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Heavy metal tolerance in contrasting ecotypes of *Alyssum montanum*

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ABSTRACT

The response of metallicolous (M) and nonmetallicolous (NM) *Alyssum montanum* ecotypes to multi-metal stress was investigated under *in vitro* condition and compared in this study. Shoot cultures were simultaneously treated with 0.7 mM $ZnSO_4$, 3.0 μM $Pb(NO_3)_2$ and 16.4 μM $CdCl_2$ for 8 weeks and evaluated for their morphogenetic and ultrastructural reaction, growth tolerance as well as ability to Zn, Pb, and Cd uptake. Moreover, tissue localization and concentrations of antioxidant compounds were determined in order to elucidate the potential role of ROS-scavenging machinery in plant tolerance to metal toxicity. The results clearly demonstrated that M specimens treated with heavy metals showed less phytotoxic symptoms and low level of lipid peroxidation than reference NM one. The enhanced tolerance of M ecotype resulted from heavy metals detoxification in trichomes and intracellular leaf compartments as well as balanced ROS accumulation. The inactivation of ROS in M plants was based on peroxidase-flavonoid system, while in NM plants such relationship was not detected and amounts of antioxidant enzymes or phenolic compounds was comparable to untreated specimens or decreased significantly. Considering the procumbent growth of such hemicryptophyte which reproduce effectively in the presence of heavy metals but is characterized by low biomass production, it is proposed to exploit M ecotype of *A. montanum* in revegetation schemes of polluted calamine wastes to provide the prompt stabilization of areas prone to erosion.

1. Introduction

Most heavy metals (HMs) constitute a severe threat to living organisms due to their high toxicity, inability to transform into less toxic forms and long-term persistence in the environment (Sidhu et al., 2017). In plants HMs induce numerous kinds of alterations which could be analyzed at the cell, tissue, and organ level. The diverse structural and functional disorders resulted either from direct interaction of toxic metals with structural components or a more indirect consequences of specific changes in metabolic processes (Jin et al., 2008; Mukhopadhyay et al., 2013; Costa et al., 2017; Feng et al., 2018). Regardless of the origin, such disturbances lead to growth and development inhibition in the range depending on the stressed genotype. One of the factors causing plant tissues injury, following exposure to HMs, is enhanced and uncontrolled production of reactive oxygen species (ROS) (Ortega et al., 2017). These highly reactive molecules contribute to lipid peroxidation, an autocatalytic process changing the structure and function of membranes, that might be evaluated by the level of thiobarbituric acid reactive substances (TBARS) (Islam et al., 2008). To protect cells against toxic oxygen intermediates, plants are equipped with the complex antioxidant defense mechanism that involves both enzymatic and non-enzymatic antioxidants. Among the most prominent enzymes superoxide dismutases (SOD), catalases (CAT) and a large number of H_2O_2-reducing peroxidases (POX), like guaiacol-type peroxidases (GOPX) or glutathione peroxidases (GPX), should be mentioned. These enzymes take part in detoxification of ROS and organic hydroperoxides formed by peroxidation of various biomolecules (Sharma et al., 2012; Sidhu et al., 2017). In turns, compounds like reduced glutathione (GSH), reduced ascorbate or phenols are important non-enzymatic antioxidants, among which phenols additionally exhibit high tendency to chelate toxic ions (Michalak, 2006; Gill and Tuteja, 2010; Corso et al., 2018). The induction of cellular antioxidant machinery can also increase plant resistance to other abiotic stresses (Hasanuzzaman et al., 2017). It might suggest that the level of cell damage under HMs stress depend on the rate of ROS formation and the efficiency of their detoxification. Nevertheless such complex mechanism of HMs tolerance is still unclear and diverse scavengers should be taken into consideration to explain their potential role in plant

Abbreviations: CAT, catalase; GOPX, guaiacol-type peroxidase; GPX, glutathione peroxidase; GST, glutathione-*S*-transferase; HMs, heavy metals; M, metallicolous ecotype; NM, nonmetallicolous ecotype; ROS, reactive oxygen species

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response against metal toxicity (Sharma and Dietz, 2008; Malar et al., 2014).

Although the negative impact of HMs on plants is well-known, some species or specialized ecotypes have been adapted to extremely high amounts of trace elements and thus such taxa are able to thrive in metallicolous environment without suffering any phytotoxic effects (Corem et al., 2009; Wójcik et al., 2015; Fernandez et al., 2017; Muszyńska et al., 2017). The high level of tolerance to ballast elements may be related to extracellular strategies that enable to reduce metal penetration into the protoplast as well as its increased internal detoxification and/or sequestration in cell compartments like cortex root vacuoles, leaf mesophyll, epidermis, trichomes or cuticle (Broadhurst et al., 2009; Maestri et al., 2010; Chandra and Kumar, 2017; Feng et al., 2018). Among metal-tolerant plants, more and more attention is currently paid on so-called 'pseudometallophytes' that represent a common species which exhibit increased tolerance to toxic level of metals in comparison to the reference populations from unpolluted areas (Dresler et al., 2014; Wójcik et al., 2015; Kabeya et al., 2018). Pseudometallophytes are treated as especially useful organisms for holistic approach during investigation the plant response to metals as well as the adaptation mechanisms to stress conditions (Wiszniewska et al., 2017; Kabeya et al., 2018). Additionally, it would be of great interest to apply such plant material in advantageous plant-based remediation technology called phytoremediation (Barzanti et al., 2011; Muszyńska et al., 2013, 2017; Chandra and Kumar, 2017).

Alyssum is a large genus within Brassicaceae, including about 195 species (Li et al., 2014). Some of them represent serpentine adapted plants, among which *Alyssum bertolonii* was the first species established as nickel accumulator and afterwards it was even called "the commercial crop of nickel" (Corem et al., 2009). Numerous species from this genus have subsequently been shown to hyperaccumulate metallic trace elements in their above-ground parts, mainly Ni ions (Pollard et al., 2014; Broadhurst and Chaney, 2016). However, there are no available literature data on the possible effect of cadmium, lead and zinc on structural integrity, growth and development of *Alyssum montanum* calamine ecotype. Furthermore, bioaccumulation factors in *A. montanum* have not been studied, so practical use of this species for phytoremediation purpose has not been assessed until now. For this reason, we investigated the morphogenetic response, growth tolerance and ability to Zn, Cd, and Pb uptake by two *A. montanum* ecotypes cultivated in the presence of elevated concentration of above-mentioned metals. We used *in vitro* culture as a convenient laboratory tool for better understanding the reaction of tested ecotypes on heavy metal stress. Such aseptic techniques provide opportunity to carry out the experiments under easily controlled conditions that allow to evaluate the straightforward effect of tested agents (Muszyńska et al., 2018). Thus, we applied proliferating shoot culture as a model to reveal *A. montanum* strategies involved in counteraction metal toxicity at the cellular and physiological level. Besides, we examined the HMs accumulation in cultured organs in order to propose appropriate utilization of examined species in phytoremediation technology.

2. Materials and methods

2.1. Plant material and culture conditions

Alyssum montanum L. (Brassicaceae) cultures were established using seeds samples collected from their natural habitats located in Poland: (1) a Zn–Pb waste heap in Bolesław near Olkusz (metallicolous ecotype, M) and (2) uncontaminated soil in Pińczów near Kielce (non-metallicolous ecotype, NM). The apical fragments of aseptically obtained seedlings were used as primary explants. The medium used to propagate shoot culture (referred as propagation medium) consisted of WPM salts and vitamins (Lloyd and McCown, 1980), 0.65 g L^{-1} calcium gluconate, 0.5 g L^{-1} PVP, 0.6 g L^{-1} activated charcoal, 0.5 g L^{-1} MES, and 20.0 g L^{-1} sucrose. 12.3 μM2iP and 5.71 μM indole-3-acetic acid (IAA) were applied as plant growth regulators. The pH of medium was adjusted to 5.6 prior to solidification with 0.75% Difco agar.

2.2. Heavy metals treatment

The tested cultures were established by placing 10 mm long secondary shoot explants onto the propagation medium supplemented with the combinations of zinc, lead and cadmium salts. The salt concentrations, that were previously described in detail by Muszyńska et al. (2013), corresponded to the amounts of soluble forms of these elements in the calamine substrate from natural habitat of metallicolous ecotype. Thus, the following concentrations of mixed salts were tested: 0.7 mM $ZnSO_4$, 3.0 μM $Pb(NO_3)_2$ and 16.4 μM $CdCl_2$. As a control, propagation medium without HMs salts was used. HMs were added to medium, prior to autoclaving, and pH was adjusted to 5.6. Five microcuttings per 200 mL flask were explanted on the respective media and each flask contained 50 mL of culture medium. Six replicates (flasks) per each treatment were used, which correspond to at least 30 explants. Cultures were maintained in a growth chamber MLR-350 (Sanyo, Tokyo, Japan) at 24 °C, under 16 h photoperiod (irradiance 80 μmol m^{-2} s^{-1}). The experiment lasted 8 weeks, with subculture after 4 weeks when entire microcuttings were transferred on the fresh medium containing the same concentration of initially applied heavy metals.

2.3. Evaluation of plant growth parameters

Shoots were measured and counted after 8 weeks of *in vitro* cultivation, and micropropagation coefficient (MC) was calculated using the formula:

MC = total number of regenerated shoots / number of primary explants.

Moreover, the percentage of rooted shoots and the number of spontaneously regenerated roots were evaluated. At the end of experiment, plant material was dried in 105 °C to the constant mass and weighted afterwards for determination of dry matter. The ability of plant material to grow in the presence of tested heavy metals was ascertained on the basis of the growth tolerance index (in %) calculated after 8 weeks of cultivation separately for shoots and roots, using the formulas:

GTI_s = (mean fresh weight of shoots developed on medium supplemented with metal salts / mean fresh weight of shoots developed on medium without metal salts) × 100%.

GTI_r = (mean fresh weight of roots developed on medium supplemented with metal salts / mean fresh weight of roots developed on medium without metal salts) × 100%.

2.4. Determination of heavy metals

2.4.1. Histochemical detections

The localization of Cd, Pb and Zn ions in leaves was examined according to histochemical method with dithizone (diphenylthiocarbazone) as described by Seregin and Kozhevnikova (2011). The series of hand-made sections from ten randomly chosen leaves were soaked in the staining solution (30 mg dithizone, 60 mL acetone, 20 mL deionized water and a few drops of acetic acid 45% (v/v) for 30 min. A metals-dithizonate complex in plant tissue was red.

2.4.2. Content determination

The content of zinc, lead and cadmium was determined after 8 weeks of HMs treatment in both regenerated shoots and roots. Plant samples, previously dried, were mineralized in 65% super pure HNO_3 (v/v) at 230 °C and pressure of 30 atm, and analyzed with the use of inductively coupled plasma mass spectrometry (ICP–MS). The translocation factor (TF) for each metals were calculated as follows:

TF = HMs content in shoots (μg g^{-1} d.w.) / HMs content in roots (μg g^{-1} d.w.).