was specific to most of other populations and especially to NM populations from Niepołomice Forest (haplotype IV),

have not been detected. This suggested that these two subgroups of NM populations have not been directly related. The analysis of nuclear markers supported this hypothesis.

3.2. Population structure and genetic differentiation of metallicolous and non–metallicolous *A. halleri* populations from southern Poland

Despite the small geographic distances, significant differentiation for microsatellite markers was found between compared subgroups. Investigation of neutral nuclear markers showed that populations of *A. halleri* in southern Poland were clustered into two groups, mainly corresponding to geographic location. The first one comprising populations from Tatra Mts. (NM sites 32, 33) and Silesia region (M sites 15, 17, 19, 30, 21), the second one from Olkusz region (M sites 22, 24, 27, 28, 29) and Niepołomice Forest (NM sites 12, 13, 14). Interestingly, M populations from Silesia region clustered distinctly the M populations from Olkusz region, in spite of their close relationship based on cpDNA. This suggests that they have diverged independently, however from common ancestor (*i.e.* Tatra Mts. populations). It should be stressed, that similary to cpDNA markers, also microsatellite diversity was lower in M populations than in NM from Tatra Mts. Reduction of gene diversity during foundation of M populations could have caused strong genetic bottleneck (Bradshaw 1984; Luikart *et al.* 1998). Thus, colonisation of metal polluted areas might have been an important feature of the recent history of *A. halleri* and could have influenced on genetic structure of species in southern Poland. On the other hand, results indicate close relationship of NM populations from Niepołomice Forests with M populations from Olkusz region (populations clustered together in this study). It is a strong support for supposed recent foundation of NM by neighborhooding M populations in studied area. This is the first evidence for an evolution in this direction (from M to NM) in *A. halleri*. Recently Pauwels *et al.* (2005) in the study at a large geographic scale on *A. halleri* population structure and history, proposed an hypothesis that M populations have been founded independently in distinct polluted areas by their nearest NM. Moreover, authors suggested that the foundation of M populations was not accompanied by strong founder events. However, their survey was performed on sampling with hundreds kilometers distances between sites, the genetic gaps among the populations occurred.

Interestingly, in the present study "newly" founded NM populations in Niepołomice Forest have reduced genetic variation, as compared to the closest M populations. It is then possible, that also during colonization of non–metalliferous area by plants from metalliferous sites, a strong genetic bottleneck might occur.

Assessment of divergence time of A. halleri populations during recent colonization of investigated sites in southern Poland

Results presented above highlighted the most probably migration routes of *A. halleri* during colonization of M and NM sites in region of southern Poland. It should be kept in mind that these data are highly dependent on the mutation rate, which was not estimated directly. Newertheless, the estimations of time of divergence were consistent with a recent colonization of metalliferous areas. In particular, in 14th century M populations in Silesia region were founded, while in 17th century M populations in Olkusz region. Recently, in 18th century M populations from Olkusz region have been split into NM populations in Niepołomice Forest.

Summing up, in the investigated area of southern Poland 2 types of NM populations were identified: (1) ancestral "ancients" NM populations in non–contaminated natural species habitats in Tatra Mts., (2) evolved recently, as a result of two successive episode of colonization: first one on metalliferous sites and second one on non–contaminated sites beyond the mountains.

IV. ZINC ACCUMULATION IN POLISH POPULATIONS OF *ARABIDOPSIS HALLERI* UNDER CONTROLLED CONDITIONS

1. Materials and methods

1.1. Plant material – greenhouse plant collection

From August to September 2005 fifteen populations of *Arabidopsis halleri* were sampled in southern Poland (Tab. 17). The physic–chemical analysis of plants' and soils' field samples (see chapter II) showed that the closely located sites (within the same region) presented similar properties and similar plant responses to heavy metals. For that reason, to the present study 12 selected populations were included. Seven of them were located in industrial areas (19, 30, 15, 17, 22, 24, 27) and were defined as metallicolous (M); four (12, 13, 14, 32) were located outside industrial areas and were defined as non–metallicolous (NM). Though site 21, located near an industrial area of Silesia region, was considered to be in particular category and qualified as NMp (non–metallicolous in a polluted region) (see chapter II 3.1).

Table 17. Studied populations of *A. halleri* in southern Poland. M – metallicolous populations; NM –non–metallicolous populations; NMp – non–metallicolous populations in industrial area.

Site	Type	Location	Geographic coordinates		Zn (mg·kg^{-1})	
			N	E	Total	Extractable
32	NM	Western Tatra Mts.	49°16'26.94"	19°52'41.76"	125 ± 19	6 ± 2
12	NM	Niepołomice Forest	50°06'24.36"	20°21'55.98"	103 ± 12	8 ± 2
13	NM	Niepołomice Forest	50°06'35.64"	20°21'40.26"	160 ± 10	18 ± 2
14	NM	Niepołomice Forest	50°06'31.80"	20°22'02.88"	169 ± 20	18 ± 4
22	M	Bukowno	50°16'58.08"	19°28'43.38"	3911 ± 340	103 ± 13
24	M	Bolesław	50°17'00.18"	19°29'05.64"	14964 ± 4395	348 ± 203
27	M	Galman	50°11'36.78"	19°32'15.12"	35942 ± 27393	3631 ± 824
15	M	Wełnowiec	50°17'12.96"	19°01'32.04"	10163 ± 5085	311 ± 200
17	M	Wełnowiec	50°16'57.12"	19°01'46.98"	10642 ± 4146	184 ± 65
19	M	Miasteczko Śląskie	50°30'12.84"	18°56'08.34"	1167 ± 732	47 ± 32
30	M	Miasteczko Śląskie	50°30'10.03"	18°56'20.02"	1481 ± 882	47 ± 23
21	NMp	Bibiela	50°29'45.66"	18°59'00.12"	327 ± 139	7 ± 3

In each study site a 60 m long transect was set out. Seeds from one plant were sampled each 3 m, to avoid clone sampling (Van Rossum *et al.* 2004). From each site, seeds of 25–30 *A. halleri* plants were obtained. Seeds were stored at 4^0C. In March 2006, seeds were sown in compost in mini–glasshouses.

After 5 weeks, seedlings presenting new leaves were transplanted individually into 1–l pots of greenhouse soil, at a rate of two clonal replicates of each plant individual. Pots were randomly distributed in the greenhouse. They were kept under controlled conditions, with a 20^0C day temperature, 15^0C night temperature and 16 h of light per day. This plant collection (195 genotypes) (Fig. 29) was used for two different phenotypical experiments: hydroponic and pot experiment.

Fig. 29. Greenhouse plant collection.

1.2. Cultivation in controlled condition

Four NM (32, 12, 13, 14), seven M (22, 24, 27, 15, 17, 19, 30) and one NMp (21) *Arabidopsis halleri* populations from greenhouse collection were included in the present study. In November 2006, 5 cuttings per plant from presented collection were generated. Then, cuttings were dipped in rooting hormone, planted in 100–ml pots with garden soil (five cuttings per pot) and grown five weeks in mini–glasshouses. After five weeks of growth in mini–glasshouses, three clonal replicates of each plant individual (three seedlings per genotype) presenting new leaves were planted out. They were transplanted into 1–l pots filled with Zn contaminated soil. Zinc concentration was chosen to provide stressing but non–toxic environment and to allow the measurements of life history traits on a large number of *Arabidopsis halleri* individuals. Contaminated soil was prepared using a garden soil. Pots containing 650 g of dry soil were complemented with zinc sulfate – heptahydrate ($ZnSO_4 \cdot 7H_2O$, Merck) (Fig. 30). Solution of Zn (50 ml; 100 mM) was added individually into each pot and soil was left until equilibrium for two days before transplantations. The final concentration of Zn in the soil was 500 $mg \cdot kg^{-1}$.

In order to homogenize, soil was thoroughly mixed up in each pot separately, before putting plant into the pot. One seedling was transplanted into each pot. Five hundred eighty five pots (195 genotypes x 3 replicates) were randomly distributed in a complete block design, placed under controlled greenhouse conditions, with optimal luminosity and normal photoperiod (Fig. 31). Plants were watered gently with deionised water twice a week.

Fig. 30. Complementing of soil with Zn.

Fig. 31. *Arabidopsis halleri* in Zn contaminated soil.

1.3. Zinc content, shoot mass production and mineralomass of tested plants

After 5 weeks of growing in Zn treatment, shoots of plants were harvested. Sampled material was washed with deionised water and dried at 60^0C for 48 h in order to keep the weight of the sample stable. Total dry weights of shoots were then measured.

The zinc content of shoots was determined using the colorimetric reagent *zincon* ({2–[ct–(2–hydroxy–5–sulfophenylazo)–benzylidene]–hydrazino} benzoic acid) with the method of Macnair and Smirnoff (1999). Dry matter was homogenized and 0.025 g samples of ground tissue were dissolved in 750 µl of 2% sulphosalicylic acid ($C_7H_6O_6S \cdot H_2O$) and left for 24 h. 4 µl of each sample was mixed with reagent *zincon* (40 µl; 0,03 %) and a buffer solution (156 µl; pH 9,6). After 5 min, absorbance of the tested sample was measured at 606 nm on a microplate absorbance reader (SUNRISE Tecan V 3.17, Austria). Two independent samples per plant were analyzed. Zinc concentrations were expressed as $mg \cdot kg^{-1}$ shoot dry weight.

The mineralomass of zinc for each plant was then determined as product of dry weight and metal concentration in shoots.

1.4. Statistical analyses

In the present study all samples were grouped into three edaphic types: metallicolous (M), non–metallicolous (NM) and "non–metallicolous in industrial area" (NMp). Firstly, because of empirical reason, related to physic and chemical analysis of field samples (see chapter II) NMp plants could not be classified neither within NM nor within M group. Then, NMp group contained only one population, nevertheless because of sample size of this population comparable with the size of remaining M and NM populations, this data set was considered as a third group in the statistical analyses.

Repeatability of performed measurements: linear regression

In order to verify method used in the present study to analyze plants' Zn content, for each tested plant Zn content was determined in two independent samples of homogenized material. Then the recurrence between measurements' results was determined by linear regression analysis using Statistica 8.0 integrated package.

Phenotypic differences between plants and part of genetic variance: analysis of variance and related analyses

For each measured trait (*i.e.* zinc accumulation abilities, shoot mass production and mineralomass of Zn), the phenotypic differences between plants

from different edaphic type (M, NM or NMp), either different populations, were analyzed with a hierarchical analysis of variance (ANOVA), after square transformation of data to respect the normality and variance homogeneity assumptions. On the measured parameters, the GLM procedure in SAS v. 9.1 (SAS Institute, 2002) was performed with the following model:

$$Y_{ijkl} = \mu + orig_i + pop(orig)_{ij} + geno(pop\ orig)_{ijk} + \varepsilon_{ijk}$$

where*:*

- μ is the common effect for the whole experiment,
- $orig_i$ is the effect of edaphic types (origin) (i = metallicolous, non–metallicolous or "non–metallicolous in industrial area"),
- $pop(orig)_{ij}$ is the effect of population *j* (*j* = 32, 12, 13, 14, 22, 24, 27, 15, 17, 19, 30, 21) nested within the origin i,
- $geno(pop\ orig)_{ijk}$ is the effect of genotype *k* (*k* = 1, …, 195) nested within the population j which is nested within the origin i,
- ε_{ijk} represents the random error.

Edaphic origin of plants was a fixed factor; population and genotype were random factors. The part of environmental variance (V_E) was estimated by dividing the value of environmental variance by the total phenotypic variance (V_P), using the mean square values (MS) from the ANOVA (MS_{error}/MS_{total}). The genetic components (V_G) of phenotypic variation were estimated as $V_P–V_E$ (Sleper & Poehlman 2006).

In order to assess the differences between accumulation abilities, shoot mass production and mineralomass of zinc between populations, the Tukey–Kramer test for multiple comparisons of means was applied ($p<0.05$) using SAS software v. 9.1 (SAS Institute, 2002).

Then, for each measured trait, a Bartlett's test was performed to compare homogeneity of variances across samples grouped among edaphic types (origin). Analyses were performed using SAS software v. 9.1 (SAS Institute, 2002).

Comparison with the field observation: linear regressions

The comparison of mean shoot zinc concentrations in the controlled conditions to that in the field of investigated *A. halleri* populations, was achieved to assess the inherited of accumulation abilities. Because of homogenized impact of Zn soil concentration in the experiment in controlled conditions, zinc bioaccumulation factor (BF) has been chosen as a field parameter, whereas zinc accumulation values were used

as experimental parameter. The bioaccumulation factor, defined as a ratio of Zn concentration in shoots to soil Zn concentration measured in the rhizospheric soil (in this study: the total Zn concentration in soil), is indeed usually used in order to estimate quantitative expression of accumulation (Deram *et al.* 2006). As the quotient form, BF can eliminate the differences in soil metal contamination impact on plants' accumulation. Such factor assessing the accumulation abilities of field collected samples allows to comparing directly metal accumulation abilities of plants originated from both contaminated as well as normal soils.

Then, to assess the influence of Zn toxicity growth conditions on Zn accumulation, the linear relationship between mean Zn accumulation in controlled conditions and the mean soil zinc concentrations for each population was achieved.

2. Results

Repeatability of Zn content measurements

The comparison of the results of measurements performed on two independent samples at each investigated plant, in term of above–ground zinc content, was made to evaluate the accuracy of chosen method. Results (Fig. 32) showed strong correlation (R^2 = 0.91; p < 0.001) between replicate samples, what proved strong repeatability of measurements. For this reason only first data set was used in all statistical analyses.

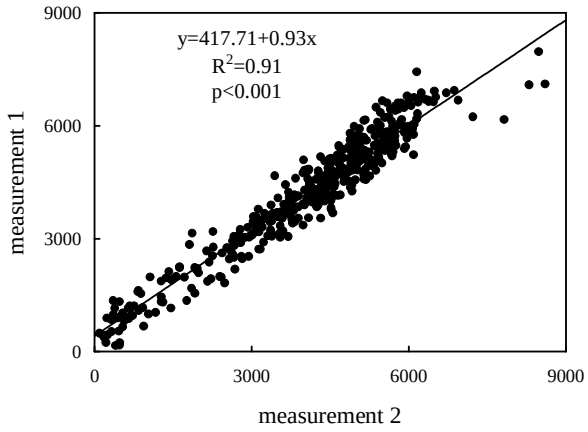


Fig. 32. Linear regression between two independent measurements of zinc concentrations in shoots of plants growing in Zn contaminated soil.

2.1. Effect of soil zinc contamination

Content of zinc in shoots, shoot mass and mineralomass of Zn, determined for plants growing in Zn treatment, were presented in Table 18.

Table 18. Zinc concentrations ($mg \cdot kg^{-1}$ d.wt) in shoots, shoot mass (g) and mineralomass (mg) of Zn in *A. halleri* plants, treated in zinc contaminated soil in greenhouse experiment ($\bar{X} \pm SD$). M – metallicolous populations; NM – non–metallicolous populations; NMp – non–metallicolous populations in polluted region; n_i – number of phenotyped individuals.

Site number	Type	n_i	Zn concentrations	Shoot mass	Mineralomass of Zn
32	NM	16	5211 ± 187	0.81 ± 0.05	4.33 ± 0.26
12	NM	14	5273 ± 198	0.54 ± 0.05	2.98 ± 0.27
13	NM	18	4452 ± 176	0.51 ± 0.05	2.43 ± 0.24
14	NM	16	4874 ± 178	0.59 ± 0.05	2.85 ± 0.25
22	M	11	4233 ± 230	0.41 ± 0.06	1.69 ± 0.32
24	M	15	4454 ± 210	0.49 ± 0.06	2.18 ± 0.29
27	M	15	4313 ± 206	0.58 ± 0.06	2.56 ± 0.29
15	M	10	2815 ± 206	0.45 ± 0.06	1.40 ± 0.29
17	M	15	3051 ± 199	0.52 ± 0.05	1.70 ± 0.28
19	M	14	4086 ± 198	0.46 ± 0.05	1.99 ± 0.28
30	M	10	4417 ± 244	0.36 ± 0.07	1.56 ± 0.34
21	NMp	10	4715 ± 231	0.80 ± 0.06	4.10 ± 0.32

Zn accumulation

An ANOVA analysis showed that metallicolous, non–metallicolous and "non–metallicolous from polluted area" *A. halleri* plants differed in shoot's zinc concentration ($p < 0.05$; Tab. 19a). No significant variation between NMp and NM populations was detected, but significantly different responses to Zn between NMp and M were found (Fig. 33a). Significant differences were found between and within populations ($p < 0.001$ and $p < 0.05$, respectively, Tab. 19a). The average zinc content of investigated populations varied from 2815 to 5273 $mg \cdot kg^{-1}$ d.wt (Tab. 18). The lowest concentrations were measured in metallicolous populations originated from Wełnowiec (populations 15 and 17; $p < 0.001$, Tukey Kramer test), while the highest concentrations of Zn were found in non–metallicolous populations from Niepołomice Forest (12) and Tatra Mts. (32) (significantly different from metallicolous populations $p < 0.01$; Tukey Kramer test; Fig. 33a). In NMp individuals from Bibiela (21) relatively high concentrations were observed, similar to those of NM plants.

The genetic variance component was 58.5 % of the value of total variance observed among individuals in case of Zn concentrations in shoots of treated plants.

Plants' growth

The three edaphic types had significantly different shoot biomass ($p < 0.05$; Tab. 19b). No significant differences were found between populations within these types, while significant variation was observed between genotypes ($p < 0.001$). The average shoot mass of investigated populations varied broadly from 0.36 to 0.81 g d.wt (Tab. 18). The lowest values were measured in population from Miasteczko Śląskie (30), while the highest shoot mass were found in populations from Bibiela (21) and Tatra Mts. (32) ($p<0.05$; Tukey Kramer test; Fig. 33 b).

For shoot mass production, the part of genetic variance over total amount of phenotypic variance was 76.9 %.

Mineralomass

The three edaphic types had significantly different mineralomass of zinc ($p<0.05$; Tab. 19 c). NMp plants' response was similar to that from NM type, but significantly different from M. No significant difference between populations within these types was found, while significant difference was observed between genotypes ($p<0.001$). The average mineralomass of Zn of investigated populations varied from 1.40 to 4.33 mg (Tab. 18). The lowest values were found in population from Wełnowiec (15), while the highest were determined for Tatra (32) and Bibiela (21) populations ($p < 0.05$; Tukey Kramer test; Fig. 33 c).

The genetic variance component was 77.9 % of the value of total variance observed among individuals, in case of mineralomass of Zn of tested plants.

Table 19. Variation of *A. halleri* plant (among edaphic origins M *vs* NM and populations) for **(a)** zinc accumulation, **(b)** shoot mass and **(c)** mineralomass of zinc. Plants were treated in zinc contaminated soil in greenhouse experiment. The table shows the ANOVA results for three measured traits. The level of significance is noted n.s. when it is not significant.

(a) Zn accumulation

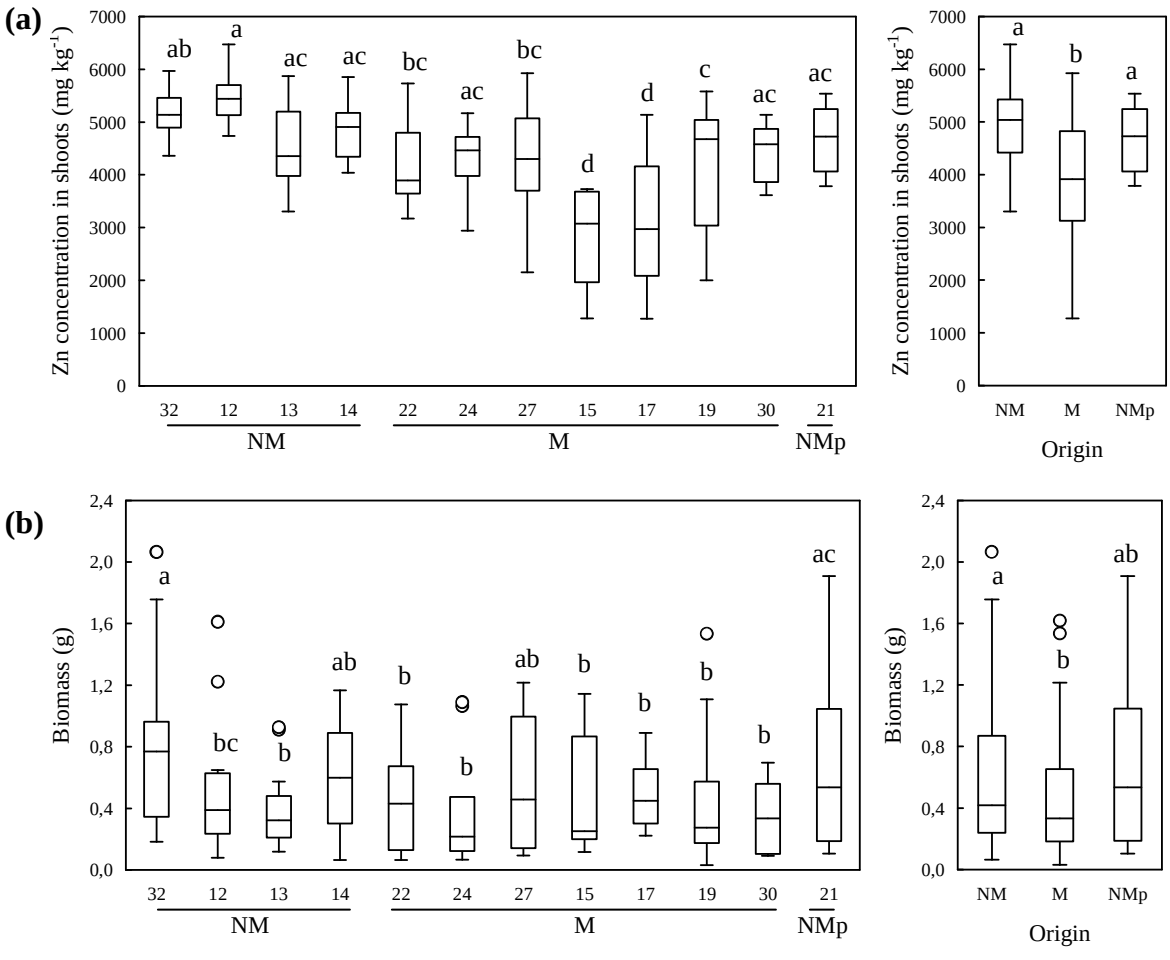
Source of variation	df	SS	MS	F	p
among origin	2	92391572	46195786	4.19	0.046
among populations within origin	9	99295054	11032784	6.45	<0.001
among genotypes within population	144	246380519	246380519	1.33	0.025
Error	254	326974127	1287299		

(b) shoot mass

Source of variation	df	SS	MS	F	p
among origin	2	0.432	0.216	4.44	0.045
among populations within origin	9	0.437	0.048	0.73	n.s.
among genotypes within population	144	9.52	0.066	5.14	<0.001
Error	254	3.27	0.013		

(c) mineralomass

Source of variation	df	SS	MS	F	p
among origin	2	82.7	41.3	7.99	0.010
among populations within origin	9	46.56	5.17	0.95	n.s.
among genotypes within population	144	780	5.42	4.61	<0.001
Error	254	298	1.17		



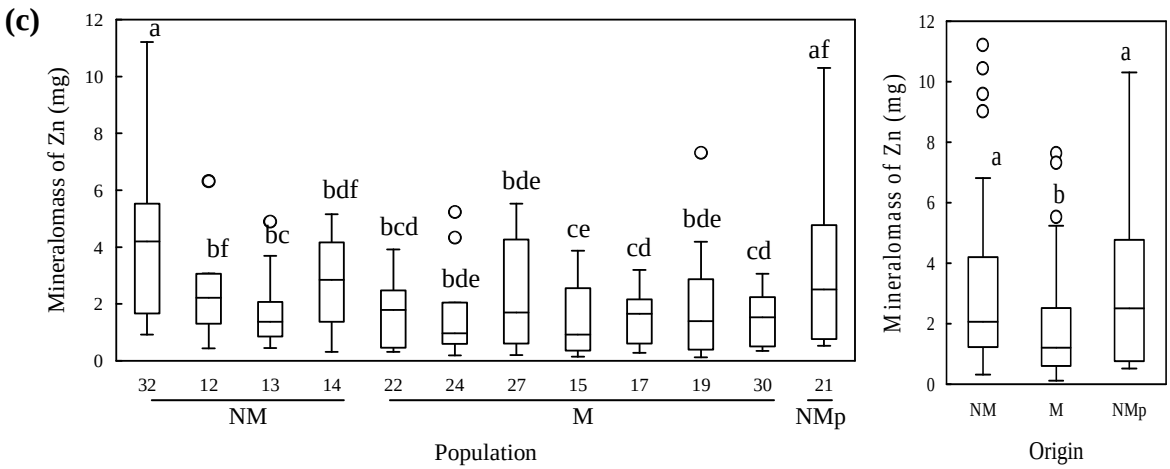


Fig. 33. Box plots of **(a)** Zn concentration in shoots ($mg \cdot kg^{-1}$), **(b)** shoot mass (g) and **(c)** mineralomass of Zn (mg), for *A. halleri* plant samples from greenhouse experiment. The boxes represent the interquartile range, with median (indicated by horizontal lines), minimum and maximum (indicated by whiskers). Circles are outliers (*i.e.* any data that is distant from the upper or lower quartile by more than 1.5 times the standard error). Different letters above the box plots indicate a significant difference at the 5% level (Tukey–Kramer test). M – metallicolous populations, NM – non–metallicolous populations, NMp – non–metallicolous populations in polluted region.

Then, the homogeneity of variance for all measured parameters was tested by the Bartlett's test (Tab. 20). In this analysis, samples were grouped by edaphic types. For Zn accumulation as well as shoot mass and mineralomass of Zn, no significant differences were found between variances across grouped samples.

Table 20. Results of the homogeneity of variance analysis, performed on **(a)** Zn concentration in shoots, **(b)** shoot mass and **(c)** mineralomass. Populations were grouped within edaphic types (origin NM and M). Analysis was carried out without population 21 (NMp). The level of significance is noted n.s. when it is not significant.

Source of variation	df	Khi 2	p
(a) Zn concentration in shoots			
among origin	2	2.678	n.s.
(b) shoot mass			
among origin	2	18.9	n.s.
(c) mineralomass of Zn			
among origin	2	65.7	n.s.

2.2. Comparison with the field observation

This experiment tested whether there was genetic variation within *A. halleri* populations for zinc accumulation. Additionally, results were compared to data concerning accumulation abilities in investigated *A. halleri* populations in the field, which were an objective of chapter II of this study. Both, field and experimental observations, in terms of phenotypic variation, could contribute to the achievement of a better knowledge of adaptive value of zinc accumulation and its phenotypic variation.

To evaluate relationships between the experimental and field data sets, the comparison of mean shoots zinc concentration of investigated *A. halleri* populations performed in presented experiment, with zinc bioaccumulation factor (BF) as a field parameter, was made. Results (Fig. 34) show weak but significant correlation ($R^2 = 0.42$; $p < 0.001$) between field and controlled condition data set.

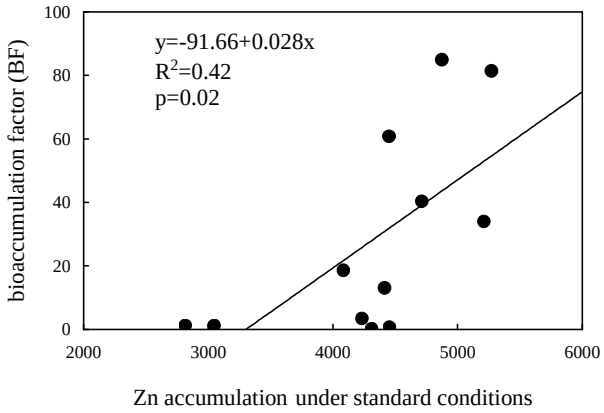


Fig. 34. Mean population zinc accumulation ($mg \cdot kg^{-1}$ d.wt) in controlled conditions plotted against the mean bioaccumulation factor (BF) in the field. BF – ratio of metal concentration in shoots to total concentration in soil (see Tab. 9).

The relationships between zinc content in plants (from presented experiment) and soils' zinc contents (from field study) were also assessed. Results (Fig. 35) show, that there is no significant trend between Zn content in plants and neither total nor available soils' zinc contents.

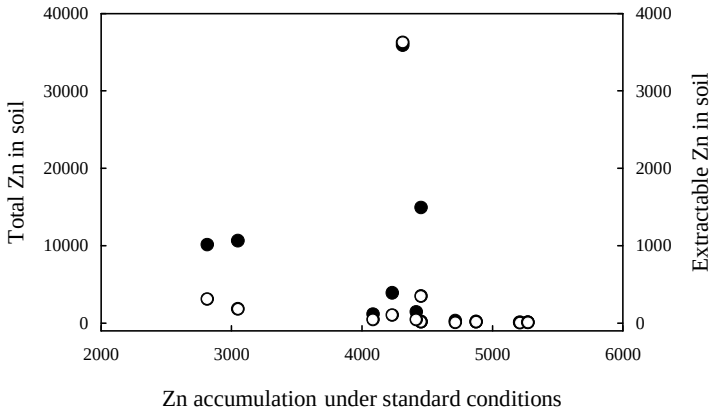


Fig. 35. Mean population zinc accumulation (mg·kg^{-1} d.wt) in controlled conditions plotted against the mean total (left axis Y) and available (right axis Y) zinc concentration (mg·kg^{-1} d.wt) of the soil of sites of respective populations. Black circles: total metal concentration; white circles: available metal concentration (see Tab. 17).

3. Discussion

3.1. The ability of *A. halleri* to accumulate zinc

In the present study the Zn accumulation analysis performed on 12 M, NM and NMp *A. halleri* populations revealed that all investigated *A. halleri* populations, independently from their edaphic type, accumulate Zn in shoots at similar and high level, when they grow on soils with Zn content. Additionally, in presented short–term controlled experiment, the slight but significant tendency to the higher accumulation level in NM populations, as compared with M, was observed. Interestingly, NMp plants exhibited relatively high level of Zn accumulation and then similar to plants of NM edaphic type. Therefore, zinc accumulation can be considered as a species–wide or constitutive property in *A. halleri,* as it was already concluded by Bert *et al.* (2000). To our knowledge, ability to accumulate Zn of *A. halleri* NMp populations has never been studied.

Until now, there have been only a few studies on variation in zinc accumulation, conducted on *A. halleri.* Bert *et al.* (2000) and Macnair (2002) showed that, when *A. halleri* from populations on both metalliferous and non–metalliferous soils are grown in standard conditions in glasshouse or solution culture, the physiological ability to hyperaccumulate is a constitutive property. However, they also demonstrated differences between *A. halleri* populations in their ability to accumulate zinc in their

shoots (Macnair *et al.* 1999; Bert *et al.* 2000; Macnair 2002). Bert *et al.* (2000) studied zinc accumulation in two populations of *A. halleri*. They found that the population from the uncontaminated site accumulated more zinc than the one from a contaminated site, what is consistent with those performed in the present study on populations from small region of southern Poland. Authors suggested that high accumulation was not disadvantageous for populations on non–metalliferous sites, as it had been already stated in *Thlaspi caerulescens* studies (Boyd & Martens 1998a; Pollard & Baker 1997). Interestingly, in terms of zinc accumulation ability of *A. halleri* populations from southern Poland, results from pot and field experiments, showed significant relation between the maternal plants collected in the field and the plants' from greenhouse experiment. These results suggest that variation in zinc accumulation between populations was heritable.

3.2. Relationship between Zn accumulation and soil Zn concentration

In the present study, as well as in the Macnair's (2002), accumulation abilities found in investigated *A. halleri* populations were unrelated to the zinc content (neither total nor available) in the soil sites' in which the populations originated. It is possible, that plant metal concentration could be relatively insensitive to soil metal concentration over a wide range of soil metal levels if the curve relating plant metal concentration to soil concentration saturates at quite a low external zinc concentration (Baker 1981). Results of genetic analysis of *A. halleri* from southern Poland (see chapter III) highlighted that some NM populations (sampled from uncontaminated sites in Niepołomice Forest) could have been recently founded by M plants from metallicolous area. Then, because of such recent colonisation, as well as constitutive character of Zn accumulation of *A. halleri*, no correlation between accumulation abilities and site contaminated property could be observed. In recently founded populations the relationship between plants and soils properties could be still stabilizing and that's why their direct comparison is not possible. Similarly, no relation between accumulation abilities of *A. halleri* populations with soil zinc content were found by Bert *et al.* (2000) as well as by Macnair (2002). Macnair (2002) showed that the Zn concentration of field collected plants was affected partly by plant genotype and not by the total content of Zn in soil around their roots. Thus, such results may simply have been due to the chance selection of the particular populations studied

(Macnair 2002) and Zn accumulation in *A. halleri* could be directly related to a factor other than Zn, and thus could be of wider ecological significance. However, it should be pointed, that detected soil Zn content, neither total nor available, might not reflect real biodisponibility of metal for investigated plants, because of physical and chemical parameters of soils influence on Zn absorption by plants.

Interestingly, results from pot and field experiments in term of zinc accumulation ability of *A. halleri* populations from southern Poland, showed significant relation between the maternal plants collected in the field and the plants from greenhouse experiment. These results might suggest that variation in zinc accumulation between populations was heritable.

3.3. Genetic variation within populations for Zn accumulation

The genotypic differences between populations are of great interest to researchers trying to understand and manipulate the genetics of accumulation (Pollard *et al.* 2002). However, in term of the evolutionary background of accumulation, it seems more important to examine variation within populations, because this is the level at which natural selection might act to favor the evolution of hyperaccumulation, if individuals accumulating more metals have greater reproductive success (Pollard 2000).

In this study, significant variations in the ability to accumulate zinc also within populations were found, which is in agreement with Macnair's (2002) results. In order to test what factors lead to this variation, plants grew in controlled environmental conditions and in the same concentrations of zinc (to reduce the environmental variance). Then, the variance observed among *A. halleri* individuals from different soil types was mostly determined by genetic components. Nevertheless, relatively high part of environmental variance could be determined by heterogeneity of cuttings (= clones from the same genotype) at the start of pot experiment and some environmental heterogeneity in the greenhouse. It appears that although the ability to hyperaccumulate is largely a pervasive property within species, significant within populations genetic variation in accumulation levels exists.

There have been only a few studies of genetics of within–population variability of this character, and the majority of studies has been conducted on the Zn / Ni / Cd hyperaccumulator *T. caerulescens* (Pollard & Baker 1996; Meerts & Van Isacker 1997;

Escarré *et al.* 2000). Recent analyses of zinc accumulation in *Arabidopsis halleri* were conducted by Macnair (2002). Macnair (2002) tested the ability of 15 *A. halleri* populations to accumulate zinc, under standard conditions, and he found considerable variation between and within populations in this character. His results indicated that selection for increased accumulating ability should be possible in discussed species.

3.4. *Arabidopsis halleri* – the Zn hyperaccumulator plant potentially useful for phytoremediation

In the present study, it was shown that plants from non–metalliferous sites appear to accumulate Zn more effectively than those from metalliferous sites, and so could represent a valuable resource of genotypes for phytoextraction. Presented results have also demonstrated that the metal accumulation does not relate to the biomass level (data not shown). However, a higher mineralomass of zinc was found in non–metallicolous populations which performed better zinc uptake. This suggests, as it was already proposed by Bert *et al.* (2002), that with increasing total Zn soil content, the Zn content of the aerial parts of the plants reaches a plateau. Genetic variation in Zn concentration may indicate that different genotypes achieve different plateaus. Hamon *et al.* (1999) suggested that a plateau response could be due to plant physiology and be explained by blocking of the translocation of metals from the roots to the shoots and /or saturation of the metal uptake mechanism at the root surface. Thus, plateau response could be a safety mechanism that limits plant metal uptake and prevents phytotoxicity at high metal soil concentration. It could be then supposed that, in non–metallicolous *A. halleri* populations this safety mechanism for Zn did not occur.

In the present study, plants from NM population from Tatra Mts. (site 32) and NMp population from Bibiela (site 21) were found as the most efficient phytoextractors. Additionally, under natural conditions, as it was already presented in chapter II related to field observation, bioaccumulation factor of these populations was relatively high (34.0 and 40.3, respectively), which confirm their potential utility in phytoextraction. On the other hand, in hydroponic culture (chapter V), plants from presented populations have been shown relatively low level of Zn tolerance, as compared with investigated M populations. Nevertheless, it should be stressed that, the controlled and rather artificial environments in performed experiments

were far from reflecting natural conditions in *A. halleri* habitats. Future works, using transplantation experiments *in situ* should be then performed in order to verify plants' response to soil Zn concentration and their efficiency in phytoextraction in the natural sites. According to results of presented study, populations from Tatra Mts. and Bibiela seem to be the best candidates and then it would be recommended to use them in preliminary tests.

V. ZINC TOLERANCE IN POLISH POPULATIONS OF *ARABIDOPSIS HALLERI* UNDER CONTROLLED CONDITIONS

1. Materials and methods

1.1. Plant material

Ten *Arabidopsis halleri* populations (Tab. 21) from greenhouse collection (see chapter IV 1.1) were included to the present study: 3 non–metallicolous (= NM: 32, 13, 14), 6 metallicolous (= M: 24, 27, 15, 17, 19, 30) and one "non–metallicolous in industrial area" (= NMp: 21). The chemical analysis of plants' and soils' field samples (see chapter II) as well as experiment of Zn accumulation ability in controlled conditions (see chapter IV) showed that the closely located sites (within the same region) presented similar properties and similar plant responses to heavy metals. For that reason, no more than two populations from each area were chosen to this experiment. For further details on investigated populations see chapter II 1.1.

Table 21. Studied populations of *A. halleri* in southern Poland. M – metallicolous populations; NM – non–metallicolous populations; NMp –non–metallicolous populations in polluted region; n_i – number of phenotyped individuals.

Site	Type	n_i	Location	Geographic coordinates	
				N	E
32	NM	14	Western Tatra Mts.	49°16'26.94"	19°52'41.76"
13	NM	10	Niepołomice Forest	50°06'35.64"	20°21'40.26"
14	NM	10	Niepołomice Forest	50°06'31.80"	20°22'02.88"
24	M	6	Bolesław	50°17'00.18"	19°29'05.64"
27	M	9	Galman	50°11'36.78"	19°32'15.12"
15	M	6	Wełnowiec	50°17'12.96"	19°01'32.04"
17	M	8	Wełnowiec	50°16'57.12"	19°01'46.98"
19	M	6	Miasteczko Śląskie	50°30'12.84"	18°56'08.34"
30	M	7	Miasteczko Śląskie	50°30'10.03"	18°56'20.02"
21	NMp	4	Bibiela	50°29'45.66"	18°59'00.12"

Whole 80 plant individuals (= genotypes) from investigated populations were tested in three independent series of experiments (due to limited available space in controlled environment growth chamber), performed from December 2006 to January 2007, from April to June 2007 and from July to September 2007 respectively. In each series, about 40 to 50 genotypes were tested. Twelve cuttings per genotype have been generated. Cuttings were directly placed in 1l polyethylene pots containing

hydroponic solution (3 cuttings per pot) and grown for rooting within the basal nutrient solution containing 10 µM of zinc ($ZnSO_4$, $7H_2O$). After a preculture period, 6 the biggest and the most homogenous rooted cuttings per genotype have been selected and included in the experiment.

It should be pointed out that in the present experiment, cuttings from population 21 did not acclimatise to hydroponic conditions and this population was represented only by 4 genotypes.

1.2. Hydroponic culture conditions

Hydroponic culture conditions were established during a preliminary screening test performed in order to define the most discriminating conditions for zinc tolerance.

After two or three weeks of preculture, performed to allow plants to root and acclimatise to hydroponic conditions in nutrient solution, selected rooted cuttings were distributed into two groups submitted to two different concentrations of Zn. Three clones per genotype were treated with a hydroponic "treatment" solution containing 2000 µM of Zn, whereas three others were grown in the "control" solution with

10 µM of Zn. Plants were transferred individually to 1l polyethylene pots and grown in suitable concentrations another six weeks. The experiments were arranged as follows: plant genotypes (80) x genotype replicates (3) in each two conditions (control solution at 10 µM Zn and Zn treatment at 2000 µM Zn). Cuttings were randomly distributed in each block (Fig. 36).

Fig. 36. Hydroponic culture experiment.

Each 1l pot was filled with slightly modified half–strength Hoagland's nutrient solution: 20 µM FeEDDHA, 500 µM $MgSO_4$, 1 mM $NH_4H_2PO_4$, 0.1 µM $(NH4)_6Mo_70_{24}$, 0.1 µM $CuSO_4$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 1 µM KCl, 0.1 µM NaCl, 2 mM $Ca(NO_3)_2$, 3 mM KNO_3, and 10 or 2000 µM Zn added as $ZnSO_4$ (Hoagland & Broyer 1936). The pH of the solution, adjusted at 5.5 with KOH, was maintained constant using 3 mM MES (2–morpholinoethanesulphonic acid), which is known to be chemically inert towards metals. The nutrient solution has been renewed once a week by fresh solution of the same composition.

The experiment was performed in a controlled growth chamber with the following conditions: photoperiod of 13–h day, temperature of 20^0C day / 17^0C night, and a relative humidity of 80 % during 2 or 3 first weeks of preculture and 65 % for the other weeks.

1.3. Assessment of Zn tolerance in tested plants

The monitored traits

At the end of the 6^{th} week of culture, the following traits have been scored for each plant: leaf width of the three largest leaves, root length, chlorophyll fluorescence and chlorophyll content. Chlorophyll fluorescence and content measurements were performed on three young leaves considered as the most typical and representative of each plant. The width of leaves was measured in the widest part of leaf laminas. Chlorophyll content and chlorophyll fluorescence were measured on the lamina, midway between the base and tip of mature leaves. Chlorophyll fluorescence was determined with field portable, pulse amplitude, modulated fluorometer (PAM–2100, Walz, Effeltrich, Germany). Fluorometer operation and data processing were conducted with a Hewlett Packard palmtop computer (HP 200LX). Relative chlorophyll content in leaf samples was determined with a hand–held chlorophyll absorbance meter (CL–01 Chlorophyll Content Meter) following the instructions recommended by the manufacturers. Plants were harvested at the end of the experiments: shoots and roots were separated, dried at 60^0C during 48 h and their respective dry weights were recorded.

Tolerance index

For each tested genotype, traits were scored in Zn treatment and control solution. On the basis of both values, a tolerance index (TI) has been calculated.

For each genotype, the value of life history traits of each clone grown in the Zn treatment was divided by the median value calculated with the values of the three clones of this genotype grown in the control solution. Due to low values of within–genotype variance in control solution (10 µM of Zn), medians have been calculated for each genotype. For leaf traits, three repetitions of measurements were performed on each clone, so that the median values of repetitions were calculated for each clone.Therefore, for each genotype and for each of 6 monitored traits, 3 values of TI were calculated according to the formula:

$$TI = \frac{\text{trait of clone in 2000 } \mu\text{M of Zn}}{\text{trait of genotype in 10 } \mu\text{M of Zn}}$$

As the quotient form, TI can eliminate most of the variation in tested plant responses unrelated to metal treatment.

1.4. Statistical analyses

In the present study, all samples were grouped into three edaphic types: metallicolous (M), non–metallicolous (NM) and "non–metallicolous in industrial area" (NMp). Firstly, because of empirical reason, related to physic and chemical analysis of field samples (see chapter II) NMp population could not be classified neither within NM nor within M group. Secondly, because of small sample size of NMp population in hydroponic experiment (only 4 genotypes), this data set was not considered as a third group and was excluded from statistical analyses.

Similarity between the three series of experiments: exact test

Plants were tested in three independent series, but observations on experimental units were computed under the same conditions. Therefore, data sets could be pooled after statistical verification of similarity between series in control solution. From all traits measured during the experiments, the total biomass *i.e.* coproduct of shoots and roots biomass, was recognized as a parameter integrating all other measured parameters and was chosen to test for the similarity of results obtained in each series of experiment. Then, in each series of experiments, the median of total biomass appointed for all plants treated in control solution was computed in M and NM edaphic types.

Because of small and unbalanced sample sizes, differences between medians were assessed using nonparametric Kruskal–Wallis exact test ($p < 0.05$). Data sets that could not be pooled were considered as different strata. Exact tests have been performed using the statistical package StatXact v.8 of the base software Cytel Studio (2008).

Share of genetic variance: analysis of variance

To estimate share of genetic variance in total phenotypic variance, a hierarchical analysis of variance (ANOVA) was performed. On the measured tolerance indexes, the GLM procedure in SAS v. 9.1 (SAS Institute, 2002) was performed, with the following model:

$$Y_{ijkl} = \mu + orig_i + pop(orig)_{ij} + geno(pop\ orig)_{ijk} + \varepsilon_{ijk}$$

where*:*

- μ is the common effect for the whole experiment,
- $orig_i$ is the effect of edaphic types (origin) (i = metallicolous or non–metallicolous),
- $pop(orig)_{ij}$ is the effect of population j (j = 19, 30, 15, 17, 24, 27, 21, 13, 14, 32) nested within the origin i,
- $geno(pop\ orig)_{ijk}$ is the effect of genotype k (k = 1, …, 119) nested within the population j which is nested within the origin i,
- ε_{ijk} represents the random error.

Edaphic origin of plants was fixed factor; population and genotype were random factors. The part of environmental variance (V_E) was estimated by dividing the value of environmental variance by the total phenotypic variance (V_P), using the mean square values (MS) from the ANOVA (MS_{error}/MS_{total}). The genetic components (V_G) of phenotypic variation were estimated as $V_P–V_E$ (Sleper & Poehlman 2006).

Assessment of Zn tolerance: exact tests and Principal Component Analysis

Because of data organization in different strata, for each appointed TI the differences between M and NM edaphic types in tolerance ability were analysed by a Wilcoxon–Mann–Whitney exact test for independent samples, using the statistical package StatXact v.8 of the base software Cytel Studio (2008).

To reduce the number of variables explaining the variance between individuals, and to appoint combination of parameters explaining differentiation between tested plants, a Pearson correlation matrix and PCA analysis based on covariance matrix were computed for all TIs using Statistica 8.0 integrated package.

Then, on the basis of the coordinates on PC1, a Bartlett's test was performed to compare homogeneity of variances across samples grouped among edaphic types (origin M, NM), using SAS software v. 9.1 (SAS Institute, 2002).

By PCA analysis the most sensitive TI accounting for difference between edaphic types was detected. Then, the differences of zinc tolerance – based on appointed TI – between populations were assessed. Because of small and unbalanced sample sizes nonparametric Kruskal–Wallis exact test ($p < 0.05$) has been performed using the statistical package StatXact v.8 of the base software Cytel Studio (2008).

2. Results

Similarity between the three series of experiments

The comparison of the results of three series of experiments, in terms of total biomass, was made to evaluate the possibility of pooling the data. Results (Fig. 37) show that series 1 is significantly different from the two others ($p < 0.05$), whereas series 2 and 3 are similar ($p > 0.05$). Because of this, the results of series 1 were regarded as distinct (stratum n° 1) whereas series 2 and 3 were pooled and considered as a single stratum (stratum n° 2) in all comparison tests (exact tests).

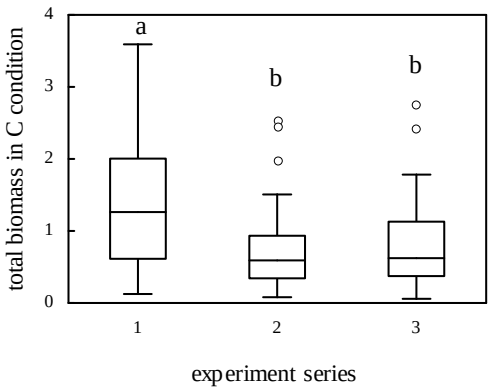


Fig. 37. Box plots of total biomass of plants grown in control (C) condition in hydroponic culture, by series of experiment. The boxes represent the interquartile range, with median (indicated by horizontal lines), minimum and maximum (indicated by whiskers). Circles are outliers (*i.e.* any data that is distant from the upper or lower quartile by more than 1.5 times the standard error). Different letters above the box plots indicate a significant difference at the 5% level (exact Kruskal–Wallis test).

2.1. The effect of zinc concentration on edaphic types

Arabidopsis halleri responses to different Zn concentrations were compared between M and NM plants, in both control and treatment solutions. Results (Fig. 38) show that zinc influences most of measured live history traits of plants from both edaphic origins.

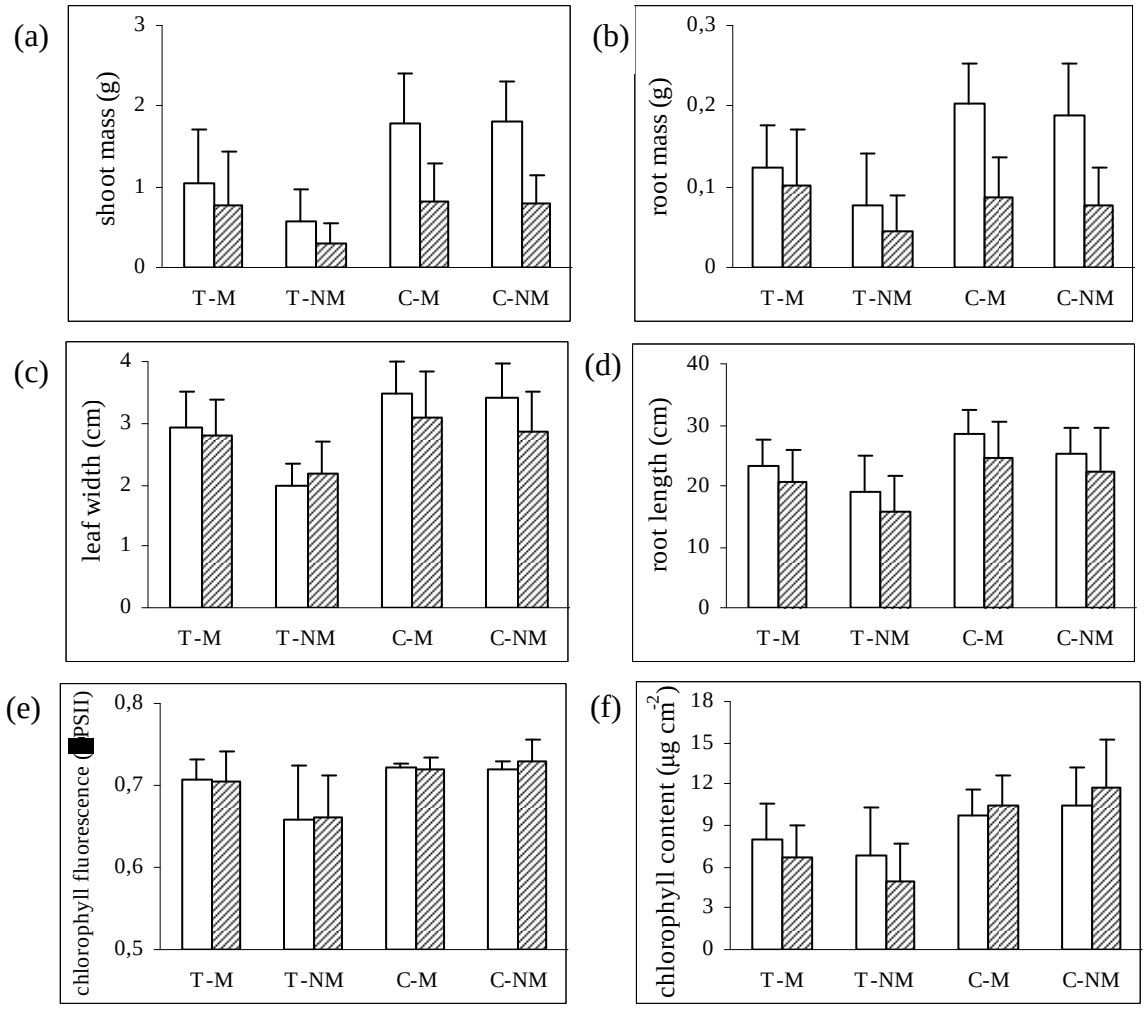


Fig. 38. Model predictions for the effect of zinc concentration on 6 (a–f) life history traits ($\overline{X}$ ± SD) for plants from stratum 1 (empty bars) and from stratum 2 (hatched bars). T–M – metallicolous plants in treatment solutions; T–NM – non–metallicolous plants in treatment solutions; C–M – metallicolous plants in control solutions; C–NM – non–metallicolous plants in control solutions.

In control condition, plants grew faster (Fig. 38 a, b, c, d) and contained more chlorophyll (Fig. 38 f) than plants in the Zn treatment solution. Zinc concentration had no effect on the chlorophyll fluorescence of metallicolous plants (Fig. 38 e),

which maintained the same performance for this trait in both levels of solution toxicity. In zinc contaminated solution, for all analyzed traits, the differences between plants of different edaphic origin were larger than in control solution. It was mainly a consequence of the fact that non–metallicolous plants suffered more from Zn toxicity. Distribution of the measured traits was comparable between strata 1 and 2, except for shoot and root mass of plants which were considerably larger in stratum 1 than in stratum 2.

2.2. Detection of the most sensitive tolerance indexes accounting for difference between edaphic types

For all analyzed traits, there was a clear trend to displaying the highest tolerance index by the metallicolous plants (Fig. 39). Distribution of tolerance indexes of M and NM plants among stratum 1 (Fig. 39 a) was comparable with their distribution among stratum 2 (Fig. 39 b). Differences in response to zinc contamination observed between M and NM plants were significant for tolerance indexes calculated for shoot and root biomass, leaf width, chlorophyll fluorescence and chlorophyll content (Tab. 22), but not for root length. For this latter trait, only a trend towards higher TI for M populations was detected ($p = 0.089$). The part of genetic component in whole variance, calculated for all tested genotypes, was given in Table 22. Only indexes for which the part of genetic variance was higher than 70 % were chosen for further analyses. Indeed, the part of genetic variance for tolerance index of shoot mass was very low (22 %). Tolerance index of shoot mass was then excluded from analyses. For all other traits, parts of genetic variations values were higher than 70 %.

Table 22. Comparison of tolerance indexes (TI) between metallicolous (M) and non–metallicolous (NM) investigated *A. halleri* populations, and the part of genetic variance (V_G) estimated for measured tolerance indexes**.**

TI	Exact Test among M and NM		Variance component
	statistic	p–value	V_G [%]
Shoot mass	30.45	< 0.001	22.0
Root mass	39.38	< 0.001	86.0
Leaf width	31.92	0.011	83.6
Root length	29.78	0.089	75.0
Chlorophyll fluorescence	34.37	< 0.001	82.7
Chlorophyll content	23.83	0.001	70.1

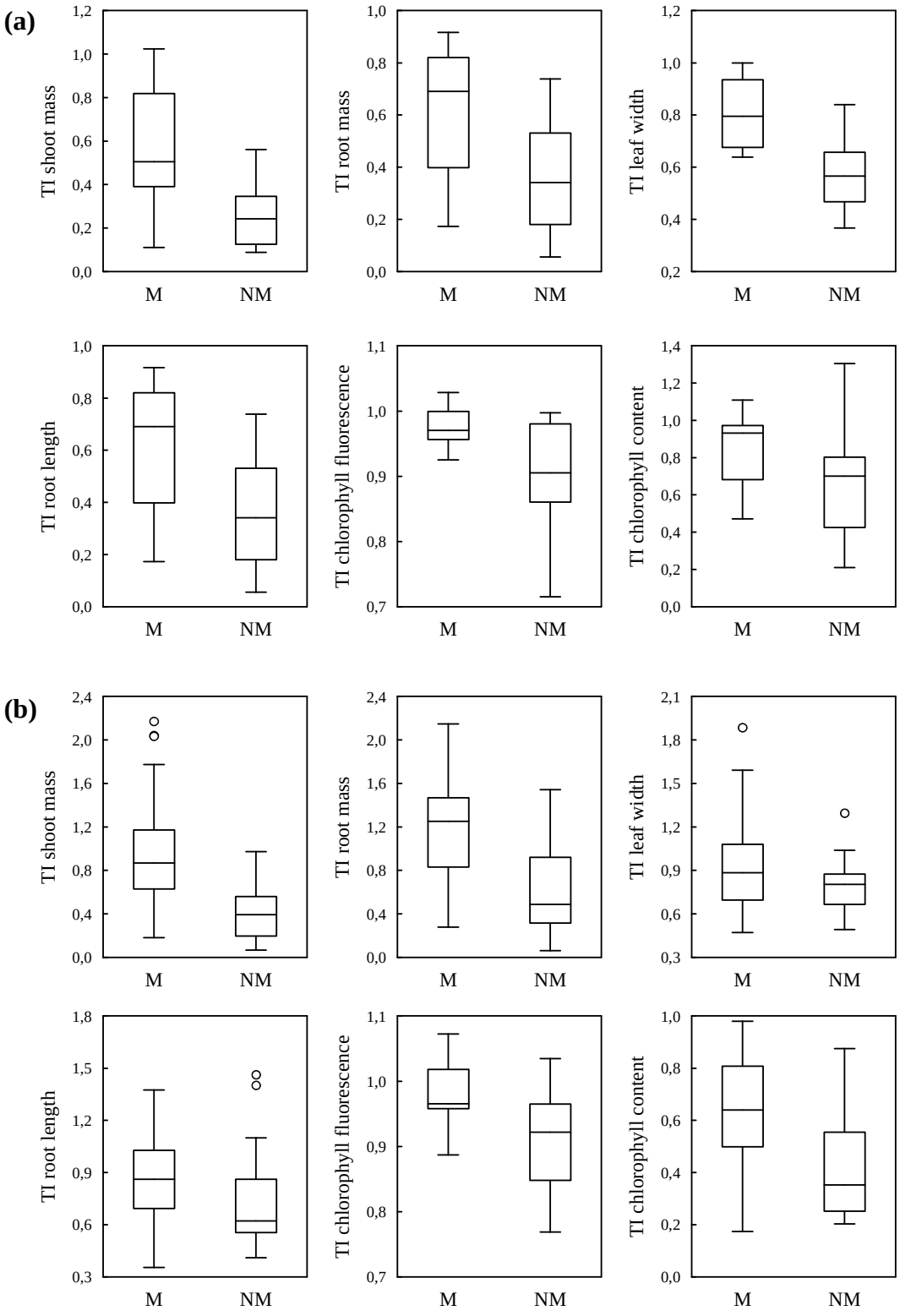


Fig. 39. Box plots of tolerance indexes of 6 measured parameters by plant origin (M or NM), for **(a)** data from stratum 1 and **(b)** stratum 2. M – metallicolous plants NM – non–metallicolous plants. The boxes represent the interquartile range, with median (indicated by horizontal lines), minimum and maximum (indicated by whiskers). Circles are outliers (*i.e.* any data that is distant from the upper or lower quartile by more than 1.5 times the standard error).

Combination of TIs to explain variance between individuals

The correlations between selected indexes were given in Table 23. All of TIs were positively correlated. The strongest correlation was observed between TI root mass and TI leaf width (0.50) and between root mass and root length (0.37).

Table 23. Results of the correlation analysis performed on five tolerance indexes (only traits for which the part of genetic variance was higher then 70 % are shown) measured on metallicolous and non–metallicolous *A. halleri* populations. The level of significance is noted * for $p < 0.05$ and n.s. when it is not significant.

TI	Leaf width	Root length	Chlorophyll fluorescence	Chlorophyll content
Root mass	0.50*	0.37*	0.34*	0.16 n.s.
Leaf width		0.19 n.s.	0.21 n.s.	0.10 n.s.
Root length			0.35*	0.19 n.s.
Chlorophyll fluorescence				0.33*

Results of the PCA of five tolerance indexes for all *Arabidopsis halleri* populations were presented in Table 24 and Figure 40. The two first principal components (PC) explained 79.61 % of the total variance (66.13 % and 13.48 % explained by PC 1 and PC 2, respectively). The first principal component separates plants with different growth strategies: lower negative values of PC 1 correspond to plant that have a big root mass, large width of leaves and long roots (Fig. 40 a). The root mass is the major source of variation along this axis. The second component was mainly related to the chlorophyll content: plant with a low negative PC 2 score contained high amount of chlorophyll (PCA loadings' values were represented graphically on the Figure 40 a: absolute values corresponded to the length of the arrow; the sign corresponded to the direction of the arrow related to the PC1 and PC2 axes)

The separation between metallicolous and non–metallicolous plants can be clearly seen in the two–dimensional space delimited by PC1 and PC2 (Fig. 40 b). The largest differences in reaction norms explained by PC 1 were found between metallicolous plants. In particular, some M individuals showed high negative values on PC1, whereas NM individuals displayed quite low negative values and a majority of positive values on this axis. Among NM, a particular category of "new"–NM populations was distinguished (see *Discussion*). Plants of this group did not show a great difference in behavior as compared to the other NM, excepting few individuals in the negative section of PC1 axis. Also NMp individuals (population 21)

were included to this analysis, in order to verify distribution of this particular group in comparison with M and NM. All investigated NMp plants displayed positive values on PC1 and then were found within the group of majority NM plants.

Table 24. PCA loadings of five (only traits for which the part of genetic variance was higher than 70 % are shown) tolerance indexes (TI) for *A. halleri* populations. PC1, PC2 – two first Principal Components.

TI	PC 1	PC 2
Root mass	–0.56	0.05
Leaf width	–0.16	0.03
Root length	–0.12	–0.15
Chlorophyll fluorescence	–0.03	–0.02
Chlorophyll content	–0.06	–0.22
Explained variation (%)	66.13	13.48

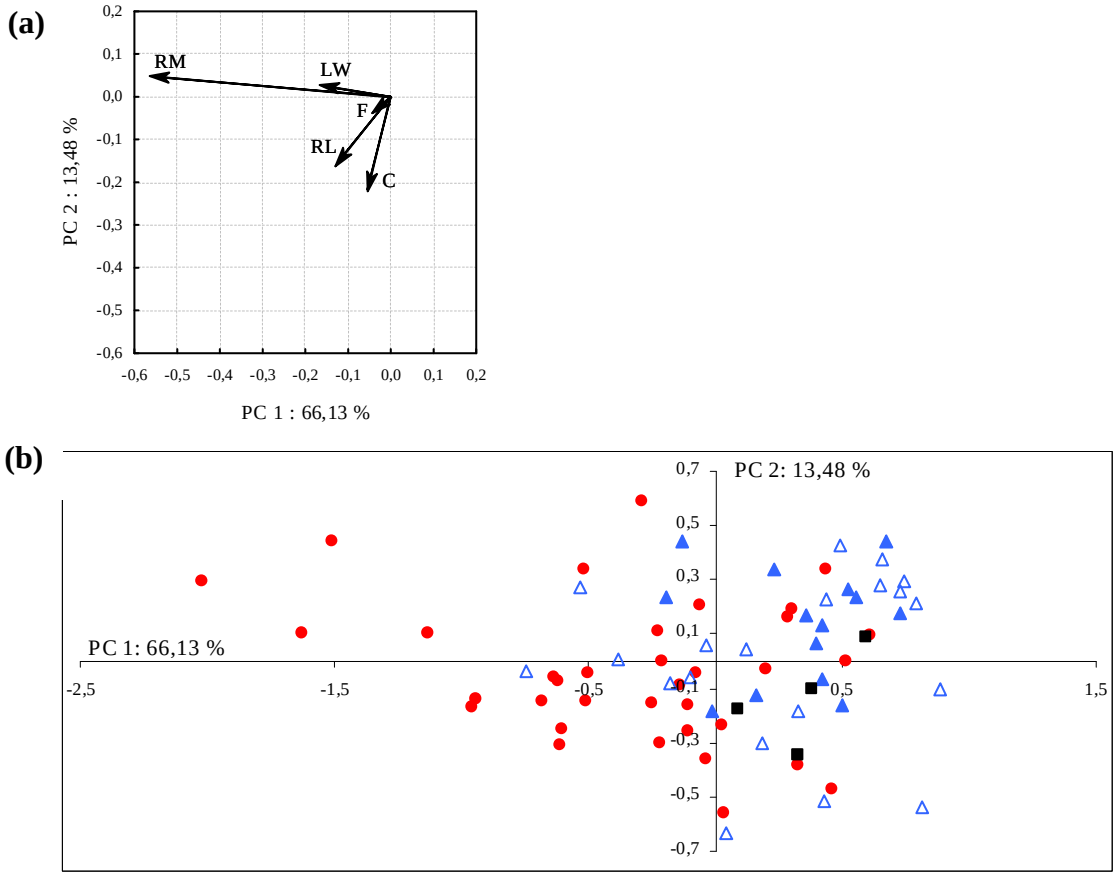


Fig. 40. (a) Correlations of five (only traits for which the part of genetic variance was higher than 70 % are shown) tolerance indexes for *A. halleri* populations and contribution of the TI root mass (RM), TI leaf width (LW), TI root length (RL), TI chlorophyll fluorescence (F) and TI chlorophyll content (C) to the first two components (as PC 1 and PC 2). **(b)** Individual scores on the first (PC 1) and the second (PC 2) principal components. Red circles represent M individuals, blue triangles – NM individuals (empty triangles – "new" NM individuals, see *Discussion* of presented chapter) and black squares – NMp individuals.

The homogeneity of variance for coordinates of the first principal component (PC 1) was tested by the Bartlett's test. In this analysis, samples were grouped within edaphic types (NM origin *vs* M). Bartlett's test affirmed higher variance within metallicolous plants than non–metallicolous ones (*df* = 1; *Khi2* = 5.436; p = 0.019).

Assessment of interpopulational variation in Zn tolerance in investigated populations

The tolerance index of root mass was the major source of total variation in presented Principal Component Analysis. Therefore it was considered to be the best trait to explain the differences between tested plants. In presented study the tolerance index based on root mass measurement varied broadly among populations (Fig. 41 a). There were no significant differences between TI of most populations. Only population 17 (Wełnowiec) was significantly different from population 32 (Tatra Mts.) ($p < 0.05$; Kruskal–Wallis test; Fig. 41 a). However, tolerance index of presented trait showed significant differences between entire metallicolous data set and entire non–metallicolous data set ($p<0.05$; Kruskal–Wallis test; Fig. 41 b). Populations NM (13, 14, 32) and NMp (21) appear to be less tolerant than the M ones (19, 30, 17, 24, 27).

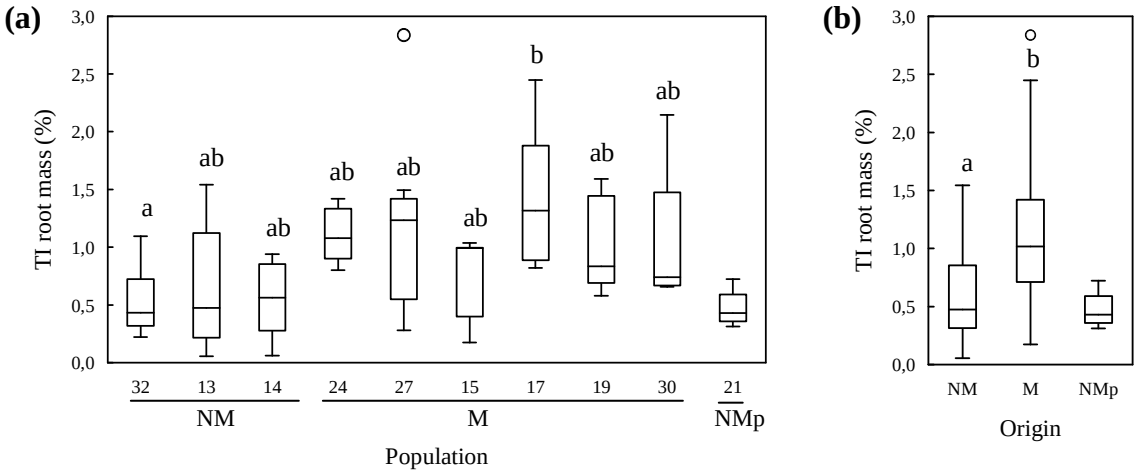


Fig. 41. Box plots showing median, minimum and maximum of tolerance indices of root mass for populations **(a)** and edaphic origin **(b)**. M – metallicolous populations, NM – non–metallicolous populations, NMp – NM populations from industrial area. Different letters above the box plots indicate a significant difference at the 5% level (Kruskal–Wallis test). Circles are outliers (*i.e.* any data that is distant from the upper or lower quartile by more than 1.5 times the standard error).

3. Discussion

3.1. Designing the best methodology to evaluate Zn tolerance in *A. halleri* populations from southern Poland

Choice of the best test

The problems linked to tolerance measurement have been and are still widely discussed (for review see Macnair 1993). As tolerance is the result of interaction between a genotype (including genes determining metal tolerance but also all other genes involved in quantitative variation of growth) and an environment it can not be unambiguously scored by comparison with a reference. One of the first approaches proposed to solve these problems in measuring tolerance is an index produced by comparing a rate of root growth in a contaminated environment with growth of the same genotype in a control environment (Wilkins 1978). However, this method requires cloning of large number of plants, which is an elaborate procedure, especially in plants without an inherent clonal growth habit. Moreover, some plant species were found to be particularly inappropriate for vegetative propagation. In order to avoid the cloning of individual plants an alternative type of tolerance test, *i.e.* the sequential exposure test (Schat & Ten Bookum 1992a) based on seedlings was established. Each individual plant was exposed to a test solution in which the metal concentration was increased in time. Tolerance was then determined as the lowest concentration (EC100) at which no new root growth was observed. This test has been recently successfully applied on seedlings from European widespread *A. halleri* populations (Pauwels *et al.* 2006).

In the present study one of the objectives of experiments in controlled conditions was to compare the variation of the tolerance and accumulation on the same individuals to determine whether a relationship exists between these two traits. To reach this objective it was necessary to work on cloned plant material. Fortunately, *A. halleri* is known to perform vegetative propagation in nature (Van Rossum *et al.* 2004) and this property has been recently successfully applied in a sequential test performed on cuttings to measure tolerance in a backcross progeny produced by interspecific cross between *A. halleri* and its non–tolerant and non–accumulated close relative *A. lyrata* (Willems *et al.* 2007). Nevertheless, because its discriminating capacities appeared

to be lower on cuttings than on seedlings (Saumitou–Laprade pers. com.) the sequential test was inappropriate to assess the variation of tolerance at the within species level.

Moreover, most of tests performed on controlled condition are based on short–term root–elongation assays (*e.g.* Wu & Antonovics 1975; Baker *et al.* 1986; Schat & Ten Bookum 1992a; Pauwels *et al.* 2006; Willems *et al.* 2007). However, hyperaccumulators like *Arabidopsis halleri* or *Thlaspi caerulescens,* store the metal mainly in their leaves, while metal concentration in roots is comparable to that in non–accumulating species (Krämer *et al.* 1996; Lasat *et al.* 1996). This fact suggests that, in case of hyperaccumulators, early responses to toxic metal exposure might appear firstly in leaves, rather than in roots. For this reason, the tests of tolerance only based on root length could be incomplete or inadequate (Assunção *et al.* 2001). Additionally, in the short–term root–elongation tests the role of genes involved in processes of internal sequestration and/or detoxification in leaves could have been underestimated or missed (Roosens 2008). Short–term tests probably do not reveal all inter– and intra–population genetic variation for plant response to metals.

Alternative approach for phenotyping hydroponic experiment has been proposed in this study, which enable to investigate the same genotypes in several independent experiments (*e.g.* accumulation test) and also to overtake uncertainties with short–term root–elongation tests in hyperaccumulator species. Additionally, proposed methodology also enable to assess genetic and environmental variance on tolerance abilities, and then to choose the most heritable traits. Such trait is essential in research of genetic adaptation. Moreover, this test when aimed to assess the variation of tolerance at the within species level, is characterised by relatively high discriminating capacities. Therefore, similar phenotyping experiment might be applied for example in studies of QTLs (Quantitative Trait Loci) for tolerance, on intra–species level (between less tolerant and very tolerant genotype).

Choice of the best trait

Both root– and shoot–based measurements were chosen as tolerance criterions, *i.e.* root biomass, production of differential root length, shoot biomass, production of differential leaf size and chlorosis (measurements of chlorophyll fluorescence and chlorophyll content). In 6–weeks test, during which plants were exposed to constant Zn treatment, for most of traits (excepting shoot biomass)

essential part of the inter– and intra–population genetic variation for plant response to metal have been revealed. Only shoot biomass was considered as inadequate to estimate Zn tolerance in investigated *A. halleri* plants, as a trait for which the variance observed among individuals was mostly determined by environmental components. This part of variance could be determined by heterogeneity of cuttings (= clones from the same genotype) at the start of hydroponic experiment. Differences between the cuttings were connected with the state of the maternal plant from greenhouse collection as well as different rooting ability. Additionally, the cuttings were formed from different parts of maternal plant, which could strongly affect the shoot mass production and differentiate clones during whole hydroponic experiment.

Presented analyses have shown that in case of *A. halleri* plants originated from a small region of southern Poland, the most relevant trait to assess populational and individual differences in responses to Zn exposure was root biomass. The advantage of this trait, in comparison of currently used root length, is that elongation growth as well as production of adventitious roots are integrated in biomass value and can be successfully measured. Nevertheless, the other traits also showed significant differences between origins of investigated populations, and among them, the chlorophyll content revealed the differences between individuals in particular. Then, the tolerance can be considered as a complex character, which is represented by many different traits. However, some of them seem to be more appropriate in a case of tolerance abilities of species investigated in the present study.

3.2. Tolerance level in tested *A. halleri* populations

A recent study, performed on whole 31 metallicolous (M), non–metallicolous (NM) and non–metallicolous from polluted area (NMp) *Arabidopsis halleri* populations, widespread in central Europe clearly established the occurrence of constitutive Zn tolerance in *A. halleri* and of quantitative variation of the degree of Zn tolerance among and within populations (Pauwels *et al.* 2006). Nevertheless, the highest mean tolerance level was observed in M populations, the lowest in NM, while NMp individuals were intermediates. The authors also pointed out, that the distribution of the polymorphism was heterogeneous and the highest phenotypic differentiation was observed within NM populations (Pauwels *et al.* 2006).

Then, in the present study performed on 15 M, NM and NMp *A. halleri* populations from southern Poland, it was expected that inter– and intra–population as well as inter– edaphic origin variation in the degree of tolerance to Zn should be found. Indeed, a higher mean tolerance level was found in M populations than in NM or NMp. Additionally, NMp plants' responses to zinc treatment seem to be similar to NM plants. However, it should be stressed, that in this study, the sample size of NMp population was very low and then the discussed differences in the response to Zn toxicity between NMp and M or NM type could be assessed cautiously.

Interestingly, no significant variation was found between populations within edaphic type, which is in contrast to data of Pauwels *et al.* (2006). Only populations with the lowest (Tatra Mts., NM site 32) and the highest level of tolerance (Wełnowiec, M site 17), showed variation in the degree of tolerance. Different arguments can be proposed to explain the discrepancy between results of presented study and Pauwels *et al.* (2006). Firstly, most of *A. halleri* genotypes used in both studies were not located in the same sites. Populations investigated in the present study originated from a small region of southern Poland, while Pauwels *et al.* (2006) study was performed on a widespread geographic area in central Europe. Then, low level of interpopulation differentiation observed in the present study could be due to relatively low geographical scale of distribution of investigated populations. Secondly, the methodologies to assess tolerance were different. In present study Zn tolerance was evaluated on the plants obtained by vegetative propagation (cuttings) at a fixed Zn concentration. The root biomass was used as a subjective measure of tolerance. On the other hand, in Pauwels *et al.* (2006) study a multiple concentration test based on the seedlings was applied. Tolerance measurements were based on root–elongation test. Indeed, the assessement of tolerance in different ways could lead to different results. The difference in tolerance assessment, as well as different propagation methods of plant material or investigation of different roots' characters to tolerance measurements, could have impact on the discrepancies observed between these two studies.

The polymorphism observed in hydroponic controlled conditions among investigated M *A. halleri* individuals was significantly higher than among NM. Higher heterogeneity among M individuals found in the present study, manifested by the farthest point on the first principal component PC 1 of the graphical presentation of ACP results (Fig. 34 b), was due to genotypes derived from several

investigated populations (*i.e.* 17, 19, 27, 30). This might suggest that this polymorphism could be a general phenomenon observed in metalliferous sites from southern Poland. This is in agreement with results from the same populations in the field (see chapter II of this study), where Polish metallicolous populations showed considerable plasticity. However, this observation is opposite to that in Pauwels *et al.* (2006), where higher within–population heterogeneity was found in NM populations than in M populations. Discrepancy between results of presented studies might be explained as above, by the different origin of populations investigated in both studies. Additionally, *A. halleri* is a pseudometallophyte species with most populations being non–metallicolous (Pauwels *et al.* 2008), which was reflected by majority of NM populations (20 out of 31) in European widespread collected sampling by Pauwels *et al.* (2006). Contrary, in the present study sampling was made in the particular area of southern Poland, where contaminated sites are frequent, and then *A. halleri* populations occur mostly on metalliferous sites. Then, in such region sampling was made mainly on M sites, so this bias towards M populations can lead to differences in results as compared to Pauwels *et al.* (2006)

3.3. Evolution of metal tolerance in *A. halleri* populations from southern Poland

In presented thesis, plants from the same populations as for the phenotypic survey have been also studied with molecular markers, in order to verify the genetic differentiation and the direction of evolution (see chapter III of the present study). Phylogenic relationships revealed that in the discussed area of southern Poland, non–metallicolous populations from Tatra Mts. were ancestral. Then, anthropogenic heavy metal polluted areas were colonised: metallicolous populations in Olkusz region (sites 22, 24, 27, 28, 29) as well as M populations in Silesia region (sites 15, 17 in Wełnowiec and 19, 30 in Miasteczko Śląskie). Recently, among the industrialized Silesia region, in low contaminated site in Bibiela, NMp population has been founded by neighborhoods M populations. Recently also, the "new" NM group in non–contaminated site in Niepołomice Forest was founded by M populations from geographically the closest metalliferous Olkusz region.

Despite the small geographic distances between investigated *A. halleri* populations, significant differentiations for microsatellite markers among populations were observed. In particular two NM populations from Tatra Mts. had the highest gene

diversity. All metallicolous populations presented intermediate values. Interestingly, NMp population from Bibiela had relatively high level of gene diversity, similar to geographically the closest M populations from Miasteczko Śląskie and Wełnowiec. The "new" NM populations from Niepołomice Forest had the lowest values for this measure.

As regard of differences in quantitative traits, in controlled conditions, the lowest tolerance abilities were observed in ancestral NM population from Tatra Mts. (site 32). This tolerance level could represent the basic level for Polish *A. halleri* populations. Oppositely, the highest tolerance abilities were found in population from Wełnowiec (site 17) from Silesia region. Population from Wełnowiec was located on a large waste heap from zinc smelter, which started to exist in the first half of 19th century. In the present study, this is a the only site situated on heavy metal smelter waste heap (except population 15, which was located on the same heap but on its top, on a mostly unfavorable dry and sunny place, so under much higher stress conditions; see chapter II). It might then be suggested that because of this kind of heavy metal origin, strong natural selection could have acted during colonization on this heap and then Zn tolerance appears to evolve secondary towards enhanced abilities, highest than in other populations. Interestingly, the most tolerant population found in the present study was also the most tolerant within sampling of Pauwels *et al.* (2005) study of 64 *A. halleri* populations widespread in central Europe. However, in the present study all other Polish M and "new" NM as well as NMp populations, represented a continuum, with a following trend towards decreasing tolerance ability: M > "new" NM > NMp. Superior tolerance to Zn found in M populations might suggest, similar to what was proposed by Pauwels *et al.* (2005) that, during colonization of polluted areas the evolution towards increased tolerance has occurred in the M populations.

As it was suggested by Pauwels *et al.* (2005, 2006), the features of NMp populations and then "new" NM should be understood with respect to the narrow genealogical relationships with surrounding M populations. Interestingly, the absence of strong metal pressure in recently colonized NM sites did not greatly changed plants tolerance capacities. Lower molecular genetic diversity found in "new" NM populations, as compared with the closest M populations, might suggest a genetic drift following a founder event. Then, comparison of patterns of variation for quantitative traits and molecular markers gives further support

to the hypothesis that, these populations may simply have inherited enhanced tolerance level and then tolerance character did not change because of probable weak "cost of tolerance" on non–metallicolous sites. In a contrary, in a particular group of NMp the average tolerance level was lower than in neighborhoods M and no differentiation between these edaphic types for neutral molecular markers was found. This could suggest a local selection on discussed sites. However, because of very low sample size of investigated NMp population, this tendency should be assessed cautiously.

VI. GENERAL DISCUSSION AND PERSPECTIVES

History and characteristics of Polish Arabidopsis halleri populations

Investigating natural populations system is of great importance to understand the phenomenon of plant adaptation to heavy metal contaminated environments. In field studies, concentrations of metals in soil and shoots represent real conditions and therefore reflect a realistic behavior of plants. In addition, study of a wide range of polymorphism in the field allows the examination of the intensity of the natural selection which may influence the populations. Differentiation between field populations may be therefore due to the local adaptation to different environments.

Variation of soil properties and population behavior within M type of sites was higher than within NM type. This may result from different histories of industrial activities in studied M site, where metals originated from different sources such as mining, Zn smelter pollution, heavy metal smelter waste heap. Zinc contamination was from soil or/and atmospheric origin. Moreover, this study showed that M and NM sites differed significantly not only in terms of heavy metal concentration but also other physic–chemical soil parameters (i.e. C_O, N, P, K, Mg, Fe). Contrasting selection regimes due to different conditions in M and NM habitats might affect behavior of plants and then differentiated populations from different sites. Therefore, local M and NM *A. halleri* populations should be specifically adapted to the local soil metal composition as well as to other sites' properties. The knowledge about properties of sites could be helpful in reconstruction of species history on studied area as well as to explain the differences observed between populations which arise from the local adaptations.

Evolutionary relationships between *A. halleri* edaphic types are poorly known. In wide European range of species it was suggested that M populations have been founded by closely located NM (Pauwels *et al.* 2005), as mine or industrial polluted sites were recently created. This is generally true because we have no evidence for pollution on ancient refuge in Tatra Mts.; however, in the present study, in low geographical scale, it was shown that foundation of NM populations by M is also possible. This was observed in Niepołomice Forest. This is the first study which reveals such an evolutionary event and shows evidence of different origins of populations within the same edaphic type. Moreover, in this study, for the first time, it was shown that NM populations from the same geographic region might be genetically

independent. The behavior of "new–NM" (= nNM) populations, recently evolved from closely located M, differs from that of ancestral NM populations. There was also another particular site, in Bibiela, considered as "non–metallicolous in a polluted region" (= NMp). Despite localization in industrial area of Silesia region, this site was slightly polluted and then considered as NM, while *A. halleri* plants from this field revealed chemical characteristic similar to M populations. Therefore populations from both, Niepołomice Forest and Bibiela sites, may differed from "true" NM ones by their abilities to exchange genes with geographically proximate M populations.

This fact suggests that populations should be studied independently, with respect of sites conditions and history of populations. While category of NMp population was already proposed by Pauwels *et al.* (2006), category of nNM has never been described before this study.

Metal accumulation capacity of Polish populations

In the field, populations from different edaphic types accumulated Zn at very different level, while in the pot experiment, under uniform controlled conditions, most of populations accumulated Zn at similar level. Moreover, it was observed that in the field some of populations showed the capacity to mobilize and concentrate metal in shoots (i.e. populations from Niepołomice Forest, Tatra Mts. and Bibiela), while the others have been limited the uptake of metal (i.e. populations from Galman, Bolesław and Wełnowiec). Interestingly, populations which accumulated the highest amounts of Zn in the field and in the controlled experiment were not the same. Moreover, accumulation ability in the field seemed not to be a direct function of soil metal content (neither total nor extractable).

This might indicate that the adaptive value of the hyperaccumulator phenotype is independent of soil metal contamination (as it was already suggested by Bert *et al.* (2000)). Thus, other environmental factors *(e.g.* organic matter content, cationic exchange capacity, water retention capacity) than metal content must influence heavy metal accumulation. It should be stressed that metals could be associated to various soil components from studied sites, so that metals' availability, closely related to their chemical speciation, might differed between sites. On the other hand, it is also possible that amount of extractable metals determined in this study was not the fraction really mobilized by plants. The relationship between metal extraction

by *Arabidopsis halleri* and the exchangeable metals from substrates amended with various metal–bearing solids were already studied by Dahmani–Muller *et al.* (2002). The results showed different concentrations of exchangeable metals after plant growth, depending on the nature of the metal–bearing solids. Therefore in studied region of southern Poland Zn–uptake by *A. halleri* might be also affected by the metal–bearing solids.

Finally, it was shown that accumulation is genetically determined, so that variance observed between populations in the field was both genetics and environment–dependent. Accumulation is then the result of interaction between genotype and environment.

Relationship between Zn accumulation and tolerance in A. halleri

In the present study, the tolerance and accumulation abilities were determined on the same individuals (on cloned plant material in experiments in controlled conditions) in order to verify whether it exists a relationship between these two traits. If hyperaccumulation is a direct mechanism of tolerance, or a pleiotropic consequence of such mechanism, hyperaccumulation ability should be expected to be strongest in plants from the most toxic soil (Pollard *et al.* 2002). However, in controlled conditions, NM populations of *A. halleri* were significantly less tolerant and accumulated Zn to significantly higher concentration as compared to M populations. A similar kind of relationships between higher tolerance and lower accumulation was also found in studies of *Thlaspi caerulescens* that populations from non–contaminated sited were able to accumulate more Zn than populations from contaminated sites (Lloyd–Thomas 1995; Meerts & Van Isacker 1997; Escarré *et al.* 2000). Limited plateau of metal accumulation observed in M populations of hyperaccumulator species might be then considered as a cause of higher tolerance as compared with NM. This might suggest some negative correlation between tolerance and accumulation. This tendency was also observed regarding general populations characteristics, but no relationship was revealed regarding traits measurements on each genotype (data not shown). This is probably because of mechanisms complexity underlying both traits at the plant level.

Up to now, Zn tolerance genetic architecture in *A. halleri* has been studied through interspecific crosses performed between *A. halleri* and its closest non–tolerant relative *A. lyrata petraea* (Macnair *et al.* 1999). Segregation of Zn tolerance

and accumulation suggested that these characters are genetically independent. Recently, by quantitative trait loci (QTL) analysis performed on one BC1 generation, three genomic regions for Zn tolerance (ZnTol1, ZnTol2 and ZnTol3), as well as Cd tolerance (CdTol1, CdTol2 and CdTol3) were identified in *A. halleri* (Filatov *et al.* 2006; Courbot *et al.* 2007; Willems *et al.* 2007; Roosens *et al.* 2008a). Interestingly these results based on two independent phenotyping experiments identified a co–localization of QTL regions ZnTol1 and CdTol1. Because calamine sites contain generally both Zn and Cd, it was more parsimonious to suppose, that metal tolerance initially evolved in *A. halleri* through the fixation of a single mutation conferring tolerance to both metals (Roosens *et al.* 2008a). This single and early event could be responsible for the constitutive tolerance observed in the whole *A. halleri* species and therefore be involved in the first processes having drive a possible ecological speciation process. After the fixation of this QTL, specific tolerance to Zn or Cd concentrations in the soil might have evolved through the fixation of additional QTLs that are particular to each population according to the specific chemistry of its metalliferous growing site (Roosens *et al.* 2008a). Interestingly, co–localization of Zn accumulation (ZnAcc1) and tolerance (ZnTol1) QTL with the major QTL for Cd tolerance (CdTol1) was also proved (Roosens *et al.* 2008b). Recently, two other studies conducted on an F2 interspecific progeny have confirmed that this common QTL confers Zn tolerance and Zn accumulation (Frérot *et al.* in prep.) as well as Cd tolerance and accumulation in *A. halleri* (Willems *et al.* in prep.). These results of genetic studies, based on interspecific crosses, suggest that, at least, a partial relationship between tolerance and hyperaccumulation is present in studied species. However, it should be stressed that such a relationship could not exist at intraspecific level.

Designing a relevant methodology to evaluate Zn tolerance in A. halleri populations from southern Poland

Because of many problems with hitherto used short–term root–elongation tests in hyperaccumulator species (for review see Macnair 1993), as well as of necessity to use cloned material, an alternative approach for phenotyping hydroponic experiment has been worked out in this study. The method's principles are following: (1) replicates of each tested genotypes are obtained by vegetative propagation; (2) three clones per genotype are treated with a hydroponic "treatment" solution (containing 2000 µM

of Zn), whereas three others are grown in the "control" solution (containing 10 µM of Zn); (3) plants are cultivated in Zn constant solutions during 6 weeks; (4) at the end of the 6th week of culture various root– and shoot–based traits are scored for each plant (in Zn treatment and control solution); (5) on the basis of both values (in Zn treatment and control solution), a tolerance index is calculated for each tested genotype. It was shown that in case of *A. halleri* plants originated from a small region of southern Poland, the root biomass have been the best trait to revealed the differences between plants' edaphic types in responses to Zn exposure.

The main advantages of proposed method are the assessment of genetic and environmental variance components on tolerance abilities, as well as the investigation of the same genotypes in several independent experiments. Moreover, it was shown that the use of this test at the within–species level is characterized by relatively high discriminating capacities. Therefore, similar phenotyping experiment might be applied for example in studies of QTLs for tolerance. Indeed, presented method may allow selecting parents with contrasted phenotypes (less tolerant and very tolerant) used in subsequent interspecific crosses, and also used in progeny phenotyping. It should be then verified whether QTLs which could be determined with such a test would be identical to QTLs already detected in previous studies, and then whether the same genes are involved in tolerance to the short and long–term plant exposure to Zn contaminated solution.

As it was already shown, the metal stress could have a more important impact on the reproductive than vegetative traits (Ernst & Nelissen 2000; Ryser & Sauder 2006). In addition, several works highlighted the diversity of the answers of the various reproductive traits to the metal stress as delay of flowering (Ryser & Sauder 2006; Brown 2001), absence of flowering (Saikkonnen *et al.* 1998; Ernst & Nelissen 2000), decrease of seeds quality (Ernst & Nelissen 2000; Izmaiłow 2002; Biskup & Izmaiłow 2004), reduction of seeds production (Brown 2001) and absence of seed (Piccini & Malavolta 1992). Indeed, the survival and the growth of plants in controlled experiments do not guarantee a survival and a reproductive success at a more advanced stage of life. It should be noted that, traits measured in controlled conditions could reflect only a part of the adaptation to metalliferous soils. Therefore, reciprocal transplantations of metallicolous and non–metallicolous genotypes, on metalliferous and non–metalliferous sites from a single geographic region, should be performed.

The measurements of life history traits (including both vegetative and reproductive traits) should reveal the whole capacity of NM and M genotypes to colonise M and NM sites and then test for local adaptation.

Is there evidence of local adaptation in Polish populations of A. halleri?

Controlled experiments with *A. halleri* populations from different edaphic types revealed differences in plants' accumulation and tolerance abilities between NM and M types. The quantitative phenotypic polymorphism observed in the present study could be explained, as it was already proposed for several pseudometallophytes, by minor effects of hypostatic modifier genes (Macnair 1993; Schat *et al.* 1993; Smith & Macnair 1998; Van Hoof *et al.* 2001). Modifier genes might have been locally selected to enhance tolerance in investigated *A. halleri* populations. Following the field (see chapter II), experimental (see chapter IV & V) and genetic survey (see chapter III) that concluded different types of sites in studied area (NM, M, NMp and nNM), different plant's behavior between and within type of sites and different origin of populations, it was further suggested that selected genes might differ in separately founded population. Molecular analysis of these populations, on neutral nuclear and chloroplast markers, shows that populations from ecologically different sites were also genetically differentiated. Gene flow between ancestral populations from Tatra Mts. and all others is weak, which suggests that strong divergent selection might shape patterns of observed phenotypic variations during colonization of M as well as NM sites outside Tatra Mts. The combination of high tolerance level and reduced genetic polymorphism in M populations support the hypothesis that, despite constitutive character of accumulation and tolerance in *A. halleri*, natural selection towards enhanced tolerance and limited metal uptake occurred during recent colonization of heavy metal contaminated sites. However, despite strong differences between M sites (in term of metal content and sites' history), no evidence supporting the existence of diversifying local adaptation patterns among M edaphic type was found. The highest tolerant and most accumulating populations were not located on the most contaminated soils, which imply that the strength of selection might be not directly related to the local degree of soil heavy metal contamination and thus tolerance and accumulation could be of wider ecological significance (6 hypotheses have been already proposed to explain the reasons for which plants accumulate high amount of heavy metals; for review see Boyd and Martens

(1992) and Boyd (1998) and chapter I 2.2 of this study). Also the gene flow between geographically closely located populations (M populations were not genetically different from each other) might strongly decrease the structuring effect of selective pressure and then selection can not act to adapt populations to local conditions in M sites.

Intermediate levels of tolerance and accumulation might be related to the recent history of nNM and NMp types of populations (see above). Lower molecular genetic diversity found in nNM populations, as compared with the closest M populations, might suggest a genetic drift following a founder event. Then, comparison of patterns of variation for quantitative traits and molecular markers gives support to the hypothesis that during colonization of NM area by plants from M sites, a strong genetic bottleneck might have occur, and then low level of metal in soil and "cost of tolerance" on NM sites could have secondarily resulted in a reduction of tolerance abilities. In the contrary, in the particular group of NMp, no differentiation between NMp and M edaphic types for neutral molecular markers was found despite the lower tolerance levels than in neighborhoods M. This could suggest a local selection on discussed sites. However, reciprocal transplantation experiments *in situ* are needed to measure the "cost of tolerance" and convincingly demonstrate local adaptation of Polish *A. halleri* populations.

Recently, field reciprocal transplantations of M and NM populations of *Thlaspi caerulescens* were performed to determine the pattern of local adaptation and to assess the cost of adaptation of M populations to a metalliferous environment (Dechamps *et al.* 2008). It was shown that an imbalance of selective forces shown between M and NM environment exists. Selection in the M environment was found to be strong and specific, and making colonization by a foreign genotype extremely difficult. In contrast, the NM ecotype did not show fitness superiority compared with the M ecotype in the NM environment. The authors suggested that colonization of the NM environment by the M ecotype is then more probable than the colonization of the M environment by the NM ecotype. These results are in agreement with genetic survey performed in the present study, when nNM *A. halleri* populations were founded in Niepołomice Forest.

Results of this study confirmed reasonableness of hypotheses have been made at the beginning of the study.

Perspectives

Results of this study showed that the *A. halleri* populations from southern Poland have a promising potential for the phytoremediation of Zn contaminated soils. Among all studied populations, two presented the highest values of mineralomass of zinc: population 32 from Tatra Mts. and 21 from Bibiela. Indeed, these populations might be considered as particularly interesting in the phytoremediation aspects.

Furthermore, in this study the most and the less tolerant and accumulating populations have been detected. This intraspecific variability can be explored in order to gain knowledge about mechanism of metal tolerance and accumulation. The most interesting populations, determined in this study, might be sources of parental genotypes to generate the intraspecific cross progeny (F2). In F2 progeny derived from reciprocal crosses between the most tolerant – the less accumulating population (pop. 17 from Wełnowiec) and the less tolerant – the most accumulating population (pop. 32 from Tatra Mts.) the segregation patterns of Zn tolerance and accumulation might be studied. Such study of interpopulation crosses will give information about the genetics and interrelationships of these traits, trough comparative transcriptome analysis and QTL mapping.

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