

# Different Response of Perennial Ryegrass – Epichloë Endophyte Symbiota to the Elevated Concentration of Heavy Metals in Soil

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## Research Article

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# Different response of perennial ryegrass – *Epichloë* endophyte symbiota to the elevated concentration of heavy metals in soil

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**Abstract:** The phenomenon of plant mutualistic symbiosis with microbes may have a positive effect on the improvement of plant tolerance to environmental stresses as well as on the ability of plants to accumulate heavy metal (HM) ions from soil. The influence of *Epichloë* fungal endophyte (*Ascomycota*, *Clavicipitaceae*) on perennial ryegrass (*Lolium perenne* L.) plants grown in the presence of elevated concentrations of HM ions (Cd<sup>2+</sup>, Pb<sup>2+</sup> and Cu<sup>2+</sup>) in soil was studied. The presence of *Epichloë* in the host grass tissues resulted in different accumulation of HM ions in the aboveground parts of the plants. In some cases endophyte infection positively affected ryegrass ability to accumulate HM ions from soil. In plants with (E+) and without (E-) endophytes the hormesis effect was induced by the 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 HM accumulation, we were able to choose the best associations of perennial ryegrass with endophytes for HM phytoremediation.

**Keywords:** *Epichloë* endophytes; heavy metals; perennial ryegrass; photosynthesis; phytoremediation; soil pollution

## Introduction

Endophytes can colonize plant tissues and live without inducing any visible symptoms of biotic stress in plants. 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-Bajas et al. 2016). Hao et al. (2010) observed that treatment of suspension cells of *Ginkgo biloba* with fungal endophytes resulted in the 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, Slaughter et al. 2018). 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)

40 (Bacon et al. 2015, Dupont et al. 2015, Rodriguez et al. 2008, Schardl et al. 2012, Schardl et  
41 al. 2013).

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

60 Species of the fungal genus *Epichloë* (*Ascomycota*, *Clavicipitaceae*) are specialized  
61 fungi of cool-season grasses that can grow throughout the aerial parts of their host plants,  
62 forming systemic and predominantly asymptomatic associations, resulting in defensive  
63 mutualism (Clay 1988, Tadych et al. 2014). The importance of *Epichloë* endophytes for  
64 ecosystems due to modulation of both below- and aboveground ecosystem processes is well  
65 recognized and accepted (Saikkonen et al. 2016).

66 Phytoremediation is increasingly used as a sustainable approach for soil remediation.  
67 However, methodology improvement is constantly forced due to the expected increase in  
68 phytoremediation efficacy as well as due to economic reasons. Due to complex biological  
69 interactions, currently used methods do not always give the demanded results, so further  
70 multidirectional studies are needed (Thijs et al. 2017).

71 The aim of this study was to describe different reaction of perennial ryegrass – *Epichloë*  
72 endophyte association to the elevated concentration of lead, cadmium, and copper in soil with  
73 further possible application in the phytoremediation process.

## 74 **Materials and Methods**

### 75 *Plant Collection*

76 Ecotypes of perennial ryegrass (*Lolium perenne* L.) were collected from 12 localities in  
77 Poland in the form of living plants from permanent grasslands in most cases used for cattle  
78 feeding. The term 'ecotype' refers to a group of plants within a species that is adapted to  
79 particular environmental conditions (locality) and therefore exhibiting structural or  
80 physiological differences from the other members of the same species. Those areas were  
81 located in Podlaskie (POD), Mazowieckie (MAZ), Lubelskie (LUB), and Świętokrzyskie  
82 (SWK) regions located on Central European Plain, in Poland. (Fig. 1, Tab. 1, Supp. Tab.1).

83 From five to ten plants were picked up in each locality, with the distances of 5–10 m  
84 from each other, to avoid collecting clones. GPS coordinates of each locality has been also  
85 noted. Average concentration of  $Pb^{2+}$ ,  $Cu^{2+}$ , and  $Cd^{2+}$  ions in soils for regions of collections  
86 sites were given after Terelak (2007).

87 Since plants in the described experiment were part of a large collection, their numbering  
88 has no ordinal values. Collected ecotypes were further replanted in a spaced nursery, with  
89 0.5 m distances between plants in Radzików, Poland (52.21 N; 20.64 E). No additional  
90 treatments (fertilization, watering, chemical weed control) were applied.

### 91 *Endophyte Detection*

92 *Epichloë* endophyte-perennial ryegrass symbiota were identified by the rapid staining  
93 method according to Saha et al. (1988). Fungal hyphae (E+), appeared as an intercellular,  
94 long and convoluted hyphae parallel to the leaf-sheath axis of the plant cell without forming  
95 haustorial structures (Clay and Holah 1999), (Supp. Fig. S1). On the bases of our recent  
96 discoveries and work done on perennial ryegrass endophytes (Wiewióra et al. 2015a,  
97 Wiewióra et al. 2015b), fungus forming fungal hyphae inside intercellular spaces were  
98 described as belonging to genus *Epichloë* (*Clavicipitaceae*). Studies with confirmed isolates  
99 describing the typical fungal hyphae in the intercellular space of infected tissues from  
100 perennial ryegrass and our own recent studies, we refer the endophyte found in Polish  
101 perennial ryegrass to *Epichloë festucae* spp. *lolii*.

102 Twelve ecotypes with E+ plants were selected as material for further studies. Seeds were  
103 collected only from E+ plants of those ecotypes grown in the nursery and again tested for the  
104 presence of the endophyte hyphae using the rose bengal staining method (Saha et al. 1988).  
105 Half of the seeds from each ecotype was treated with Tebuconazole (placing the seeds in a  
106 liquid suspension), a triazole fungicide to remove the endophyte from seed bulk (E-). Both:  
107 E+ and E- seeds were sown on filter paper and seedlings were transferred to 0.5 l pots filled

108 with mixture (1:2) of sterilized sand and peat. Seedlings were grown in pots for 4 weeks, with  
109 frequent watering and without additional fertilization.

110 The presence/absence of the endophyte hyphae was again confirmed on 3-4 weeks old  
111 seedlings by rose bengal staining before microscopic examination of 3 tillers per each plant.  
112 For each ecotype 12 E- and 12 E+ plants were vegetatively propagated: half of each set was  
113 intended for HM treatment and half remained as a control (no HM). As a result of the final  
114 round of vegetative propagation 24 plants per ecotype E+ and the same number per E- were  
115 used in the experiment run in fourfold repetitions per 3 plants each. Again, the endophyte  
116 status (E+/E-) was checked.

#### 117 *Pot Experiment*

118 From each ecotype for both E+ and E- forms, 24 plants were planted, 3 in one 1.5 L pot  
119 containing a mixture (1:2) of sterilized sand and peat substrate of the final content of: 95.1  
120 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  
121 substrate]; pH= 6.1 and 13% of soil organic carbon (SOC).

122 The pot experiment has been arranged into randomized complete block design with 4  
123 blocks, where each ecotype was grown in 4 pots per block (3 plants per pot): two pots with  
124 E+ plants and two pots with E- plants. From those four pots, two were treated with HM  
125 solution (see below) and two were control. Pots in blocks were re-arranged during the  
126 experiment to reduce the positional effect and reduce the residual or pot-to-pot variance.  
127 Therefore, two factors were used in the mentioned experiment: the first – endophyte infection  
128 (E+ and E- plants) and the second – HM treatment.

129 The experiment was run in a glasshouse, starting from late spring for 16 weeks in total,  
130 with the first 7 weeks of HM treatment. Seedlings were planted into pots and after three  
131 weeks of growth in the glasshouse the first watering was applied, then watering was applied  
132 9 times during the next 36 days of growth. Control pots were watered with distilled water.  
133 Intervals between watering usually were 4 – 5 days. The whole watering brought in total 20  
134 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,  
135 HM ions concentration in the substrate, as determined by Regional Agrochemical Station in  
136 Warsaw (accredited laboratory acc. PN-EN ISO/IEC 17025:2005), reached: 15.5 Cd<sup>2+</sup>; 550.9  
137 Pb<sup>2+</sup>; 546.0 Cu<sup>2+</sup> [mg·kg<sup>-1</sup>].

#### 138 *Analysis of Biomass Yields, Relative Chlorophyll Contents and Chl a Fluorescence* 139 *Parameters*

140 Biometric phenotyping of the aboveground part of plants was done to determine the rate  
141 of plant growth. Three cuts of plants from all experimental pots were done after 1, 2, and 4

142 months of plant growth in pots since planting, followed by drying at 70°C for 3 days for  
143 determination of dry matter yield. Dry biomass from each pot was collected to determine HM  
144 concentration in plants.

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

148 Chlorophyll *a* (Chl *a*) fluorescence was measured using PocketPEA portable fluorimeter  
149 (Hansatech Instruments, King's Lynn, Norfolk, UK). Three measurements per plant (3 plants  
150 per ecotype per replication per variant) were done. Fluorescence was induced by saturating,  
151 red actinic light with energy of  $3.500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Measured and calculated parameters  
152 were used for the interpretation of endophyte-plant interaction in the presence of HM ions  
153 (Paunov et al. 2018). Measured parameters:  $F_0 \approx F_{50\mu\text{s}}$  [minimal fluorescence];  $F_M = F_P$   
154 [maximal recorded fluorescence];  $T_{FM}$  [time (in ms) to reach the maximal fluorescence,  $F_M$ ];  
155 Area [total complementary area between the fluorescence induction curve and  $F_M$  of OJIP  
156 curve]. Parameters calculated and listed by PocketPEA software:  $F_V$  [maximal variable  
157 fluorescence calculated as  $F_M - F_0$ ];  $F_V/F_M$  [force of the light reactions]; RC/ABS [the  
158 amount of active reaction centers per absorption];  $(1-V_J)/V_J$  [measure of forward electron  
159 transport];  $PI_{ABS}$  [performance index]. The above measurements (CCI and Chl *a*) were done  
160 2 weeks after the last HM ions dosing.

#### 161 *Chemical Analysis*

162 Determination of HM concentration in plants and soil were done as described previously  
163 (Żurek et al. 2014) by Regional Agrochemical Station in Warsaw (accredited laboratory acc.  
164 PN-EN ISO/IEC 17025:2005). Plant material was washed with tap water and then with  
165 deionized water in an ultrasonic washer to remove all soil particles followed by drying at  
166 70°C for 3 days. Three hundred [mg] of dried, ground plant material was wet-washed using  
167 concentrated nitric acid (Merck) in a microwave system (MDS 2000, CEM, USA).

168 For determination of total HM ions ( $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$  and  $\text{Cu}^{2+}$ ) concentration in soil, extraction  
169 of air-dried soil samples was taken at the end of the experiment from each pot, ground to  
170  $<0.25$  mm and extracted with concentrated perchloric ( $\text{HClO}_4$ ) and fluoric (HF) acids. The  
171 amount of  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ , and  $\text{Cu}^{2+}$  ions were measured using inductively coupled plasma  
172 spectrometry (ICP-AES, Spectro Analytical Instruments GmbH, Kleve, Germany).

#### 173 *Statistical Analysis*

174 All calculations were made with STATISTICA® 12 for Windows (StatSoft, 2014). The  
175 significance of differences was accepted with a 95% probability. Two-way factorial ANOVA

176 analysis was performed with ecotypes, presence of HM in soil, and endophyte presence in  
177 plants applied as main factors. Least significant differences (LSD) were calculated according  
178 to the Fisher test. T-tests were performed at independent samples mode for HM ions contents  
179 in leaves of E+ and E-. Principal Component Analysis (PCA) based on the correlation matrix  
180 algorithm was performed for all chlorophyll fluorescence traits measured and calculated for  
181 all ecotypes.

182

## 183 **Results**

### 184 *Plant Collection Sites*

185 Most of the soil beneath meadows from which perennial ryegrass plants were derived,  
186 were of mineral or organic type, with medium or low soil moisture content, mainly with  
187 medium or low-intensity usage as pastures or for cutting (Supp. Table 1). All regions except  
188 one (SWK) were characterized by relatively low concentrations of HM ions in soil: Pb<sup>2+</sup>- c.a.  
189 9.6, Cd<sup>2+</sup>- 0.17 and Cu<sup>2+</sup>- 4.3 [mg·kg<sup>-1</sup>]. Much higher (almost doubled) concentrations of HM  
190 ions have been reported by Terelak (2007) for the SWK region: Pb<sup>2+</sup>- c.a. 17.8, Cd<sup>2+</sup>- 0.37  
191 and Cu<sup>2+</sup>- 7.6 [mg·kg<sup>-1</sup>] (Fig. 1, Supp. Table 1).

### 192 *Analysis of Biomass Yields, Relative Chlorophyll Contents and Chl a Fluorescence* 193 *Parameters*

194 Biomass yields were significantly affected by the ecotype and HM treatment throughout  
195 the whole experiment whereas the main effect of the endophyte was significant only for the  
196 first (after a month) and second cuts (after two months) (Table 2).

197 Generally, for plants grown in the presence of HM ions, dry matter yields together for  
198 three cuts were higher (3,1 g/plants) than for control plants (1,3-1,5g/plants) irrespective of  
199 endophyte presence in plants (Fig. 2, Supp. Fig. 2). The yield of plants grown in the presence  
200 of HM, despite the presence of endophyte in plants, was 48% higher than control at 1st cut,  
201 342% at 2nd and 143% at 3rd cut on average for the whole experiment the total yield from  
202 HM treated plants was 115% higher than that of the control plants. Mentioned difference was  
203 statistically significant (p=0.0000; F=387.26).

204 Elevated concentrations of the HM in the soil as well as the provenance of the tested  
205 ecotypes were the main sources of variation for the relative chlorophyll content, expressed  
206 as CCI. In contrast, neither endophyte presence nor its interaction with the plant provenance  
207 and HM gave a significant effect on the CCI (Table 2). The CCI in HM treated ecotypes was  
208 on average higher than in non-HM treated ones (Fig. 3) and above difference was also  
209 significant (p=0.0000; F=86.21).

210 Elevated concentration of the HM in the soil was also the main source of variation of  
211 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, Supp. Fig.  
212 3).

213 Neither the ecotype nor endophyte status resulted in a significant effect of any of the  
214 above- mentioned Chl *a* fluorescence parameters. However, a significant interaction between  
215 HM presence in soil and endophyte presence in plants has been calculated for  $F_0$ ,  $F_M$ ,  $F_V$ ,  
216  $F_V/F_M$ ,  $F_V/F_0$ , and Area (Table 3, Figure 4). For the parameters  $T_{FM}$ , RC/ABS, and  $PI_{ABS}$ ,  
217 none of the main sources of variation nor interactions were significant, therefore they were  
218 not listed in Table 3 and Fig. 4.

219 Considering interactions presented in Figure 4, perennial ryegrass plants, if grown  
220 without the addition of HM, exhibited some negative effects of endophyte presence in tissues,  
221 as reflected in lower values of  $F_M$ ,  $F_V$ , and higher for Area. When HM was added to the soil  
222 medium, values of the mentioned parameters increased in the presence of endophytes.  
223 However, the value of the parameter reflecting the force of light reactions of PS II ( $F_V/F_M$ )  
224 was significantly lower in the presence of HM in soil and endophytes in plant tissues.  
225 Therefore, whether E+ plants score higher or lower values of mentioned Chl *a* parameters  
226 than E- plants, depends on the addition of HM to the soil medium.

227 Measured parameters of Chl *a* ( $F_0$ ,  $F_M$ ,  $F_V$ ) were influenced by HM treatment (Table 3,  
228 Supp. Fig. 3). Interestingly, E+ plants collected in more northern localities were characterized  
229 by a more visible decline of  $F_V/F_M$  and  $F_V/F_0$  ratios. And, as in the case of measured  
230 parameters, E+ ecotype 730 reacted differently, by their slight increase. The ratio of  $F_V/F_0$   
231 was  $\leq 4.0$  in E- plants, whereas in E+ plants in 3 cases the ratio exceeded 4 (ecotypes 45, 87  
232 and 873). Parameter  $(1-V_j)/V_j$ , the measure of forward electron transport, seemed to be  
233 slightly affected by HM, especially in the leaves of E+ plants.

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

239 Ecotypes with endophytes, grown in soil without HM were separated on the left side of  
240 the graph, as opposed to E+ grow with the addition of HM. Negative values of factor 1, which  
241 is negatively correlated with  $F_0$ ,  $F_V$ ,  $F_M$ , and Area, were ascribed to increased values of the  
242 mentioned Chl *a* parameters. On the right side of the OX axis, along with decreasing values



243 of Chl *a* parameter, points representing E+ plants grown with the addition of HM were  
244 located. This is another presentation of the interaction between HM and endophyte presence.

#### 245 *HM Ions Content in E+ and E- Ecotypes*

246 Analysis of variance for the data of HM ions concentration in the plant tissue revealed a  
247 statistically significant influence of both: plant provenance and endophyte presence in the  
248 host plant as well as their interaction in case of Cd<sup>2+</sup> and Cu<sup>2+</sup> ions concentration (Table 4).

249 The highest concentration of HM ions (sum of Pb<sup>2+</sup>, Cd<sup>2+</sup> and Cu<sup>2+</sup>) was detected in the  
250 leaves of E+ variant of ecotype 160 (102 mg·kg<sup>-1</sup>), whereas in the leaves of the E- plants, the  
251 concentration of HM was low (44 mg·kg<sup>-1</sup>) (Tab. 5). Differences in the particular ions  
252 concentration of the above-mentioned ecotype were as follows: almost two-fold higher  
253 concentration of Pb<sup>2+</sup> and Cd<sup>2+</sup> ions and threefold of Cu<sup>2+</sup> in E+ plants as compared to E-.

254 The highest concentration of Pb<sup>2+</sup> (43.9 mg·kg<sup>-1</sup>) was detected in the E- plants of ecotype  
255 50 from POD region, whereas the lowest (10.2 mg·kg<sup>-1</sup>), in the E+ variant of ecotype 730  
256 from MAZ region. The average value of Pb<sup>2+</sup> for regions was the lowest for E- variants of  
257 plants from SWK region (16.9 mg·kg<sup>-1</sup>), but it was also the highest (26.6 mg·kg<sup>-1</sup>) for E+  
258 plants from the same region. Considering E+ plants, the highest Pb<sup>2+</sup> concentration (40.7  
259 mg·kg<sup>-1</sup>) was detected in ecotype 160, and was also high in ecotypes 685 and 873 (33.2 and  
260 32.7 mg·kg<sup>-1</sup>, respectively). For all those three mentioned ecotypes, Pb<sup>2+</sup> concentration in E+  
261 plants was significantly higher than in E- plants. But at the same time, for other ecotypes (50,  
262 131, 730, 45 and 87), the Pb<sup>2+</sup> ions concentration was higher in E- plants than in E+.

263 Cadmium concentration in aerial parts of E+ ecotypes was the highest in ecotype 801  
264 (19.8 mg kg<sup>-1</sup>) as well as in ecotypes: 45 and 685 (16.2 and 15.1 mg kg<sup>-1</sup>, respectively) (Tab.  
265 5). In a manner similar to relations described above for Pb<sup>2+</sup> concentration, for all three  
266 ecotypes with relatively high Cd<sup>2+</sup> concentration in E+ plants, the Cd<sup>2+</sup> ions concentration  
267 was significantly higher than the concentration values found in E- plants. Average  
268 concentration of Cd<sup>2+</sup> in plants was similar between regions of ecotype provenance, and it  
269 ranged from 9.1 – 11.8 for E- plants and 11.8 to 16.2 for E+ plants. For three from 12 tested  
270 ecotypes there were no significant difference between Cd<sup>2+</sup> concentration in E+ and E- plants.

271 High copper concentration was found in aerial parts of E+ ecotypes 160, 273 and 873  
272 (47.9, 40.6 and 37.4 mg·kg<sup>-1</sup>, respectively). All mentioned values were significantly higher  
273 than in leaves of corresponding E- plants. The average high concentration of Cu<sup>2+</sup> ions in E+  
274 plants (ca. 30 mg·kg<sup>-1</sup>) was noted for central and southern regions i.e. LUB and SWK. But  
275 the highest concentration of Cu<sup>2+</sup> (34.7 mg·kg<sup>-1</sup>) was noted in E- plants of ecotype 50, from  
276 POD region, which was the northern most exposed.

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

- 279 (i) E+ plants accumulated less HM ions from the soil than E- plants. In the  
280 experiment there were following ecotypes: 131 (all HM ions), 50 (Pb<sup>2+</sup> and Cu<sup>2+</sup>  
281 ions), 730 and 87 (Pb<sup>2+</sup> ions), 273 and 227 (Cd<sup>2+</sup> ions);
- 282 (ii) E+ and E- plants accumulated the same amounts of HM ions (no significant  
283 difference). Such was the case of ecotypes: 87 (Cd<sup>2+</sup> and Cu<sup>2+</sup> ions), 801 (Pb<sup>2+</sup>  
284 and Cu<sup>2+</sup> ions);
- 285 (iii) E+ plants accumulated a higher amount of HM ions from soil than E- plants:  
286 Ecotypes 60, 129 and 685 for all HM ions; ecotypes 45, 227, 273 and 873 for  
287 two different HM ions;
- 288 (iv) variable interaction depending on HM ion: Ecotype 730 higher concentration in  
289 E- for Pb<sup>2+</sup>, higher concentration in E+ for Cd<sup>2+</sup>, no difference between E+ and E- for  
290 Cu<sup>2+</sup>.

291

## 292 **Discussion**

293 There is increasing evidence that interactions of plants and microbes (including  
294 endophytes) play a critical role in metal phytoextraction and metal-mining, as they mediate  
295 different physicochemical and biological activities to facilitate ecological performances of  
296 the host plant (Muehe et al. 2015). The results of our studies revealed considerable variation  
297 in terms of the grass-fungus association's ability to cope with elevated concentration of HM  
298 ions in the soil. Mentioned 'variation' should be ascribed to the natural variation between  
299 host (perennial ryegrass), fungus and to their interaction. Spatial variation of mutualistic  
300 interactions between a host organism (grass plant) and infecting fungus (endophyte) through  
301 its intensity (endophyte frequency per locality) and production of toxic metabolite i.e.  
302 ergovaline, has been previously described (Żurek et al. 2013, Żurek et al. 2017).

303 Plants subjected to increased HM contents in soil were characterized by significantly  
304 higher values of the CCI and plant biomass - this could be explained based on soil fertility.  
305 The soil used for this experiment contained a low level of Cu<sup>2+</sup> ions (2.4 mg·kg<sup>-1</sup>) and high  
306 amounts of soil organic carbon (SOC), 13%. The natural content of Cu<sup>2+</sup> in soil was in the  
307 range of 15 to 40 mg·kg<sup>-1</sup> in the 0 – 20 cm soil horizon and concentration of Cu<sup>2+</sup> below 3.0  
308 mg·kg<sup>-1</sup> is usually defined as a deficit for grass species (Olszewska et al. 2008, Wyszowska  
309 et al. 2013). In the presence of high organic matter content in the soil, the Cu<sup>2+</sup> deficit for  
310 plants is quite frequent. Moreover, monocotyledonous plants (e.g. grasses) are particularly

311 sensitive to  $\text{Cu}^{2+}$  deficit (Yamasaki et al. 2008). Unfortunately all these three facts together  
312 were met together in our experiment, therefore the addition of Cu to soil medium yielded  
313 better growth of HM treated plants, which was manifested in higher CCI values. The  
314 differences were not statistically significant for ecotypes collected from the southern region,  
315 except for 1 ecotype of E+ and 2 ecotypes of E- which could be the result of adaptation to  
316 naturally occurring conditions of increased HM content in soil (Rodriguez et al. 2008).

317 Chl *a* fluorescence detection and parameters analyses ( $F_0$ ,  $F_M$ ,  $F_V$ ,  $F_V/F_M$ ,  $F_V/F_0$ ,  
318 RCB/ABS, Area,  $(1-V_j)/V_j$ ,  $P_1$ ) are simple and widely recognized methods to assess the stress  
319 influence on plants (Žurek et al. 2014, Kalaji et al. 2016). Among fluorescence parameters  
320 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  
321 found significantly influenced by both HM ions addition and its interaction with endophyte  
322 status. As a reaction to stress,  $F_0$  value mostly increases, which is interpreted as lower  
323 efficiency of energy transfer between chlorophyll antennas in PS II, and our data follow  
324 reports in the literature (Prasad and Strzałka 1999). Although the increase of  $F_0$  was detected  
325 in the case of the majority of studied ecotypes, the E+ ecotypes, as compared to E-, were  
326 characterized by lower values of this parameter pointing to the positive influence of *Epichloë*  
327 in the host plants, as was shown in studies on host orchard grass as well (Rozpądek et al.  
328 2015). The  $F_M$  is decreasing in response to stresses due to the fact that not all electron  
329 acceptors in PS II can be reduced. Considering results obtained in our experiment, endophyte  
330 presence in plant tissues seems to induce stress to a plant, as reflected by a decrease of  $F_M$ .  
331 The  $F_V/F_0$  ratio, also used for the detection of PS II destruction upon stress can descend from  
332 values of 4-5 down to 1. According to the results obtained in this experiment, a slight but  
333 significant (< 5% in average) decrease of  $F_V/F_0$  ratio was detected, showing that the stress  
334 did not influence the photosynthetic machinery too much (Kalaji and Łoboda 2010).

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

340 The distribution of points on the PCA graph indicates that the presence of HM in soil  
341 increased stress for plants as reflected by the Chl *a* parameter describing the efficiency of PS  
342 II. Points representing the efficiency of PS II in the presence or absence of HM in soil for E+  
343 plants were separated over the OX axis. Considering the negative sign of correlation  
344 coefficients between factor 1, factor 2, and Chl *a* parameters, points on the left side of the

345 OX axis (negative values of factor 1) represent the better status of plants than points on the  
346 right (positive values of factor 1). In the case of the absence of endophyte in host plants, there  
347 is also no clear separation of points representing the efficiency of PS II in the presence or  
348 absence of HM in soil.

349 Increased nutrient content due to endophyte presence was observed by many authors  
350 (Soto-Barajas et al. 2016, Malinowski et al. 2004, Zabalgogezcoa et al. 2006). In contrast,  
351 an absence of endophyte effect for total N (Lewis et al. 1996) and Zn concentration (Monnet  
352 et al. 2005) was reported with a single perennial ryegrass genotype evaluated. In the current  
353 experiment, we have observed the whole range of possible reactions: from E+ plants  
354 accumulating less HM than E- plants, through no effect, to increased accumulation of one,  
355 two, or three HM ions from the soil by E+ plants. Detected differences resulted, probably not  
356 only from differences in the endophyte activities but also from strong interactions between  
357 the fungus and the host plant, which arose as a result of particular conditions in an origin site.  
358 In the current research, spatial aggregation of E+ plants able to uptake relatively higher  
359 amounts of the HM from the soil has been found for Pb<sup>2+</sup> accumulation. Perennial ryegrass  
360 ecotypes collected from the SWK region (locations below the latitude 50.84 N) demonstrated  
361 the ability for accumulation of relatively higher concentration of Pb<sup>2+</sup> ions in E+ plants than  
362 those from the other regions. It could be presumed that it is in line with the natural  
363 concentration of Pb<sup>2+</sup> in the soils from this region which was concentrated in average of 17.8  
364 mg·kg<sup>-1</sup> of soil as compared to 9.4-10.2 mg·kg<sup>-1</sup> of the soils from other sampling sites in our  
365 experiment (Table S1). Hesse et. al. (2003, 2004) concluded that plant-endophyte  
366 associations are adapted to their native habitats via natural selection. As we have mentioned  
367 before, the natural content of the HM, especially Pb<sup>2+</sup> ions, in soil was higher in the SWK  
368 region than in other regions. Probably symbiote of this origin used to accumulate more Pb<sup>2+</sup>  
369 than those coming from areas of low Pb<sup>2+</sup> concentration. This could be further hypothesized  
370 that the whole microbiome of plants that came from soils of high Pb<sup>2+</sup> concentration could  
371 be different from soils of low Pb<sup>2+</sup> concentration. The role of the microbiome on plant health  
372 and HM tolerance has been recently widely analyzed and discussed (Dongchu et al. 2019,  
373 Ikram et al. 2018, Seneviratne et al. 2017).

374 The presence of HM tolerant endophytes could improve metals uptake and accumulation  
375 in hosting plants (Li et al. 2012). Endophyte colonization promoted Cd<sup>2+</sup> ions accumulation  
376 in tall fescue (Ren et al. 2011) and also improved Cd<sup>2+</sup> transport from the root to the shoot.  
377 Hesse et al. (2003, 2004) have also found higher abundances of infected perennial ryegrass  
378 genotypes on dry sites compared to wet sites and this has been confirmed in our previous

379 research (Żurek et al. 2013, Żurek et al. 2017). An abundance of endophyte-infected  
380 perennial ryegrass plants was significantly and negatively correlated with annual as well as  
381 winter precipitation (multi-annual averages, 1950–2000) at localities of their origin.  
382 Considering the habitat of symbiota origin, for example, Dobrindt et al. (2013) reported  
383 higher incidences of *Neotyphodium lolii* at sites of limestone bedrock. Therefore, conditions  
384 at the place of host plant origin (both climatic and soil) may influence its ability to cope with  
385 abiotic stress (drought, soil acidity, toxic metals in soil). Differences observed between the  
386 host plants appear to depend on the endophyte and the host life histories, as well as on fungal  
387 and plant genotypes, abiotic and biotic environmental conditions, and their interactions  
388 (Saikkonen et al. 2013). Specific genotypic combinations of both host and endophyte  
389 determine the morphology and physiology of endophyte colonized grasses, as well as  
390 regulates how selective pressure acts on them (Hill et al. 1996).

391

## 392 **Conclusions**

393 Tested associations (fungus + host) exerted wide variation in response to the presence of  
394 an elevated concentration of lead, cadmium, and copper in the soil. In some cases, the  
395 presence of *Epichloë* sp. in perennial ryegrass tissues resulted in the increase of accumulation  
396 of above mentioned heavy metals in aerial parts of the host plants. Generally, in the presence  
397 of endophyte mycelium, an increased accumulation of cadmium and copper was found, but  
398 not for lead.

399 The phyto-beneficial effect of endophytes was strongly dependent on specific host–  
400 fungus associations, which in turn could be the effect of the host plant’s i.e. ecotype  
401 provenance. However, results obtained in the experiment described above are not sufficient  
402 to draw conclusions on the relationship between the provenance of symbiota and their ability  
403 to accumulate heavy metals from the soil.

404 To achieve the best result of the phytoremediation of heavy metals, the choice of the  
405 most effective perennial ryegrass-*Epichloë* symbiosis should be based on their laboratory  
406 evaluation.

407

## 408 **Declarations**

409

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411 **Conflicts of Interest/Competing interests:** The authors declare no conflict of interest.

412 **Availability of data and material:** All supporting data are included within the article and  
413 its additional files.

414 **Code availability:** Not applicable

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

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423

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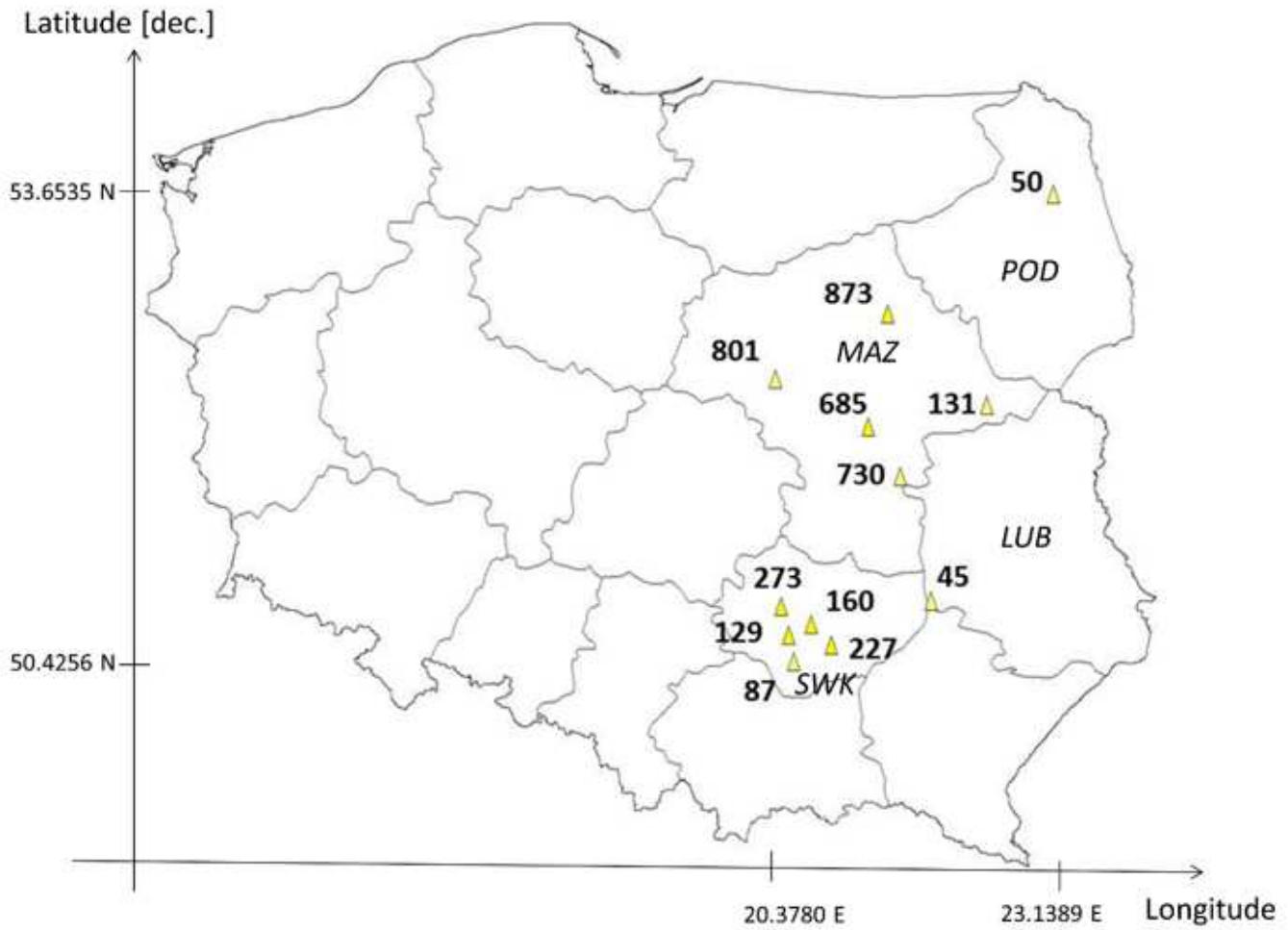
## 604 Abbreviations

ANOVA	analysis of variance
Area	total complementary area between the fluorescence induction curve and F <sub>M</sub> of OJIP curve
CCI	chlorophyll content index
Chl <i>a</i>	chlorophyll <i>a</i>
E	east

E+	grass-endophyte association
E-	endophyte-free grass (non-colonized by endophyte)
F <sub>0</sub>	minimal fluorescence
F <sub>M</sub>	maximal recorded fluorescence
F <sub>V</sub>	maximal variable fluorescence (F <sub>M</sub> -F <sub>0</sub> )
F <sub>V</sub> /F <sub>M</sub>	maximum quantum efficiency of PSII photochemistry
F <sub>V</sub> /F <sub>0</sub>	driving force of light reactions
(1-V <sub>j</sub> )/V <sub>j</sub>	measure of forward electron transport
HM	heavy metal
h.	high
l.	low
LUB	Lubelskie region
m.	medium
m.a.s.l.	meters above sea level
MAZ	Mazowieckie region
min.	mineral
n	north
org.	organic
POD	Podlaskie region
PCA	Principal Component Analysis
PI <sub>ABS</sub>	performance index
PS II	photosystem II
RC/ABS	amount of active reaction centers per absorption
SWK	Świętokrzyskie region
t	t statistic
T <sub>FM</sub>	time needed to reach the maximal fluorescence

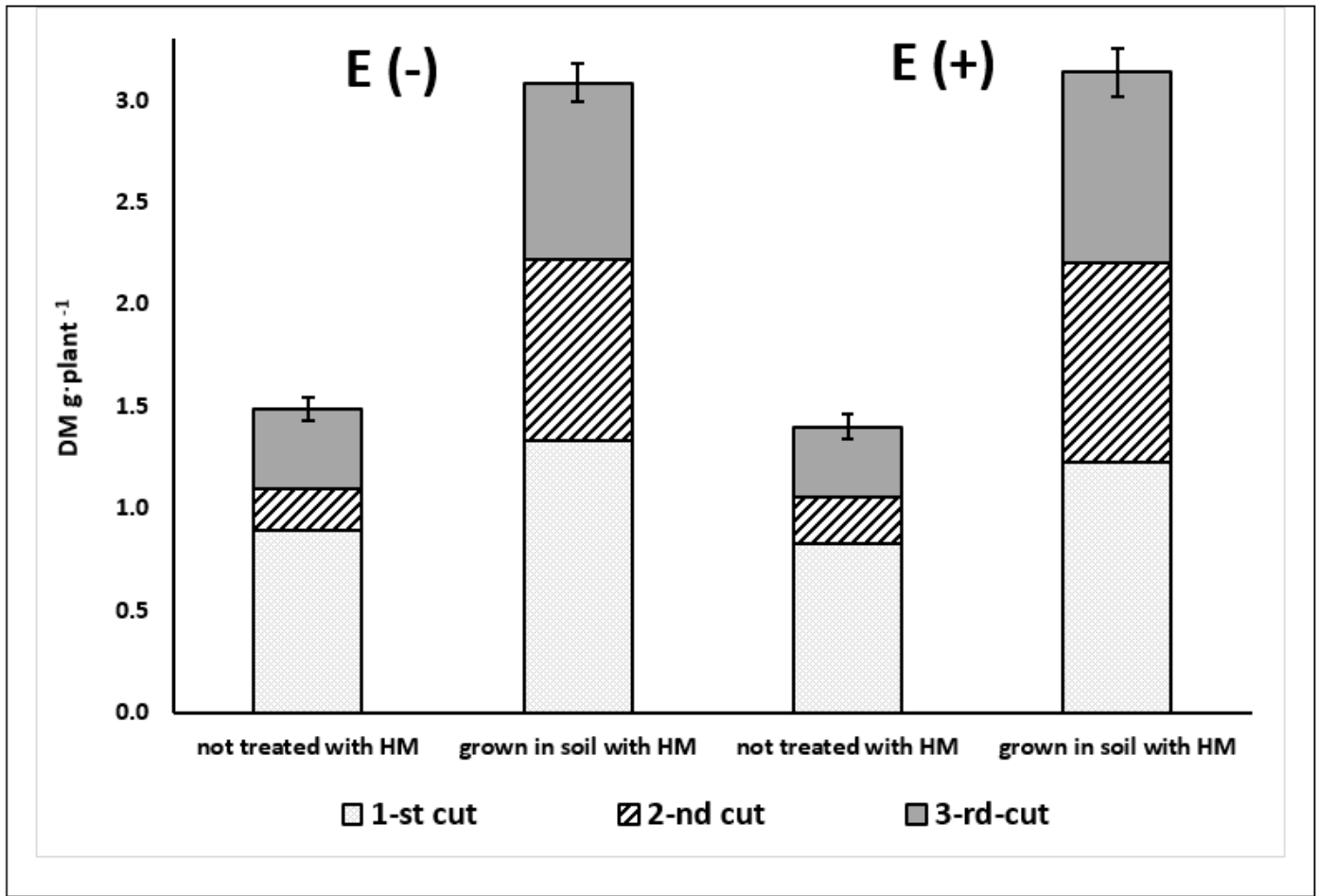
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# Figures



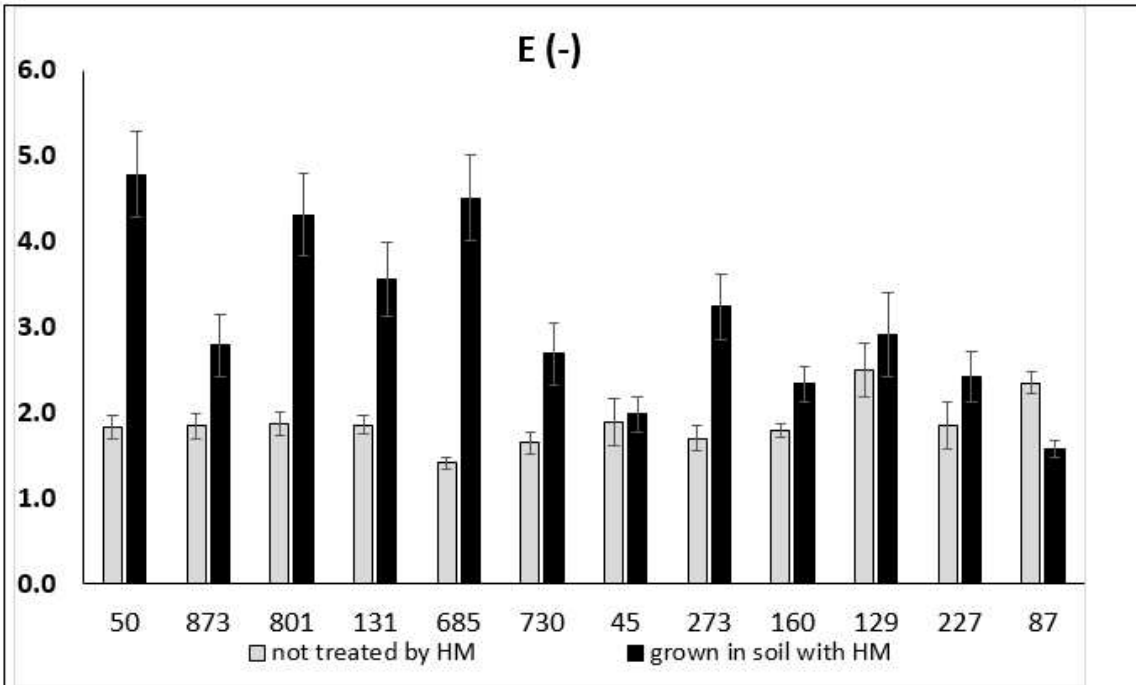
**Figure 1**

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

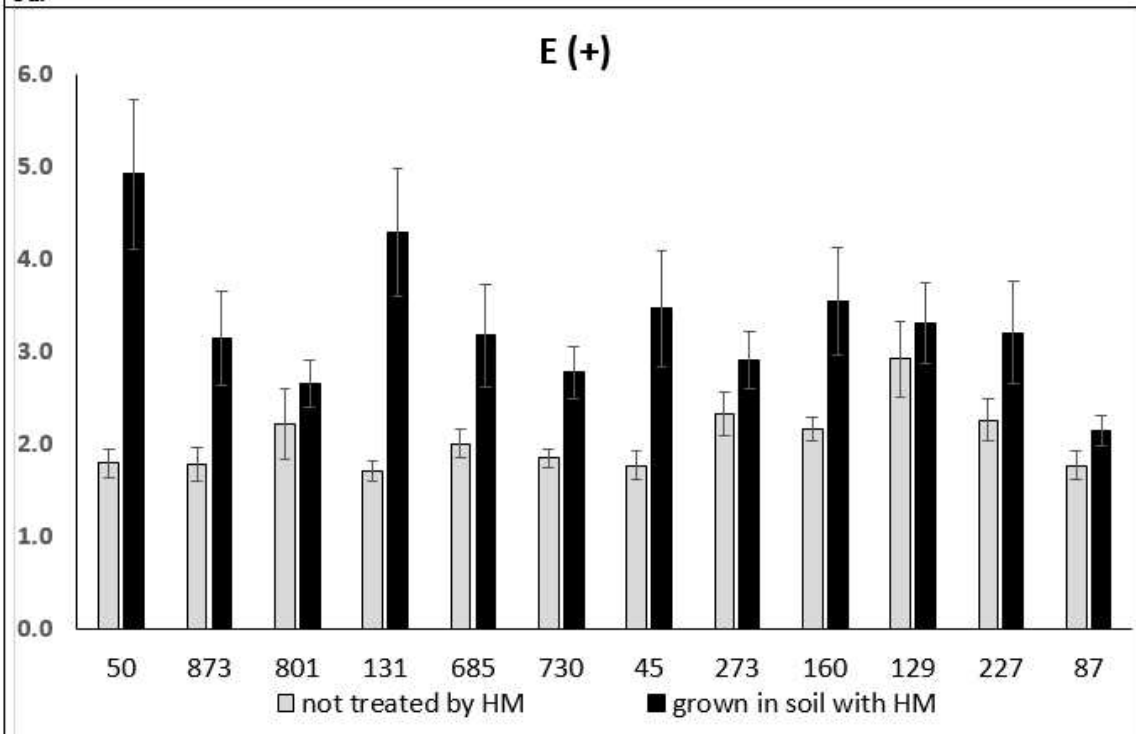


**Figure 2**

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. Error bar for the sum of 3 cuts.



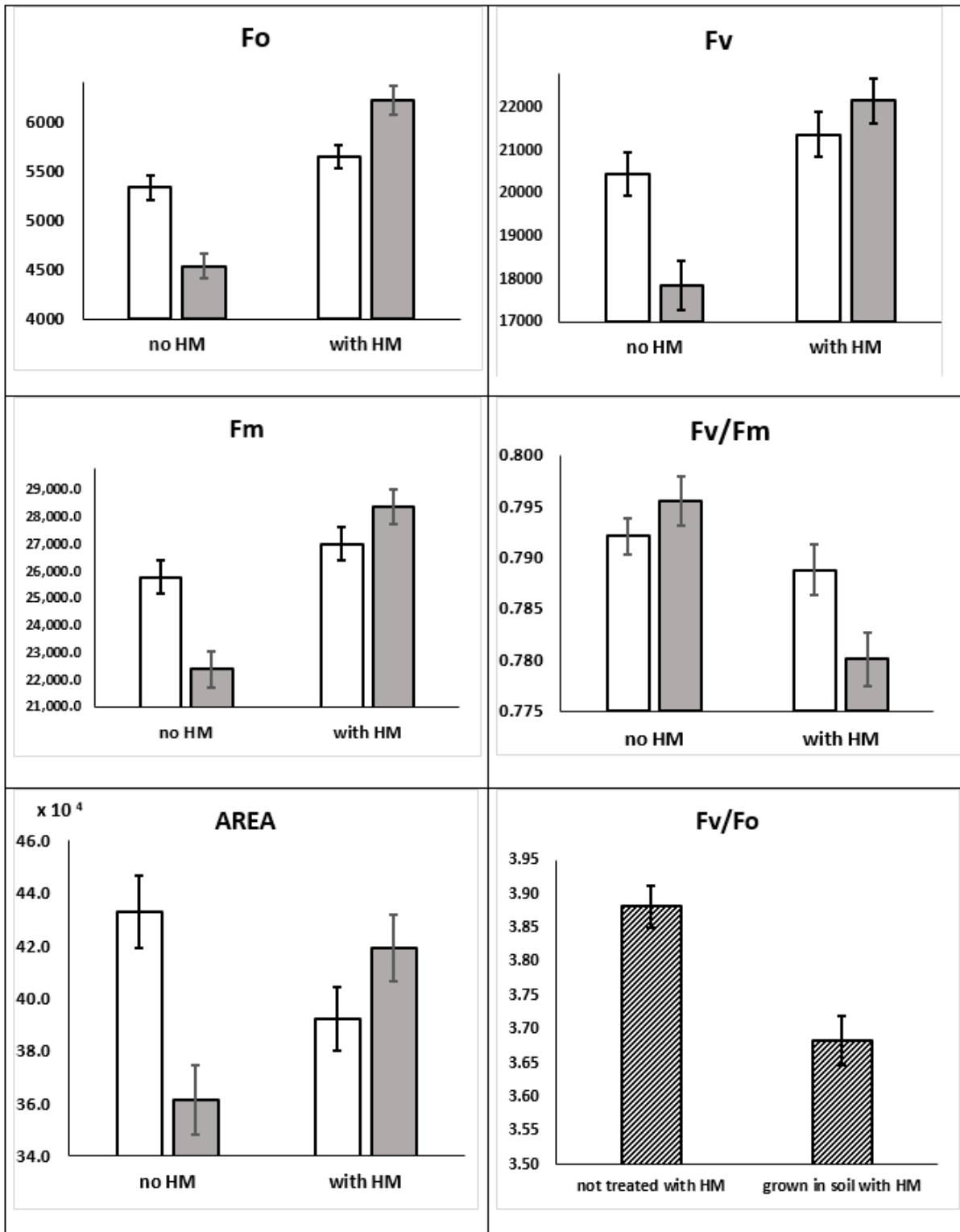
3a.



3b.

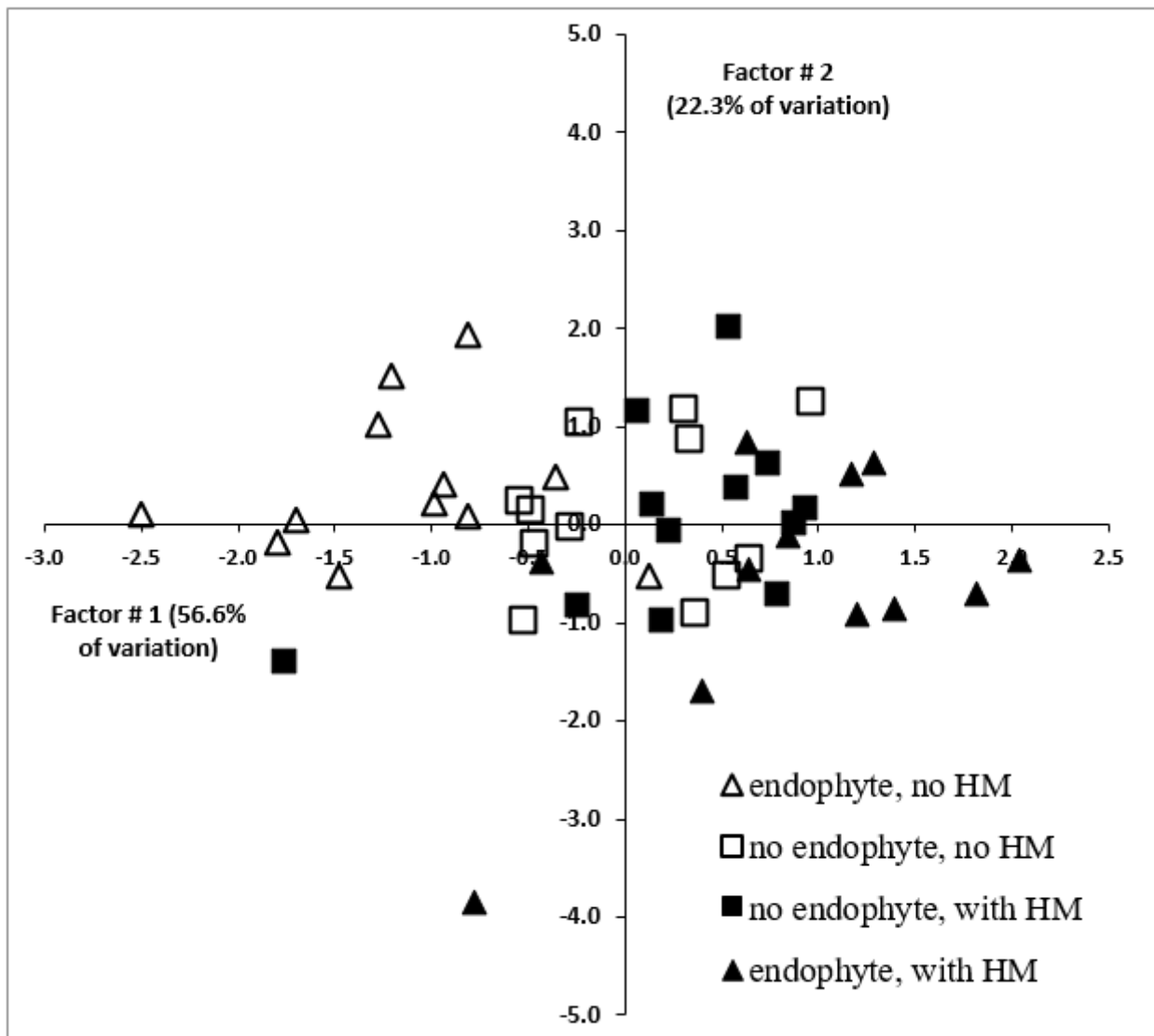
**Figure 3**

Mean values of chlorophyll contents index (CCI) in perennial ryegrass (E-) plants and Epichloë-perennial ryegrass symbiota (E+) grown in control conditions (left bar for each ecotype) and in the presence of HM ions (right bar for each ecotype)



**Figure 4**

The effect of HM treatment of perennial ryegrass plants on chlorophyll a fluorescence parameters, which differed in statistically important manner: Fo, Fv, Fm, Area, Fv/Fm, Fv/Fo. Bars on graph indicate standard error of means. For Fo, Fv, Fm 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 with or without HM supplementation. Each data point represents a different ecotype.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.docx](#)
- [Tables.pdf](#)