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Research article

Antioxidative enzymes and expression of *rbcL* gene as tools to monitor heavy metal-related stress in plants

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ABSTRACT

The aim of the study was to evaluate sensitivity and potential applications of selected biomarkers in phytoremediation under complex heavy metal contamination in *Sinapis alba* L., *Robinia pseudoacacia* L. and *Lupinus luteus* L as a potential tools in effective phytoremediation management. The toxicity assessment was conducted using selected measurement endpoints, both classical and advanced, i.e., germination index, roots length, guaiacol peroxidase activity (GPX), chlorophyll and protein content, the amount of total phenolic compounds (TPC) and level of expression of one of the ribulose-bisphosphate carboxylase genes (*rbcL*). Moreover, the influence of organic additives: cattle, horse manure, and vermicompost on lowering plant abiotic stress caused by complex heavy metal contamination was studied to assess the possible applications of selected stress markers in large scale phytoremediation planning. The results demonstrated the beneficial effects of selected soil additives on plant development. The 5% difference in the quantity of applied amendment caused statistically significant differences in GPX, TPC, chlorophyll content and expression level of *rbcL*. Among all endpoints, GPX activity, chlorophyll, and phenolic compounds content, as well as the expression of *rbcL*, turned out to be the most reliable assays for determination of the type and dosage of selected soil amendments (fertilizers) in the assisted phytoremediation process. Selected markers can be used to achieve the desired level of plant abiotic stress and consequently photosynthesis efficiency and CO_2 sequestration. The results showed, that presented assays can be used in different taxonomical groups such as *Fabaceae* for planning effective phytoremediation process.

1. Introduction

The pollution of soil is becoming a fast-rising issue worldwide due to a vast presence of anthropogenic actions such as mining and manufacturing (Bolan et al., 2014; Pant and Tripathi, 2014). Industrial wastes make soil useless for many years by lingering at its surface. Moreover, agriculture was recognized as the second largest contributor to soil degradation (Motuzova et al., 2014). Therefore, pollutants seep into the deeper layers of the ground and pose a threat to groundwater reservoirs (Tóth et al., 2016; Mahar et al., 2016). In addition, soil pollution can increase oxidative stress in plants and thus decrease plant growth gradually, causing a significant reduction in photosynthesis efficiency (Chabukdhara and Nema, 2013; Caverzan et al., 2014). Organic fertilizers, such as compost or manure, can provide a broad spectrum of nutrients, which prevent symptoms of deficiencies in plants. Moreover, such supplementation of soil can decrease the level of oxidative stress by increasing plant resistance to pollutants and also improving the efficiency of phytoremediation, sequestration and overall quality of yield and soil (Grobelak., 2016). Therefore, the use of organic fertilizers such as compost or manure is a common practice for sustaining economically viable crop production with minimal environmental pollution as an alternative to chemical fertilizers (Włóka et al., 2017). Organic fertilizers can also promote microbiological activity in soil, leading to the enhancement of soil quality since plants depend on microorganisms to mineralize organic matter (Grobelak et al., 2015). Beneficial effects of using manure were reported mainly concerning drought stress in plants (Kakoei and Salehi, 2013; Wang et al., 2012) but also to heavy metals exposure, e.g., lead (Qiao and Libault, 2013). Among organic amendments, manure can have potential applications in phytoremediation of degraded soils, because of its abundance,

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accessibility and low price.

Phytoextraction, one of the most commonly used phytoremediation methods, implies the use of hyperaccumulators to extract and accumulate heavy metals from soils, followed by harvesting plant biomass until the concentration of specific contaminant decreases to an acceptable level (Dimkpa et al., 2009; Ullah et al., 2015). Several hyperaccumulator plants have been identified, and many are still being investigated for continuous removal of heavy metals from soil and water (Ent et al., 2012; Chibuike and Obiora, 2014). For instance, *Fabaceae* plants such as *Robinia pseudoacacia* L. and *Lupinus luteus* L. create a symbiosis with nitrogen-fixing bacteria, leading to an improvement in soil quality and allow other, less resistant species to sprout on such areas over time (Alavi et al., 2014). Other studies indicate the plant growth promoting rhizobacteria (PGPR) in improving plant resistance to abiotic factors (Ullah et al., 2015). Nevertheless, still little is known about the specificity of those stress biomarkers and their proper practical application. One of the most commonly used biomarkers of oxidative stress in plants can be divided into two major groups (Murtaza and Asghar, 2013). The first one uses biochemical markers that include measuring the activity of specific enzymes such as superoxide dismutase (SOD), catalases or peroxidases (POD) (Alscher, 2002). The content of chlorophyll, phenolic compounds, and overall proteins can also be used to assess the influence of different pollutants or fertilizers on plant metabolism since they are highly sensitive to changes in the environment. The second group uses a vast range of genetic markers to assay such effects on the growth and development of plants (Pal et al., 2010).

In the presented study, the biomarkers mentioned above functioned as tools to assess the possibility and predictability of organic soil amendments application for phytoremediation of areas highly polluted by metallic trace elements. In those terms, the main aim of the study was to: (1) assess the impact of particular organic additives (cow/horse manure, vermicompost) on selected parameters involved in immune defence (potential biomarker), namely content of phenolics, chlorophyll, proteins, expression of the *rbcL* gene in three selected plant species; (2) select the biomarkers of stress for reliable assay in determining the most effective methodology of large scale phytoremediation.

2. Materials and methods

2.1. Substrates characteristics

Soil used in this experiment was derived from highly degraded and heavy metal contaminated areas in Poland (GPS: 50°30′N 18°56′E). Soil samples were collected from the surface layer (0–30 cm) of the landless area contaminated mostly from metallurgical industry. Besides the very high content of heavy metals, mainly cadmium (Cd), lead (Pb) and zinc (Zn), the soil exhibited deficient microbiological activity, fertility, and sorption capacity (Table 1). Before planting seeds, the soil was dried, sieved and thoroughly mixed with selected organic fertilizers: cattle manure, horse manure and vermicompost in particular concentrations: 10, 15, 20% by dry weight. The doses of manure and vermicompost used in the experiment were selected to meet the EU nitrogen standards (170 kg of nitrogen/year/ha) (EUR-Lex - 31991L0676 – EN). The difference between applied doses - 5% was intended to estimate the sensitivity of selected stress markers between slight differences in dosages of soil additives. The control group consisted of organic peat soil without any contamination providing optimum growth conditions to fully assess the influence of contaminated soil on plant development in comparison to their natural capabilities on clean soil without any abiotic stressors. Organic additives used in the experiment were derived from commercially available sources (FLORMIX, Poland) and were tested for their chemical and physical properties (Table 1). Cattle manure used in the experiment was characterized by a nitrogen content of approximately 5.3 g kg^{-1} dry weight, phosphorus - P – 1.1 g kg^{-1} dry weight and potassium – 4.1 g kg^{-1} dry weight. Horse manure contained a higher content of NPK, including approximately 6.4 g kg^{-1} dry weight of nitrogen, 2.3 g kg^{-1} dry weight of phosphorus and 4.9 g kg^{-1} dry weight of potassium. In Vermicompost the content of nitrogen was approximately 6.9 g kg^{-1} dry weight, the content of phosphorus – 2.2 g kg^{-1} dry weight and 5.1 g kg^{-1} dry weight of potassium.

After preparation of soil mixtures, samples were collected to perform physical and chemical assays. The pH values of the soil samples were measured in distilled water and 1 M solution of KCl according to ISO 10390:2005. For pH determination, standard laboratory pH-meter was used (Cole Parmer Model No. 59002–00). Cation-exchange capacity (CEC) was determined by the Kappen method (Kappen et al., 1995). The content of total organic nitrogen in samples was established by the Kjeldahl method (PN-ISO 11261:2002) (Bradstreet, 1940), while the content of phosphorus and potassium by the Egner method (Egnér et al., 1960). A concentration of heavy metals was measured by ICP-OES (ICP-OES; Thermo apparatus, USA). Samples were digested in a microwave digestion system according to the EPA method 3051.

2.2. Experiment procedure

Three plant species: *S. alba*, *R. pseudoacacia* and *L. luteus*, were grown separately in 11 different soil mixtures, in three replicates. Commercially available, certified and high-quality seeds were used (Flormix, Poland). Each pot (H – 15 cm, a – 12 cm) contained 300 g soil and 20 seeds sown approximately two cm deep in the soil. Plants were then incubated for 28 days in a growth chamber (Biogenet FS360, Poland) under controlled conditions: photoperiod: 16 h light 8 h dark, temperature during the day 21 °C, night 18 °C, light intensity 4000 lx (photosynthetic LED light). After 28 days, plants were extracted from soil and biomass was stored in −80 °C for further analyses. Germination was recorded every 24 h for 14 days and was considered to occur when radicles were at least 2 mm long. All seedlings with short, deformed or spiral formed hypocotyls were considered as abnormally germinated. Germination index was after calculated as a percentage of properly developed seedlings. Plant growth parameters like biomass, foliar surface, chlorosis or signs of fungi infection have been assessed. No significant variation between conditions was observed (data not shown).

2.3. Guaiacol peroxidase activity assay

The activity of GPX was established by the Asada method (Asada, 2006) with modifications (Sharkey, 2005, Caverzan et al., 2014). Briefly, GPX activity was estimated by a level of guaiacol oxidation in the presence of hydrogen peroxide within the 2-min period. Approximately 100 mg of each plant shoot material was mixed with 3 mL of phosphate buffer (pH 6) and homogenized in a mortar. The homogenate was centrifuged at 11000 g for 5 min. A guaiacol reaction mixture prepared in plastic cuvettes for spectrophotometric analysis contained 3 mL of guaiacol reagent (100 mM potassium phosphate buffer, pH 7.4 and 0.35% guaiacol). For each sample, 10 μL of the supernatant obtained after homogenization and 10 μL of 30% hydrogen peroxide was added, mixed and immediately measured spectrophotometrically. Absorbance was measured at λ 430 nm after 2 min of guaiacol oxidation. Peroxidase activity was defined as an amount of GPX that produces a change in absorbance at 430 nm of 0.021/min (HACH DR/4000V, USA). Results were normalized to U/mg of determined protein concentration in plant biomass.