

# The origin of *Epichloë* endophyte - perennial ryegrass symbionts modify plant reactions to elevated concentration of $Pb^{2+}$ , $Cd^{2+}$ and $Cu^{2+}$ ions in soil

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## Research article

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## Abstract

**Background:** The phenomenon of plant mutualistic symbiosis with microbes may have a positive effect on improvement of plant tolerance to environmental stresses. The influence of fungal endophyte of the *Epichloë* sp. (*Clavicipitaceae*) on perennial ryegrass (*Lolium perenne* L.) plants grown in presence of elevated concentration of heavy metal (HM) ions: Cd<sup>2+</sup>, Pb<sup>2+</sup> and Cu<sup>2+</sup>, in soil was studied.

**Results:** The presence of *Epichloë* in the host grass tissues resulted in increased accumulation of HM ions in aerial parts of plants and was dependent on host genotypes related to host plant origin. In plants with (E+) and without (E-) endophytes the hormesis effect was induced by elevated concentration of Cu<sup>2+</sup> ions, resulting in better growth and photosynthesis, as examined by measurements of Chl a fluorescence. The obtained results indicate that based on the laboratory evaluation of the efficiency of the symbiosis, we were able to choose the best associations of perennial ryegrass with endophytes for HM phytoremediation.

**Conclusions:** The presence of *Epichloë* endophytes positively affected ryegrass ability to accumulate HM ions and this accumulation was associated with the origin of *Epichloë*-ryegrass symbionts.

## Background

Endophytes are able to colonize plant tissues and live without inducing any visible symptoms of biotic stress in plants. The *Epichloë* species (*Clavicipitaceae*, *Hypocreales*), are specialized fungi of cool-season grasses which can grow throughout the aerial parts of their host plants, forming systemic and predominantly asymptomatic associations, resulting in defensive mutualism (Tadych et al., 2014). In general, as a consequence of host plant-microbe interactions, these endophytes produce a range of alkaloids and stimulate the host plant for enhanced synthesis of primary and secondary metabolites, e.g. free sugars, sugar alcohols, proline, glutamic acid, phospholipids, proteins and polysaccharides (Avila et al., 2012; Bush et al., 1997; Nagabhyru et al., 2013; Porter, 1994; Rasmussen et al., 2008; Soto-Barajas et al., 2016). In addition to the *Epichloë* endophytes, there are many other endophytic fungi inhabiting both aerial parts and plant roots that positively affect the life processes of their hosts. Hao et al. (2010) observed an activation of plant hormones prior to metabolome changes. Treatment of suspension cells of *Ginkgo biloba* with fungal endophytes resulted in accumulation of flavonoids, increased abscisic acid (ABA) production and activation of phenylalanine ammonia-lyase (PAL). Also the roots metabolism is altered in response to colonization of the aboveground parts of plants (Strehmel et al., 2016). Altogether, the mutual associations lead to changes in host plant gene expression and improve plant adaptations to environmental stresses, both: biotic (e.g. insects, herbivore animals, diseases) and abiotic (e.g. drought) (Bacon et al., 2015; Dupont et al., 2015; Rodriguez et al., 2008; Schardl et al., 2012, 2013).

The basic physiological aspect of the endophyte host interactions is poorly understood (Kaul et al., 2016). Dupont et al. (2015) have shown that in controlled conditions the *Epichloë*-ryegrass association induced more than one third of host plant genes, that was ten times more than in other plant-fungi symbiotic interactions and nearly twice more than in the case of plants infected by pathogens, as detected by microarray and RNAseq. 'Endophyte-induced' genes of the host were mostly responsible for the reprogramming of secondary metabolism at a cost to primary metabolism. Among a quarter of genes which were down regulated were genes of Calvin cycle and the tetrapyrrole pathway of chlorophyll synthesis; also the slowdown of photosynthesis was detected. In described experiment the *Epichloë* free seeds were germinated prior to infection under controlled conditions

(Dupont et al., 2015). On the other hand Rozpądek et al. (2015) has shown that in symbiosis of *Epichloë typhina*– *Dactylis glomerata*, the photochemistry of photosystem II (PSII) and carbon assimilation were slightly improved. Earlier studies also documented weak or no influence of endophytes on photosynthesis in non-stressed hosts and defensive mutualism of the *Epichloë* endophyte in *Achnatherum inebrians* against *Blumeria graminis* (Clay, 1988; Bacon, 1993; Richardson et al., 1993; Xia et al., 2016).

Inhibition of photosynthesis by heavy metals (HM) has been well documented (Clijsters and van Asche, 1985; Prasad et al., 1999; Singh et al., 2011). HM stress induces a series of biochemical and physiological modifications in plant tissues that display common characteristics with those induced by drought (Barceló and Poschenrieder, 1990). Membrane damage and altered enzyme activities lead to a wide range of secondary effects that concern practically all the physiological processes. Photosynthesis is very sensitive process due to several structural and metabolic disturbances: direct interactions of HM ions with thiol-, histidyl- and carboxyl-groups of cell proteins; induction of reactive oxygen species (ROS) formation; displacement of essential cations in protein active centers (Hall, 2002; Hossain et al., 2012; Farid et al. 2013). Some ions such as  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  or  $\text{Zn}^{2+}$  may substitute the central  $\text{Mg}^{2+}$  ion in chlorophyll molecules, forming complexes lowering the quantum efficiency of PSII (van Asche and Clijsters, 1990; Sharma and Dietz, 2009). These circumstances affect most of parameters of chlorophyll *a* (Chl *a*) fluorescence detected by so called JIP test (Žurek et al. 2014). However it has been demonstrated that endophytes play a key role in host plant adaptation to polluted environments and that they can enhance phytoremediation by mobilizing/degrading or immobilizing contaminants in the soil, promoting plant growth, decreasing phytotoxicity and improving plants' HM ions tolerance (Soleimani et al. 2010, Li et al. 2012a, Li et al. 2016). The importance of endophytic *Epichloë* species for ecosystems due to modulation of both below- and aboveground ecosystem processes is well recognized and accepted (Saikkonen et al., 2016).

The settlement of the polluted environment by plant-microorganisms symbionts for phytoremediation purposes has been defined as a '*low-input biotechnology for the ecosystem revitalization*' (Abhilash et al., 2012).

Phytoremediation is increasingly used as a sustainable approach for soil remediation. However, methodology improvement is constantly forced due to the expected increase in phytoremediation efficacy as well as due to economic reasons. Due to complex biological interactions, currently used methods do not always give the demanded results, so further multidirectional studies are needed (Thijs et al. 2017). The research hypothesis presented in this paper indicates that the origin of *Epichloë* endophyte - perennial ryegrass symbionts modify plant reactions to elevated concentration of  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Cu}^{2+}$  ions in soil.

## Results

It is well known that the presence of endophytes in plants can have a positive effect on improving plant tolerance to environmental stress. The research undertaken in the work focused on the impact of the presence of *Epichloë* endophytes in perennial ryegrass plants on their growth in conditions of elevated concentration of  $\text{Pb}^{+2}$ ,  $\text{Cu}^{+2}$  and  $\text{Cd}^{+2}$  ions in soil.

### Plant collection sites

Most of the soils on which meadows were located and from which perennial ryegrass plants were derived, were of mineral or organic type, with medium or low soil moisture content, mainly with medium or low intensity usage as pastures or for cutting (Suppl. Table 1). All regions but the last one (SWK) were characterized by relatively low concentrations of HM ions in soil:  $Pb^{2+}$ - c.a. 9.6,  $Cd^{2+}$ - 0.17 and  $Cu^{2+}$ - 4.3 [ $mg \cdot kg^{-1}$ ]. The SWK region was characterized by almost twice higher content of HM ions:  $Pb^{2+}$ - c.a. 17.8,  $Cd^{2+}$ - 0.37 and  $Cu^{2+}$ - 7.6 [ $mg \cdot kg^{-1}$ ] (Terelak, 2007) (Fig. 1, Suppl. Table 1).

### Endophyte detection

The average endophyte incidence in perennial ryegrass plants was 79.7% (Table 1). The lowest endophyte incidence was noted for site at the most northern position - #50 (POD region). However, relatively low values were also noted for sites from other regions (# 801 and # 730 from MAZ and #227 from SWI). More sites of high endophyte incidence, above 90% were noted for LUB and SWI than for MAZ and POD.

### Phenotyping of endophyte-infected (E+) and endophyte-free (E-) ecotypes responses to HM ions

By using term 'ecotype' we mention a group of plants within a species that is adapted to particular environmental conditions (locality) and therefore exhibits structural or physiological differences from the other members of the same species. Biomass yields were significantly affected by the ecotype and HM treatment throughout the whole experiment whereas the main effect of the endophyte was significant only for the first (after a month) and second cuts (after two months) (Table 2). Generally, plants grown in the presence of HM ions yielded much better than control plants (Fig. 2, Suppl. Fig. 2). The yield of plants grown in the presence of HM, despite the endophyte status of plants, were 148% of control at 1<sup>st</sup> cut, 442%, at 2<sup>nd</sup> and 243% at 3<sup>rd</sup> cut, in average for the whole experiment total yield from HM treated plants was 215% higher than from control plants.

Elevated concentration of the HM in the soil as well as the origin of the tested ecotypes were the main sources of variation for the relative chlorophyll content, expressed as CCI. In contrast, neither endophyte presence nor its interaction with the plant origin and HM gave a significant effect on the CCI (Table 2). The CCI in HM treated ecotypes was in average higher than in non HM treated ones (Fig. 3). The differences were higher for the ecotypes originated from the northern areas (# 50, #801, #131, #685) than from the southern ones (#227, #87) (Fig. 3).

Elevated concentration of the HM in the soil was also the main source of variation of Chl *a* fluorescence parameters:  $F_0$ ,  $F_M$ ,  $F_V$ ,  $F_V/F_M$ ,  $F_V/F_0$  and  $(1-V_j)/V_j$  (Table 3). Not the ecotype nor endophyte status resulted in significant effect of any from above mentioned Chl *a* fluorescence parameters. However, significant interaction between HM presence in soil and endophyte presence in plants has been calculated for  $F_0$ ,  $F_M$ ,  $F_V$ ,  $F_V/F_M$ ,  $F_V/F_0$  and Area (Table 3, Figure 4). For parameters:  $T_{FM}$ , RC/ABS and  $PI_{ABS}$  none of main sources of variation nor interactions were significant, therefore they were not listed in Table 3 nor on Fig. 4.

Considering interactions presented on Figure 4, perennial ryegrass plants, if grown without addition of HM, exposed some negative effects of endophyte presence in tissues, as reflected in lower values of  $F_M$ ,  $F_V$  and Area. When HM was added to the soil medium, values of mentioned parameters increased in the presence of endophyte. However, value of the parameter reflecting force of light reactions of PS II ( $F_V/F_M$ ) was significantly lower in the presence of HM in soil and endophyte in plant tissues.

Measured parameters of Chl *a* ( $F_0$ ,  $F_M$ ,  $F_V$ ) were higher in E-, and were also only slightly influenced by HM treatment, as it has been explained by the analysis of the data (Table 3, Suppl. Fig. 3). In leaves of E+ plants, higher values of Chl *a* fluorescence measured parameters were detected in the ecotypes collected from more northerly localized sites (higher latitude values), for which weaker positive reactions to HM ions were detected (Suppl. Fig. 3). Only one E+ ecotype, #730, was characterized by decrease of measured parameters. That ecotype was collected from the halfway between most north and most south locations. Two other E+ ecotypes collected south from that point (#45 and #273) were characterized by about twofold increase of measured parameters in presence of HM in the soil. E+ plants, from southern locations (in order north-south: #160, #129, #227, #87) were characterized by nearly the same changes of measured parameters in a response to HM (Suppl. Fig. 3).

On the other hand, proportions of measured parameters slightly (less than 5%) decreased in leaves of most plants grown in the presence of HM ions. Interestingly, E+ plants collected in more northern localities were characterized by more visible decline of  $F_V/F_M$  and  $F_V/F_0$  ratios. And, as in the case of measured parameters, E+ ecotype #730 reacted differently, by their slight increase. The ratio of  $F_V/F_0$  was  $\leq 4.0$  in E- plants, whereas in E+ plants in 3 cases the ratio exceeded 4 (# 45, #87, #873). Parameter  $(1-V_j)/V_j$ , the measure of forward electron transport, seemed to be slightly affected by HM, especially in the leaves of E+ plants.

The PCA (Principal Component Analysis) run on bases of Chl *a* fluorescence parameters has shown distribution of ecotypes depending on the endophyte presence mostly over the OX axis (first factor) (Fig. 5, Sup. Tab 2), which means, that mostly measured parameters, significantly correlated with the first factor ( $F_0$ ,  $F_V$ ,  $F_M$ , and Area) influenced such grouping.

### HM ions content in E+ and E- ecotypes

Analysis of variance for the data of HM ions concentration in the plant tissue revealed statistically strong influence of both, plant origin and endophyte presence in the host plant as well as their interaction. The exception was the influence of endophyte presence and  $Pb^{2+}$  ions concentration in plant leaves (Table 4 and 5, Fig. 6).

The highest concentration of HM ions (sum of  $Pb^{2+}$ ,  $Cd^{2+}$  and  $Cu^{2+}$ ) was detected in the leaves of #160 E+ ecotype ( $102 \text{ mg}\cdot\text{kg}^{-1}$ ), whereas in the leaves of the E- plants, the concentration of HM was low ( $44 \text{ mg}\cdot\text{kg}^{-1}$ ). Differences in the particular ions concentration of above mentioned ecotype were as follows: almost two fold higher concentration of  $Pb^{2+}$  and  $Cd^{2+}$  ions and three fold of  $Cu^{2+}$  in E+ plants as compared to E-. As for the E- plants, the highest concentration of  $Pb^{2+}$  was detected in the ecotype #50, ( $43.9 \text{ mg}\cdot\text{kg}^{-1}$ ) whereas the lowest in the ecotype #227 ( $10.4 \text{ mg}\cdot\text{kg}^{-1}$ ). Considering E+ plants, the highest  $Pb^{2+}$  concentration ( $40.7 \text{ mg}\cdot\text{kg}^{-1}$ ) was detected in above mentioned ecotype #160, and also high in # 685 and # 873 ( $33.2$  and  $32.7 \text{ mg}\cdot\text{kg}^{-1}$ , respectively). For all those three ecotypes  $Pb^{2+}$  concentration in E+ plants was significantly higher than in E- plants.

Cadmium concentration in aerial parts of E+ ecotypes was the highest in #801 ecotype ( $19.8 \text{ mg kg}^{-1}$ ) as well as in #45 and #685 ( $16.2$  and  $15.1 \text{ mg kg}^{-1}$ , respectively) (Table 5). Similarly to relations described above for  $Pb^{2+}$  concentration, for all three ecotypes with relatively high  $Cd^{2+}$  concentration in E+ plants the  $Cd^{2+}$  ions

concentration was significantly higher than the concentration values found in E- plants. High copper concentration was found in aerial parts of E+ ecotypes #160, 273 and 873 (47.9, 40.6 and 37.4 mg·kg<sup>-1</sup>, respectively). All mentioned values were significantly higher than in leaves of corresponding E- plants. On the other hand for some ecotypes, the Pb<sup>2+</sup> ions concentration was higher in E- plants as compared to E+. The relatively low Pb<sup>2+</sup> concentration, observed in E+ ecotypes #730 - 10.2 [mg·kg<sup>-1</sup>], #131 - 11.0 [mg·kg<sup>-1</sup>] and #50 - 15.7 [mg·kg<sup>-1</sup>] were found to be significantly lower than in the corresponding E- plants. Similar relations were registered from above mentioned ecotypes for Cu<sup>2+</sup> ions. Concentration of Cu<sup>2+</sup> ions in E+ plants of ecotypes #730, 131 and 50 was 14.6, 13.8 4.6 [mg·kg<sup>-1</sup>], respectively. For Cd<sup>2+</sup> no such relations was confirmed.

E+ plants from different regions were identified as having different efficiency in HM uptake from the soil. Four plant-endophyte symbionts out of five collected in SWK region accumulated Pb<sup>2+</sup> ions about twice more intensively than E- plants. Mean efficiency of Pb<sup>2+</sup> uptake by *Epichloë* perennial ryegrass symbionts collected from SWK region was c.a. 170%. Moreover, all *Epichloë* perennial ryegrass symbionts from SWK region accumulated up to 200% Cu<sup>2+</sup> more than E- plants. Mean efficiency of Cu<sup>2+</sup> ions uptake by E+ ecotypes from MAZ region was 150% higher than by E- ones, from LUB region it was 120%. The highest values of Cd efficiency uptake were noted for the E+ ecotypes from MAZ region: up to 200% higher than in E- plants, with mean for the region of about 130% (Table 5).

The effect of endophyte presence in perennial ryegrass plants resulted in different types of E+ plant reactions to elevated concentration of HM ions in the soil:

- E+ plants accumulated less HM ions from the soil than E- plants. In our experiment there were two ecotypes: #131 and 50;
- no significant difference between E+ and E-. It was in case of # 87 (Cd<sup>2+</sup> and Cu<sup>2+</sup> ions), # 801 (Pb<sup>2+</sup> and Cu<sup>2+</sup> ions);
- E+ plants accumulated higher amount of HM ions from the soil than E- plants. In our experiment there were three ecotypes #160, 129 and 685 which accumulated all HM ions in higher concentration in E+ than E-. Following ecotypes: #45, 227, 273 and 873 ecotypes accumulated two different HM ions in higher concentration in E+ than E-;
- all above relations between HM ions concentration in E+ and E- plants in one ecotype - #730.

Four E+ ecotypes, which were the most effective in extraction of HM ions from polluted soil (#160 and 227, 129, 273) originated from SWK region.

## Discussion

There is increasing evidence that interactions of plants and microbes (including endophytes) play a critical role in metal phytoextraction and metal-mining, as they mediate different physicochemical and biological activities to facilitate ecological performances of the host plant (Muehe et al., 2015).

The results of our studies revealed considerable variation in terms of the grass-fungus association's ability to cope with elevated concentration of heavy metals ions in soil. We suggest that origin of ecotypes (i.e. place where associations grow and were shaped by natural conditions) affected their ability to accumulate heavy

metal ions in aerial parts of plants. Spatial variation of mutualistic interactions between a host organism (grass plant) and infecting fungus (endophyte) by means of its intensity (endophyte frequency per locality) and production of toxic metabolite i.e. ergovaline, has been previously described (Žurek et al., 2013; Žurek et al., 2017). It is therefore possible that other plant-fungus responses could depend on other factors present in place of association origin.

Plants subjected to increased HM contents in soil were characterized by significantly higher values of the CCI and this could be explained on the basis of soil fertility. The soil used for this experiment contained a low level of  $\text{Cu}^{2+}$  ions ( $2.4 \text{ mg}\cdot\text{kg}^{-1}$ ) and high amounts of soil organic carbon (SOC), 13%. Natural content of  $\text{Cu}^{2+}$  in soil is in the range from 15 to  $40 \text{ mg}\cdot\text{kg}^{-1}$  in the 0 – 20 cm soil horizon and concentration of  $\text{Cu}^{2+}$  below  $3.0 \text{ mg}\cdot\text{kg}^{-1}$  is usually defined as deficit for grass species (Olszewska et al., 2008; Wyszowska et al., 2013). In the presence of high organic matter content in the soil,  $\text{Cu}^{2+}$  deficit for plants is quite frequent. Moreover, monocotyledonous plants (e.g. grasses) are particularly sensitive to  $\text{Cu}^{2+}$  deficit (Yamasaki et al., 2008). All those three elements unfortunately met together in our experiment, therefore addition of Cu to soil medium yielded better growth of HM treated plants, which was manifested in higher CCI values. The differences were statistically insignificant for ecotypes collected from southern region, with the exception of 1 ecotype of E+ and 2 ecotypes of E-, which could be the result of adaptation to naturally occurring conditions of increased HM content in soil (Rodriguez et al., 2008).

Chl *a* fluorescence detection and parameters analyses are simple and widely recognized methods to assess the stress influence on plants (Žurek et al. 2014; Dąbrowski et al., 2016; Kalaji et al., 2016). Among fluorescence parameters measured in our experiment,  $F_0$ ,  $F_M$ ,  $F_V$  as well as the  $F_V/F_M$ ,  $F_V/F_0$  and  $(1-V_j)/V_j$  were found significantly influenced by both HM ions addition and its interaction with endophyte status. As a reaction to stress  $F_0$  value is mostly increasing, which is interpreted as lower efficiency of energy transfer between chlorophyll antennas in PS II, and our data are in accordance with literature reports (Prasad et al., 1999). Although the increase of  $F_0$  was detected in majority of studied ecotypes, the E+ ecotypes were characterized by lower values of this parameter pointing to positive influence of *Epichloë* in the host plants, as was shown in studies on host orchard grass as well (Rozpądek et al., 2015). The  $F_M$  is decreasing in response to stresses due to a fact that not all electron acceptors in PS II can be reduced. Considering results obtained in our experiment, endophyte presence in plant tissues seems to induce stress to plant, as reflected by decrease of  $F_M$ . The  $F_V/F_0$  ratio, also used for detection of PS II destruction upon stress can descend from values of 4-5 down to 1. According to the results obtained in this experiment, significant but slight (< 5% in average) decrease of  $F_V/F_0$  ratio was detected, showing that the stress did not influence the photosynthetic machinery too much (Kalaji et al., 2016; Kalaji and Łoboda, 2010).

The parameter  $F_V/F_M$  is one of the most commonly used in evaluation of plant physiological status on bases of fluorescence characteristics. For most healthy plants it oscillates between 0.80-0.83. In our experiment it fluctuated in 0.78 and 0.81 ranges. Interestingly, HM ions induced statistically important drop down of that parameter in E+ ecotypes originated from northern latitudes.

Increased nutrient content due to endophyte presence was observed by many authors (Soto-Barajas et al., 2016; Malinowski et al., 2004; Zabalgoceazcoa et al., 2006). In contrast, an absence of endophyte effect for total N (Lewis et al., 1996) and Zn concentration (Monnet et al., 2005) was reported with a single perennial ryegrass

genotype evaluated. In the current experiment we have observed the whole range of possible reactions: from E+ plants accumulating less HM than E- plants, through no effect, to increased accumulation of one, two or three HM ions from the soil by E+ plants. Detected differences resulted, probably, not only from differences in the endophyte activities but also from strong interactions between the fungus and the host plant, which arose as a result of particular conditions in an origin site. In current research, spatial aggregation of E+ plants able to uptake relatively higher amounts of the HM from the soil has been found for  $Pb^{2+}$  accumulation. Perennial ryegrass ecotypes collected from the SWK region (locations below the latitude 50.84 N) demonstrated the ability for accumulation of relatively higher concentration of  $Pb^{2+}$  ions in E+ plants than those from the other regions. It could be presumed that it is in line with natural concentration of  $Pb^{2+}$  in the soils from this region which was concentrated in average of  $17.8 \text{ mg}\cdot\text{kg}^{-1}$  of soil as compared to  $9.4\text{-}10.2 \text{ mg}\cdot\text{kg}^{-1}$  of the soils from other sampling sites in our experiment (see Suppl. Table 1). Hesse et al. (2003, 2004) concluded that plant- endophyte associations are adapted to their native habitats via natural selection. As we have mentioned before, natural content of the HM, especially  $Pb^{2+}$  ions, in soil was higher in the SWK region than in other regions. Probably symbionts of this origin used to accumulate more  $Pb^{2+}$  than those coming from areas of low  $Pb^{2+}$  concentration.

The presence of HM tolerant endophytes could improve metals uptake and accumulation in hosting plants (Li et al., 2012b). Endophyte colonization promoted  $Cd^{2+}$  ions accumulation in tall fescue (Ren et al., 2011) and also improved  $Cd^{2+}$  transport from the root to the shoot. Hesse et al. (2003, 2004) have also found higher abundances of infected perennial ryegrass genotypes on dry sites compared to wet sites and this has been confirmed in our previous research (Žurek et al., 2013, 2017). Abundance of endophyte- infected perennial ryegrass plants was significantly and negatively correlated with annual as well as winter precipitation (multi-annual averages, 1950–2000) at localities of their origin. Considering habitat of symbiont origin, for example Dobrindt et al. (2013) reported higher incidences of *Neotyphodium lolii* at sites of limestone bedrock. Therefore, conditions at place of host plant origin (both climatic and soil) may influence its ability to cope with abiotic stress (drought, soil acidity, toxic metals in soil). Differences observed between the host plants appear to depend on the endophyte and the host life histories, as well as on fungal and plant genotypes, abiotic and biotic environmental conditions, and their interactions (Saikkonen et al., 2013). Specific genotypic combinations of both host and endophyte determine the morphology and physiology of endophyte colonized grasses, as well as regulates how selective pressure acts on them (Hill et al., 1996).

The economic significance of grass-endophyte associations has been studied intensively in New Zealand in aspect of grazing ruminants (Cosgrove et al., 2010). The benefits of grass-endophyte associations can be improved by selection, and such selection can substantially alter the profile of the secondary metabolites produced by the symbiont. There are clearly established precedents for selecting endophytic fungal strains for beneficial purposes as for example disease, pests and drought resistance (Easton et al., 2001; Easton and Tapper, 2005). Many different types of endophyte-grass symbionts are used for improvement of agricultural production in New Zealand due to increased resistance to environmental stresses and consequently a better yield (Johnson et al., 2013).

## Conclusions

Tested associations (fungus + host) exposed wide variation in response to the presence of elevated concentration of lead, cadmium and copper in soil. The presence of *Epichloë* sp. in perennial ryegrass tissues resulted in the increase of accumulation of cadmium and copper in aerial parts of the host plants. Phyto-beneficial effect of endophytes was strongly dependent on specific host–fungal mutual associations, which in turn were the effect of the host plant's origin. To achieve the best result of phytoremediation of heavy metals, the choice of the most effective perennial ryegrass-*Epichloë* symbiosis should be based on their laboratory evaluation.

## Methods

### Plant collection sites

Ecotypes of perennial ryegrass (*Lolium perenne* L.) were collected from 12 localities in Poland in a form of living plants from permanent grasslands in most cases used for cattle feeding. Different contents of HM in soil from mentioned localities has been reported by Terelak (2007). Those areas were located in Podlaskie (POD), Mazowieckie (MAZ) and Lubelskie (LUB) and Świętokrzyskie (SWK) regions located on Central European Plain, in Poland, between northern parallels 50.4° - 53.7° and eastern meridians 20.4° - 23.1°, on elevations 70 - 300 meters above the sea level (Fig. 1, Table 1). None from 12 localities were protected area, therefore no written permission were required for collection of perennial ryegrass, which is a common and not protected species. However, at each locality, field owners, managers etc., were asked for collecting permission. This is in line with our country official regulations concerning plant genetic resources collecting at *in situ* conditions.

Each locality, apart from GPS coordinates, was described during plant collection in terms of: general habitat description, soil moisture (high, medium, low), type and intensity of usage and soil type (mineral, organic or mineral-organic), based on observations and local farmers inquiries. Average concentration of Pb<sup>2+</sup>, Cu<sup>2+</sup> and Cd<sup>2+</sup> ions in soils for regions of collections sites were given on the basis of Terelak (2007).

### Plant collection

From five to ten plants were picked up in each locality, with the distances of 5–10 m from each other, in order to avoid clones collection. Since plants in described experiment were part of a large collection, their numbering has no ordinal values. Ecotype #50 was picked up at Podlaskie (POD) region; ecotypes numbered: 131, 685, 730, 801 and 873 at Mazowieckie (MAZ); #45 at Lubelskie (LUB) and ecotypes numbered: 87, 129, 160, 227 and 273 were collected at Świętokrzyskie (SWK) region. Collected ecotypes were replanted in a spaced nursery, with 0.5 m distances between plants in Radzików, Poland (52.21 N; 20.64 E). No additional treatments (fertilization, watering, chemical weed control) were applied.

### Endophyte detection

*Epichloë* endophyte-perennial ryegrass symbionts were identified by rapid staining method according to Saha et al. (1988). Ten tillers from each ecotype grown in the nursery were investigated. Small epidermal strips were

peeled off the adaxial surface of the leaf sheaths and placed into a drop staining solution: 0.5 % Rose bengal in 5% of ethyl alcohol to be examined under light microscope (magnitude of x100) for the presence of fungal hyphae (E+), which appeared as an intercellular, long and convoluted hyphae parallel to the leaf-sheath axis of the plant cell without forming haustorial structures (Clay and Holah, 1999; Suppl. Fig. 1). On bases of our recent discoveries and work done on perennial ryegrass endophytes (Wiewióra et al., 2015a, 2015b), fungus forming fungal hyphae inside intercellular spaces was described as belonging to genus *Epichloë* (*Clavicipitaceae*). For each location a percentage share (Ee [%]) of *Epichloë*-ryegrass symbionts in total number of ecotypes collected from particular site were calculated.

Twelve E+ ecotypes were selected as material for further studies. Seeds were collected from plants of those ecotypes grown in the nursery and again tested for the presence of the endophyte hyphae using Rose bengal staining method (Saha et al. 1988). Half of the seeds from each ecotype was treated with Tebuconazole (placing the seeds in a liquid suspension), a triazole fungicide to remove the endophyte from seed bulk (E-). Both: E+ and E- seeds were sown on filter paper and seedlings were transferred to 0.5 l pots filled with mixture (1:2) of sterilized sand and peat. Seedlings were grown in pots for next 4 weeks, with frequent watering and without additional fertilization.

The presence/absence of the endophyte hyphae was again confirmed on 3-4 weeks old seedlings by Rose bengal staining prior to microscopic examination of 3 tillers per each plant. For each ecotype 12 E- and 12 E+ plants were reproduced on a vegetative way: half of each set was intended for HM treatment and half remained as a control (no HM). As a result of the final round of vegetative reproduction 24 plants per ecotype E+ and the same number per E- were used in the experiment run in fourfold repetitions per 3 plants each. Again, the endophyte status (E+/E-) was checked.

## Pot experiment

From each ecotype for both E+ and E- forms 24 plants were planted, 3 in one 1.5 l pot containing mixture (1:2) of sterilized sand and peat substrate of the final content of: 95.1 N; 150.2 P<sub>2</sub>O<sub>5</sub>; 153.3 K<sub>2</sub>O; 55.5 MgO; 7.7 Pb<sup>2+</sup>; 0.2 Cd<sup>2+</sup> and 2.4 Cu<sup>2+</sup> [mg·kg<sup>-1</sup> of dry substrate]; pH= 6.1 and 13% of soil organic carbon (SOC).

Pot experiment has been arranged into randomized complete block design with 4 blocks, where each ecotype was grown in 4 pots per block (3 plants per pot): two pots with E+ plants and two pots with E- plants. From those four pots, two were treated with HM solution (see below) and two were control. Pots in blocks were re-arranged during the course of the experiment due to reduce positional effect and reduce the residual or pot-to-pot variance.

Experiment was run in unheated glasshouse, starting from late spring for 16 weeks in total, with first 7 weeks of HM treatment. Seedlings were planted into pots and after three weeks of growth in unheated glasshouse first watering was applied, than watering was applied 9 times during next 36 days of vegetation. Intervals between watering usually were 4 – 5 days. The whole watering brought in total 20 mg of Cd<sup>2+</sup> and 700 mg of both Pb<sup>2+</sup> and Cu<sup>2+</sup> ions in 1 kg<sup>-1</sup> of the used substrate. Finally, HM ions concentration in the substrate, as extracted by water, reached: 15.5 Cd<sup>2+</sup>; 550.9 Pb<sup>2+</sup>; 546.0 Cu<sup>2+</sup> [mg·kg<sup>-1</sup>].

## Phenotyping of E+ and E- ecotypes responses to HM ions

Biometric phenotyping of aboveground part of plants was done in order to determine the rate of plant growth. Three cuts of plants from all experimental pots were done after 1, 2 and 4 months of plant growth in pots since planting, followed by drying at 70° C for 3 days for determination of dry matter yield. Dry biomass from each pot was collected to determine HM concentration in plants.

Chlorophyll Content Index (CCI) was measured with CCM200 Plus (PSI, Brno, Czech Republic), on 3 leaves per plant for total 24 plants of both forms E+ and E- of each ecotype. The single result consisted of five single measurements per leaf.

Chlorophyll *a* (Chl *a*) fluorescence was measured using PocketPEA portable fluorimeter (Hansatech Instruments, King's Lynn, Norfolk, UK). Three measurements per plant (3 plants per ecotype per replication per variant) were done. Fluorescence was induced by saturating, red actinic light with energy of 3.500  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Measured and calculated parameters were used for interpretation of endophyte-plant interaction in the presence of HM ions. Measured parameters:  $F_0 \approx F_{50\mu\text{s}}$  [minimal fluorescence];  $F_M = F_P$  [maximal recorded fluorescence];  $t_{FM}$  [time (in ms) to reach the maximal fluorescence,  $F_M$ ]; Area [total complementary area between the fluorescence induction curve and  $F_M$  of OJIP curve]. Parameters calculated and listed by PocketPEA software:  $F_V$  [maximal variable fluorescence calculated as  $F_M - F_0$ ];  $F_V/F_M$  [force of the light reactions]; RC/ABS [the amount of active reaction centers per absorption];  $(1-V_J)/V_J$  [measure of forward electron transport];  $PI_{\text{ABS}}$  [performance index]. Above measurements (CCI and Chl *a*) were done 2 weeks after last HM ions dosing.

## Chemical analysis

Determination of HM concentration in plants and soil were done as described previously (Żurek et al., 2014) by Regional Agrochemical Station in Warsaw (accredited laboratory acc. PN-EN ISO/IEC 17025:2005). Plant material was washed with tap water and then with deionized water in an ultrasonic washer to remove all soil particles followed by drying at 70° C for 3 days. Three hundred [mg] of dried, ground plant material was wet-washed using concentrated nitric acid (Merck) in a microwave system (MDS 2000, CEM, USA).

For determination of total HM ions ( $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Cu}^{2+}$ ) concentration in soil, extraction of air-dried soil samples taken at the end of the experiment from each pot, ground to <0.25 mm and extracted with concentrated perchloric ( $\text{HClO}_4$ ) and fluoric (HF) acids and. Amount of  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Cu}^{2+}$  ions were measured using inductive coupled plasma spectrometry (ICP-AES). (Spectro Analytical Instruments GmbH, Kleve, Germany).

## Statistical Analysis

All calculations were made with STATISTICA® 12 for Windows (StatSoft, 2014). Significance of differences were accepted with 95% of probability. Least significant differences (LSD) were calculated according to Fisher test. T-tests were performed at independent samples mode. Two-way factorial ANOVA analysis were performed with ecotypes, presence of HM in soil and endophyte presence in plants applied as main factors. Principal

Component Analysis (PCA) analysis, based on correlation matrix algorithm were performed for all chlorophyll fluorescence *a* traits measured and calculated for all ecotypes.

## Abbreviations

ANOVA	analysis of variance
CCI	chlorophyll content index
Chl <i>a</i>	chlorophyll <i>a</i>
E	east
E+	ecotype-endophyte association
E-	ecotype endophyte free (non-colonized by endophyte)
HM	heavy metal
h.	high
l.	low
LUB	Lubelskie region
m.	medium
MASL	meters above sea level
MAZ	Mazowieckie region
min.	mineral
n	north
org.	organic
POD	Podlaskie region
SWK	Świętokrzyskie region
t	t statistic
PCA	Principal Component Analysis
PS II	photosystem II

## Declarations

**Ethics approval and consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and material:** The data sets supporting the results of this article are included within the article and its supplementary materials.

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**Author Contributions:** B.W. and G.Ż. conceived, designed and performed the experiments, data analysis and paper writing; K.R. fluorescence studies planning and data interpretation, data analysis and paper writing; K.P. fluorescence studies and data collection. All authors have read and approved the manuscript.

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## Tables

Region (code)	Ecotype number	Coordinates [decimal]		Elevation [m.a.s.l.]	Ee [%]
		latitude [N]	longitude [E]		
Podlaskie (POD)	50	53.653	23.138	118	57.4
Mazowieckie (MAZ)	873	52.826	21.494	100	98.1
	801	52.374	20.378	69	61.1
	131	52.195	22.482	150	87.0
	685	52.046	21.301	80	70.4
	730	51.705	21.617	128	64.8
Lubelskie (LUB)	45	50.840	21.924	155	94.4
Świętokrzyskie (SWK)	273	50.802	20.435	283	70.4
	160	50.682	20.732	284	98.1
	129	50.604	20.506	187	100.0
	227	50.539	20.935	185	64.2
	87	50.425	20.559	172	90.6

Table 1

Geographical description of ecotype collection sites: decimal coordinates of northern latitude [N] and eastern longitude [E], elevation above sea level [m.a.s.l.]. The percentage share of endophyte colonized plants (Ee [%]) is shown in the last column. Ecotypes are identified by reference numbers the same across the whole manuscript.

Source of variation	Aboveground biomass collected in:				CCI
	1-st cut	2-nd cut	3-rd cut	all cuts (sum)	
Main effects:					
ecotype [1]	2.75 **	3.64 ***	0.86 ns	1.94 **	2.94 ***
HM in soil [2]	97.05 ***	867.79 ***	205.01 ***	455.9 ***	98.90 ***
endophyte presence [3]	4.03 **	5.86 **	0.05 ns	0.05 ns	2.41 ns
Interactions:					
ecotype x HM	2.36 **	1.90 **	0.91 ns	1.72 ns	4.05 ***
ecotype x endophyte pres.	0.85 ns	1.62 ns	1.15 ns	1.39 ns	0.95 ns
HM x endophyte pres.	0.30 ns	2.60 ns	2.06 ns	0.78 ns	0.01 ns
[1] x [2] x [3]	1.23 ns	1.62 ns	0.81 ns	1.34 ns	1.78 ns

Table 2

Analysis of variance for the effect of ecotype, HM presence in soil and endophyte on yield of aerial parts of plants and CCI values. F-values were given and significance of the effects and interactions with probability higher than 99.9% (\*\*\*) or 95% (\*\*).

Source of variation	Chl a fluorescence parameters						
	F <sub>O</sub>	F <sub>M</sub>	F <sub>V</sub>	F <sub>V</sub> /F <sub>M</sub>	F <sub>V</sub> /F <sub>O</sub>	(1-V <sub>j</sub> )/V <sub>j</sub>	Area
Main effects:							
ecotype [1]	ns	ns	ns	ns	ns	ns	ns
HM in soil [2]	57.67 ***	31.48 ***	24.66 ***	16.70 ***	18.25 ***	9.23 **	ns
endophyte presence [3]	ns	ns	ns	ns	ns	ns	ns
Interactions:							
[1] x [2]	ns	ns	ns	ns	ns	ns	ns
[1] x [3]	ns	ns	ns	ns	ns	ns	ns
[2] x [3]	27.16 ***	13.66 ***	10.36 **	7.10 **	9.51 **	ns	15.47 ***
[1] x [2] x [3]	ns	ns	ns	1.90 **	ns	2.19 **	ns

Table 3

Analysis of variance for the effect of ecotype, HM presence in soil and endophyte presence in plants on selected parameters of Chl *a* fluorescence (F<sub>O</sub>, F<sub>M</sub>, F<sub>V</sub>, F<sub>V</sub>/F<sub>M</sub>, F<sub>V</sub>/F<sub>O</sub>, (1-V<sub>j</sub>)/V<sub>j</sub>). F-values were given and significance of the effects and interactions with probability higher than 99.9% (\*\*\*) or 95% (\*\*).

For T<sub>FM</sub>, RC/ABS and PI<sub>ABS</sub>, F-values for none of main effects or their interactions were significant.

HM ions content			
Source of variation	Pb <sup>+2</sup>	Cd <sup>+2</sup>	Cu <sup>+2</sup>
ecotype [1]	124.94 ***	31.26 ***	47.87 ***
endophyte presence [2]	ns	139.48 ***	180.79 ***
interaction [1] x [2]	210.84***	39.22***	95.03***

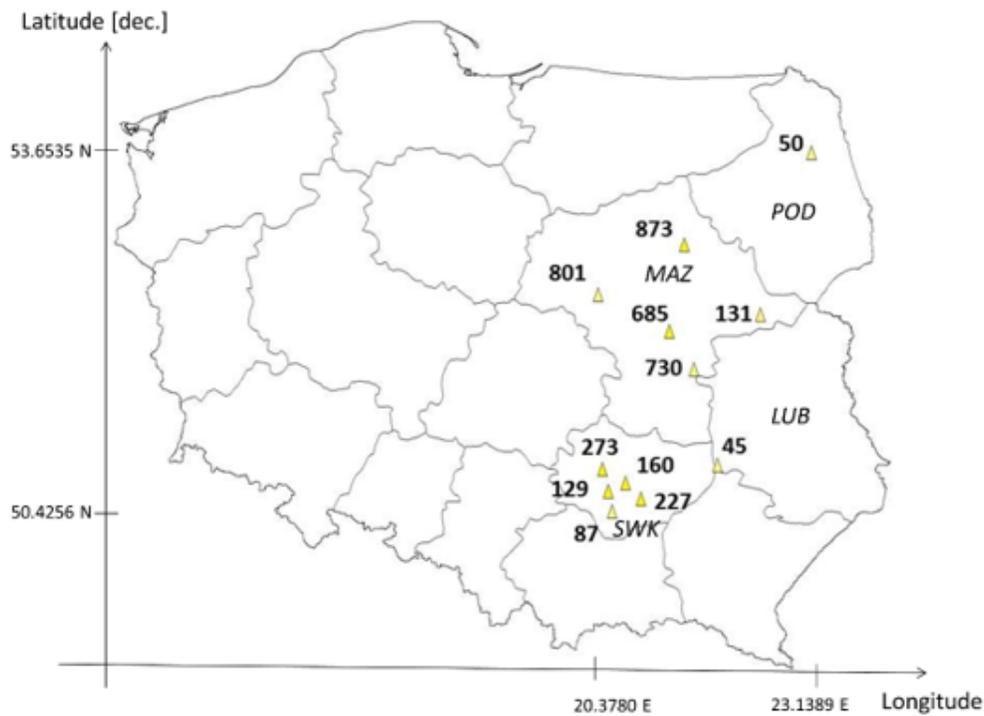
Table 4

Analysis of variance for the effect of ecotypes, endophyte presence in the host plant and their interaction on the content of HM ions in leaves of E+ (perennial ryegrass colonized by *Epichloë* endophyte) and E- (endophyte free perennial ryegrass). F-values were given and significance of the effects, with probability higher than 99.9% (\*\*\*).

Table 5. Mean values of the HM ions (Pb<sup>+2</sup>, Cd<sup>+2</sup>, Cu<sup>+2</sup>; mg•kg<sup>-1</sup>) contents in leaves of E+ (perennial grass colonized by *Epichloë* sp. endophyte) and E- (endophyte free perennial ryegrass) plants and the results of t-test for independent samples. Ecotypes were listed in decreasing order of collection sites latitude.

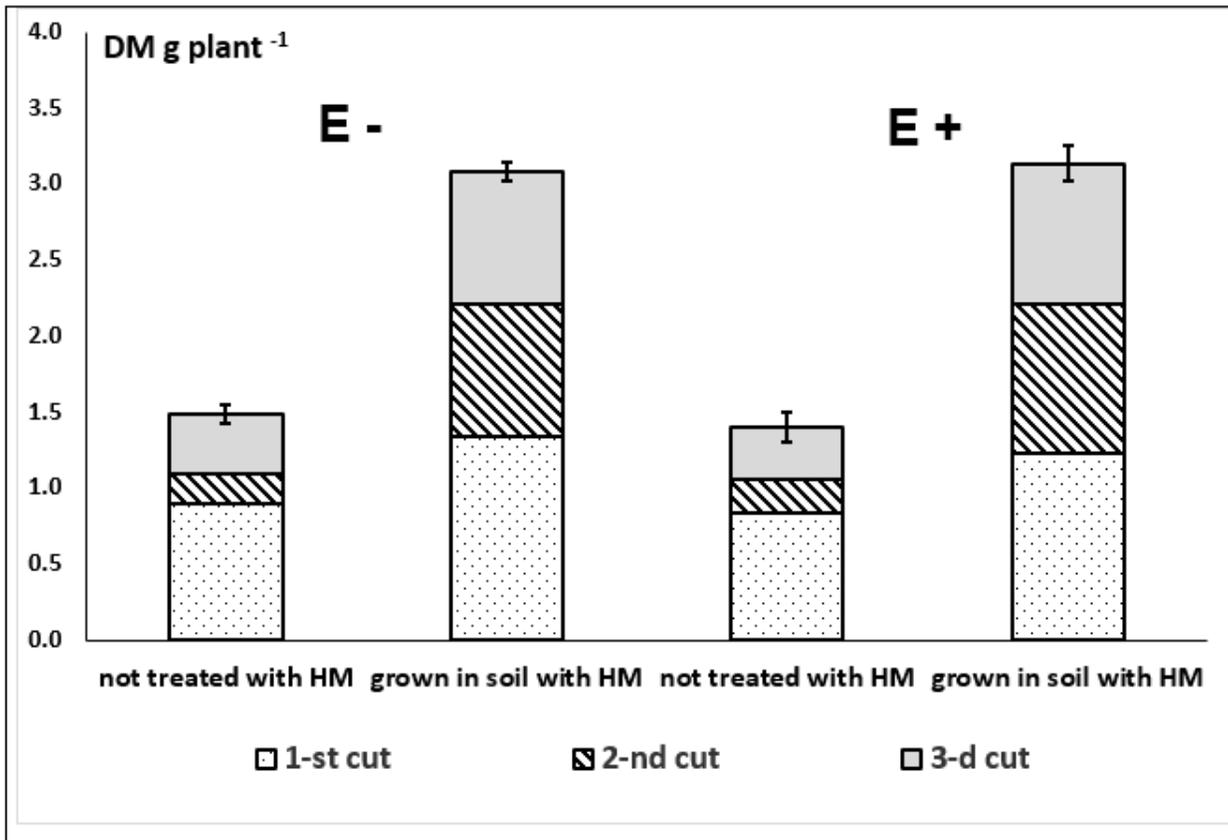
Region (code)	Ecotype number	Pb <sup>+2</sup>			Cd <sup>+2</sup>			Cu <sup>+2</sup>		
		E-	E+	t	E-	E+	t	E-	E+	t
POD	50	43.9	15.7	154.5 ***	11.8	12.3	n.s.	34.7	14.6	8.0 **
MAZ	873	16.5	32.7	- 6.8 **	8.4	8.8	n.s.	10.1	37.4	- 47.1 ***
	801	21.0	21.4	n.s.	10.3	19.8	- 18.3 ***	15.4	15.1	n.s.
	131	29.3	11.0	42.1 ***	14.8	10.3	7.2 **	23.1	13.8	9.8 **
	685	29.9	33.2	- 5.0 **	8.0	15.1	- 34.6 ***	20.9	26.5	- 2.9 *
	730	20.8	10.2	27.5 ***	7.1	8.1	- 6.7 **	15.1	14.6	n.s.
LUB	45	32.6	23.3	12.1 ***	9.1	16.2	- 5.2 **	25.7	30.8	- 3.7 **
SWK	273	16.0	28.8	- 23.8 ***	11.8	10.8	3.4 *	14.7	40.6	- 18.4 ***
	160	20.2	40.7	- 15.3 ***	7.4	13.4	- 9.2 ***	16.2	47.9	- 17.5 ***
	129	13.4	24.4	- 10.7 ***	10.3	13.4	- 11.2 ***	19.7	20.9	- 2.8 *
	227	10.4	18.9	- 14.0 ***	12.1	9.1	9.5 ***	14.0	19.6	- 6.4 **
	87	24.6	20.2	6.0 **	10.2	12.3	n.s.	21.3	22.1	n.s.
	mean	23.2	23.4	n.s.	10.1	12.4	- 3.44 ***	19.2	25.8	- 2.8 ***

## Figures



**Figure 1**

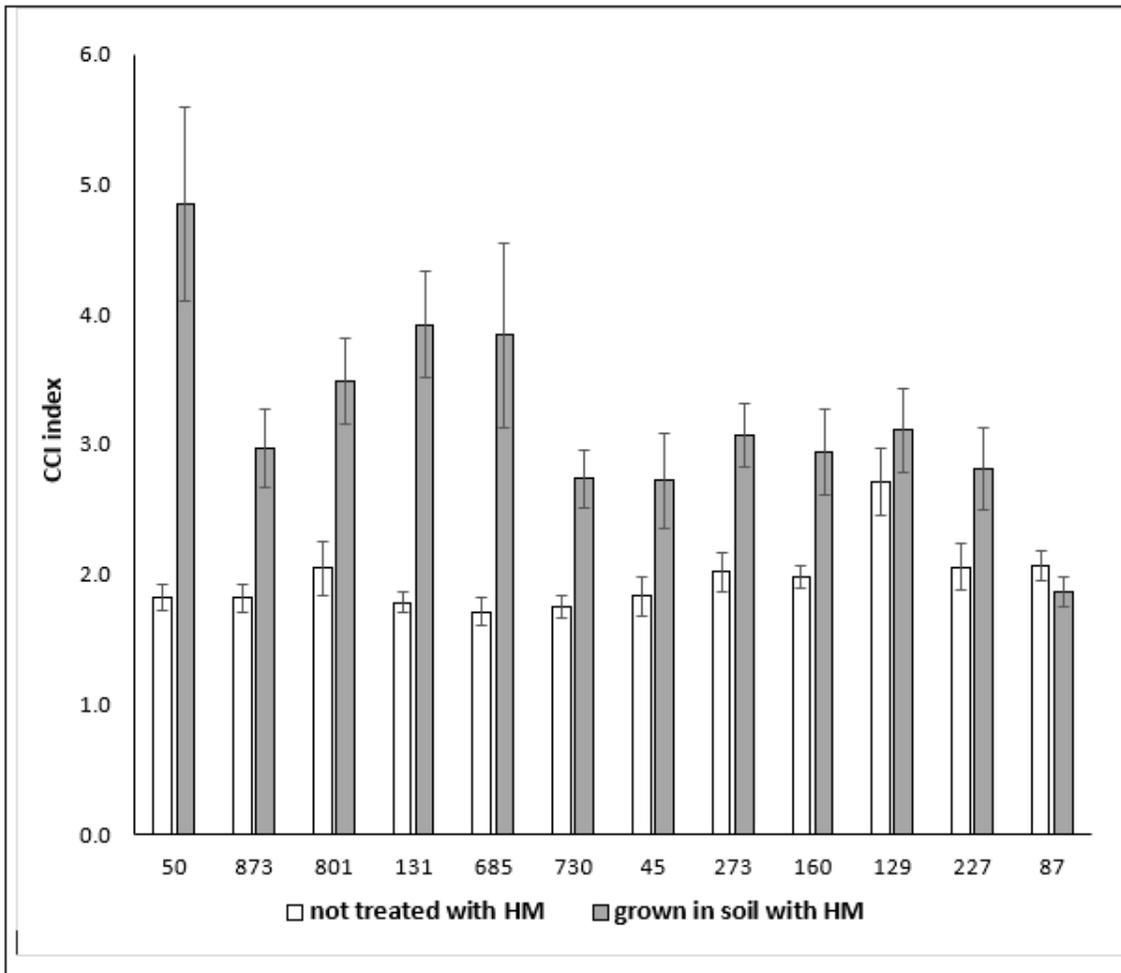
Map of the distribution of ecotype collection sites in regions: POD (Podlaskie), MAZ (Ma-zowieckie), LUB (Lubelskie), SWK (Świętokrzyskie). Ecotypes are identified by reference numbers the same across the whole manuscript. Map drawn with DIVA-GIS ver. 7.1.7 soft-ware (<http://www.diva-gis.org>)



Legend: E+ plants of perennial ryegrass with endophytes, E- plants of perennial ryegrass without endophytes, HM - heavy metals. Vertical bars – standard error for the sum of all 3 cuts.

**Figure 2**

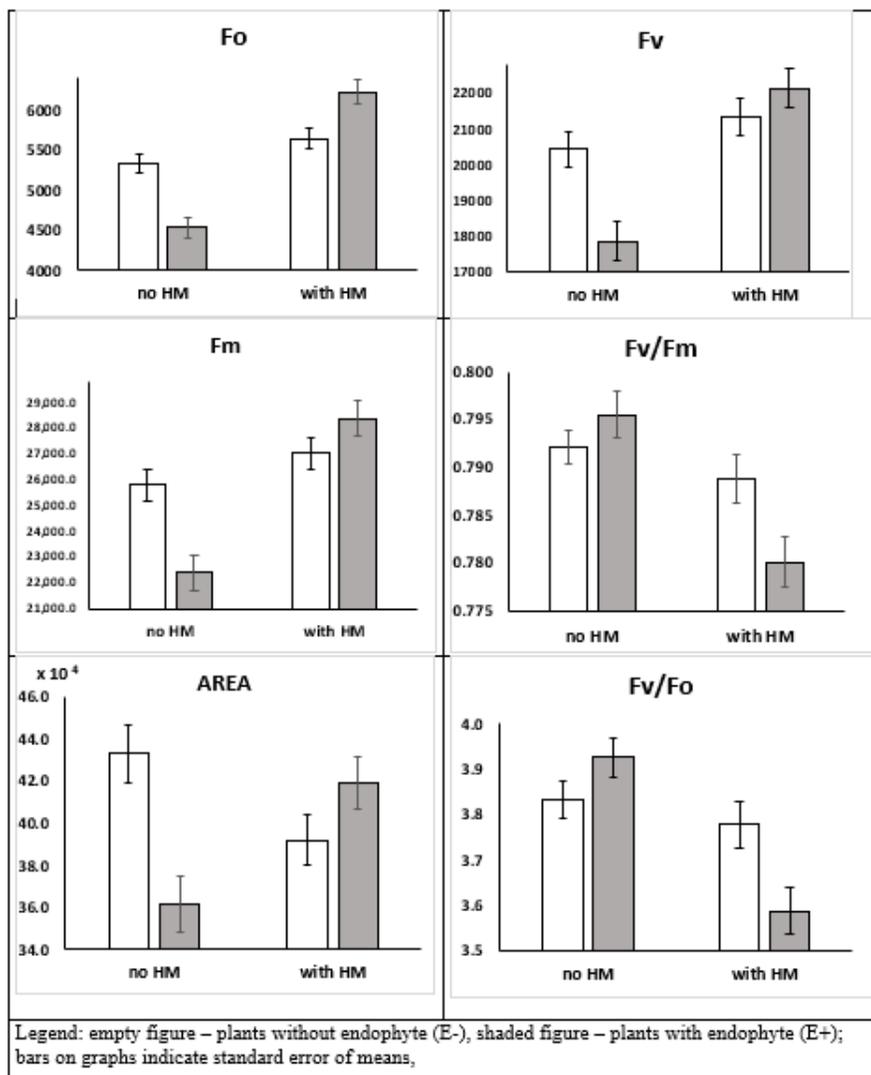
Average yields of dry biomass collected from young plants of perennial ryegrass with (E+) and without (E-) endophytes, grown in soil treated or not treated with HM.



Legend: vertical bars - standard errors of means

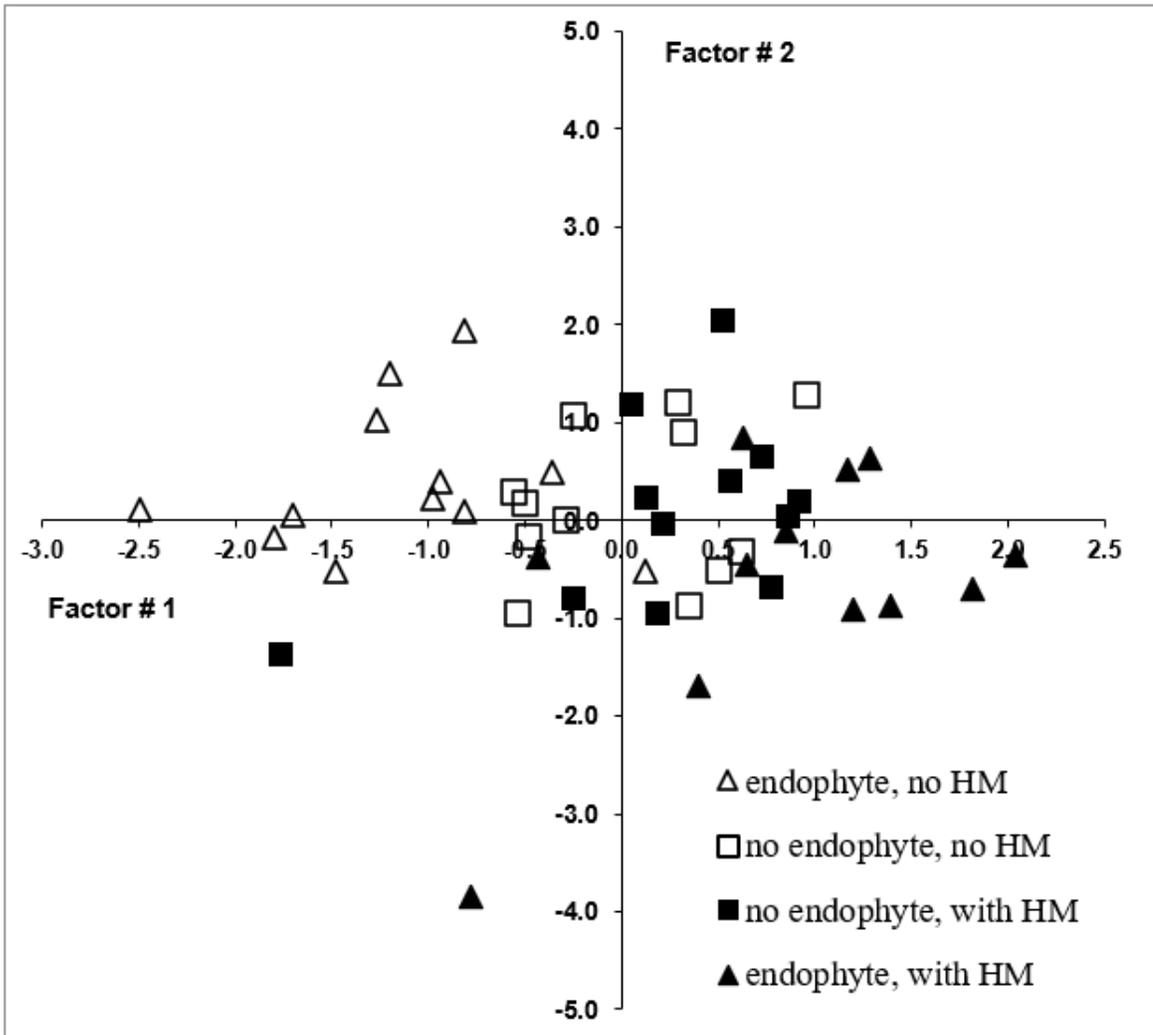
**Figure 3**

Mean values of chlorophyll contents index (CCI) in perennial ryegrass (E-) plants and Epich-loë-perennial ryegrass symbionts (E+) grown in control conditions (left bar for each ecotype) and in the presence of HM ions (right bar for each ecotype). Ecotypes organized on OX axis according to North-South location of collection sites. Left side of axis – most North locations, right side – most South locations.



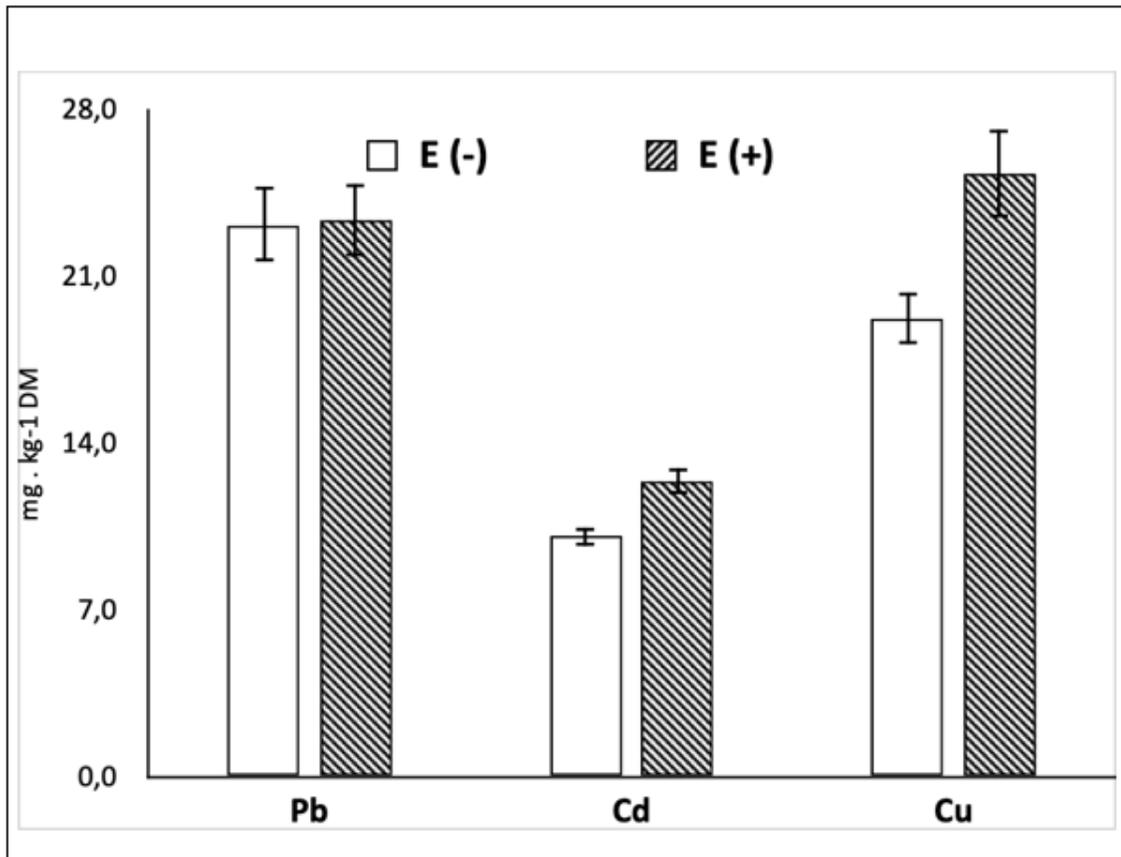
**Figure 4**

The effect of HM treatment of perennial ryegrass plants on chlorophyll a fluorescence parameters, were statistically significant interaction between HM in soils and endophyte presence were calculated: F<sub>0</sub>, F<sub>v</sub>, F<sub>m</sub>, F<sub>v</sub>/F<sub>m</sub>, F<sub>v</sub>/F<sub>0</sub> and Area. Bars on graph indicate standard error of means. For F<sub>0</sub>, F<sub>v</sub>, F<sub>m</sub> arbitrary units were used.



**Figure 5**

The graphical presentation of PCA analysis results based on measurements of Chl a parameters for ryegrass-Epichloë symbionts, grown in with or without HM supplementation.



Legend: E+ plants with endophytes, E- plants without endophytes, Pb- lead, Cd- cadmium, Cu- copper. Vertical bars – standard error of means.

**Figure 6**

Mean values of Pb<sup>2+</sup>, Cd<sup>2+</sup> and Cu<sup>2+</sup> contents in aerial parts of perennial ryegrass plants with (E+) and without (E-) endophytes. Vertical bars – standard error of means.

## Supplementary Files

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