

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

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

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12 **Abstract:** The phenomenon of plant mutualistic symbiosis with microbes may have a positive effect
13 on the improvement of plant tolerance to environmental stresses as well as on the ability of plants
14 to accumulate heavy metal (HM) ions from soil. The influence of *Epichloë* fungal endophyte
15 (*Ascomycota, Clavicipitaceae*) on perennial ryegrass (*Lolium perenne* L.) plants grown in the
16 presence of elevated concentrations of HM ions (Cd^{2+} , Pb^{2+} and Cu^{2+}) in soil was studied. The
17 presence of *Epichloë* in the host grass tissues resulted in different accumulation of HM ions in the
18 aboveground parts of the plants. In some cases endophyte infection positively affected ryegrass
19 ability to accumulate HM ions from soil. In plants with (E+) and without (E-) endophytes the
20 hormesis effect was induced by the elevated concentration of Cu^{2+} ions, resulting in better growth
21 and photosynthesis, as examined by measurements of Chl *a* fluorescence. The obtained results
22 indicate that based on the laboratory evaluation of the efficiency of HM accumulation, we were able
23 to choose the best associations of perennial ryegrass with endophytes for HM phytoremediation.

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

26 **Introduction**

27 Endophytes can colonize plant tissues and live without inducing any visible symptoms
28 of biotic stress in plants. In general, as a consequence of host plant-microbe interactions,
29 these endophytes produce a range of alkaloids and stimulate the host plant for enhanced
30 synthesis of primary and secondary metabolites, e.g. free sugars, sugar alcohols, proline,
31 glutamic acid, phospholipids, proteins and polysaccharides (Avila et al. 2012, Bush et al.
32 1997, Nagabhyru et al. 2013, Porter 1994, Rasmussen et al. 2008, Soto-Bajas et al. 2016).
33 Hao et al. (2010) observed that treatment of suspension cells of *Ginkgo biloba* with fungal
34 endophytes resulted in the accumulation of flavonoids, increased abscisic acid (ABA)
35 production, and activation of phenylalanine ammonia-lyase (PAL). Also, the roots
36 metabolism is altered in response to colonization of the aboveground parts of plants
37 (Strehmel et al. 2016, Slaughter et al. 2018). Altogether, the mutual associations lead to
38 changes in host plant gene expression and improve plant adaptations to environmental
39 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 the 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 Pb²⁺, Cu²⁺, and Cd²⁺ 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 P₂O₅; 153.3 K₂O; 55.5 MgO; 7.7 Pb²⁺; 0.2 Cd²⁺ and 2.4 Cu²⁺ [mg·kg⁻¹ 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 Cd²⁺ and 700 mg of both Pb²⁺ and Cu²⁺ ions in 1 kg⁻¹ 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 Cd²⁺; 550.9
137 Pb²⁺; 546.0 Cu²⁺ [mg·kg⁻¹].

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 (Cd^{2+} , Pb^{2+} and 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 (HClO_4) and fluoric (HF) acids. The
171 amount of Cd^{2+} , Pb^{2+} , and 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: Pb²⁺- c.a.
189 9.6, Cd²⁺- 0.17 and Cu²⁺- 4.3 [mg·kg⁻¹]. Much higher (almost doubled) concentrations of HM
190 ions have been reported by Terelak (2007) for the SWK region: Pb²⁺- c.a. 17.8, Cd²⁺- 0.37
191 and Cu²⁺- 7.6 [mg·kg⁻¹] (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 α 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 α 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 α parameters
226 than E- plants, depends on the addition of HM to the soil medium.

227 Measured parameters of Chl α (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 ≤ 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 α 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 α 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⁺² and Cu⁺² ions concentration (Table 4).

249 The highest concentration of HM ions (sum of Pb²⁺, Cd²⁺ and Cu²⁺) was detected in the
250 leaves of E+ variant of ecotype 160 ($102 \text{ mg}\cdot\text{kg}^{-1}$), whereas in the leaves of the E- plants, the
251 concentration of HM was low ($44 \text{ mg}\cdot\text{kg}^{-1}$) (Tab. 5). Differences in the particular ions
252 concentration of the above-mentioned ecotype were as follows: almost two-fold higher
253 concentration of Pb²⁺ and Cd²⁺ ions and threefold of Cu²⁺ in E+ plants as compared to E-.

254 The highest concentration of Pb²⁺ ($43.9 \text{ mg}\cdot\text{kg}^{-1}$) was detected in the E- plants of ecotype
255 50 from POD region, whereas the lowest ($10.2 \text{ mg}\cdot\text{kg}^{-1}$), in the E+ variant of ecotype 730
256 from MAZ region. The average value of Pb²⁺ for regions was the lowest for E- variants of
257 plants from SWK region ($16.9 \text{ mg}\cdot\text{kg}^{-1}$), but it was also the highest ($26.6 \text{ mg}\cdot\text{kg}^{-1}$) for E+
258 plants from the same region. Considering E+ plants, the highest Pb²⁺ concentration (40.7
259 $\text{mg}\cdot\text{kg}^{-1}$) was detected in ecotype 160, and was also high in ecotypes 685 and 873 (33.2 and
260 $32.7 \text{ mg}\cdot\text{kg}^{-1}$, respectively). For all those three mentioned ecotypes, Pb²⁺ 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 Pb²⁺ 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^{-1}) as well as in ecotypes: 45 and 685 (16.2 and 15.1 mg kg^{-1} , respectively) (Tab.
265 5). In a manner similar to relations described above for Pb²⁺ concentration, for all three
266 ecotypes with relatively high Cd²⁺ concentration in E+ plants, the Cd²⁺ ions concentration
267 was significantly higher than the concentration values found in E- plants. Average
268 concentration of Cd⁺² 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⁺² 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 $37.4 \text{ mg}\cdot\text{kg}^{-1}$, respectively). All mentioned values were significantly higher
273 than in leaves of corresponding E- plants. The average high concentration of Cu²⁺ ions in E+
274 plants (ca. $30 \text{ mg}\cdot\text{kg}^{-1}$) was noted for central and southern regions i.e. LUB and SWK. But
275 the highest concentration of Cu²⁺ ($34.7 \text{ mg}\cdot\text{kg}^{-1}$) 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^{2+} and Cu^{2+}
281 ions), 730 and 87 (Pb^{2+} ions), 273 and 227 (Cd^{2+} 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^{2+} and Cu^{2+} ions), 801 (Pb^{2+}
284 and Cu^{2+} 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^{2+} , higher concentration in E+ for Cd^{2+} , no difference between E+ and E- for
290 Cu^{2+} .

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^{2+} ions ($2.4 \text{ mg}\cdot\text{kg}^{-1}$) and high
306 amounts of soil organic carbon (SOC), 13%. The natural content of Cu^{2+} in soil was in the
307 range of 15 to $40 \text{ mg}\cdot\text{kg}^{-1}$ in the 0 – 20 cm soil horizon and concentration of Cu^{2+} below 3.0
308 $\text{mg}\cdot\text{kg}^{-1}$ is usually defined as a deficit for grass species (Olszewska et al. 2008, Wyszkowska
309 et al. 2013). In the presence of high organic matter content in the soil, the Cu^{2+} deficit for
310 plants is quite frequent. Moreover, monocotyledonous plants (e.g. grasses) are particularly

311 sensitive to Cu²⁺ 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 α fluorescence detection and parameters analyses (F_o , F_M , F_v , F_v/F_M , F_v/F_o ,
318 RCB/ABS, Area, $(1-V_j)/V_j$, P_l) are simple and widely recognized methods to assess the stress
319 influence on plants (Żurek et al. 2014, Kalaji et al. 2016). Among fluorescence parameters
320 measured in our experiment, F_o , F_M , F_v , as well as the F_v/F_M , F_v/F_o 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_o 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_o 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 (Rozpałek et el.
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_o 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_o 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 α 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 α 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, Zabalgogeazcoa 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 Pb²⁺ 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 Pb²⁺ 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 Pb²⁺ in the soils from this region which was concentrated in average of 17.8
364 mg·kg⁻¹ of soil as compared to 9.4-10.2 mg·kg⁻¹ 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 Pb²⁺ ions, in soil was higher in the SWK
368 region than in other regions. Probably symbiota of this origin used to accumulate more Pb²⁺
369 than those coming from areas of low Pb²⁺ concentration. This could be further hypothesized
370 that the whole microbiome of plants that came from soils of high Pb²⁺ concentration could
371 be different from soils of low Pb²⁺ 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 Cd²⁺ ions accumulation
376 in tall fescue (Ren et al. 2011) and also improved Cd²⁺ 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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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.Ż. 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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420 **Consent to participate:** Not applicable
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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_M of OJIP curve
CC1	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 _O	minimal fluorescence
F _M	maximal recorded fluorescence
F _V	maximal variable fluorescence ($F_M - F_O$)
F _V /F _M	maximum quantum efficiency of PSII photochemistry
F _V /F _O	driving force of light reactions
(1-V _J)/V _J	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 _{ABS}	performance index
PS II	photosystem II
RC/ABS	amount of active reaction centers per absorption
SWK	Świętokrzyskie region
t	t statistic
T _{FM}	time needed to reach the maximal fluorescence

Figures

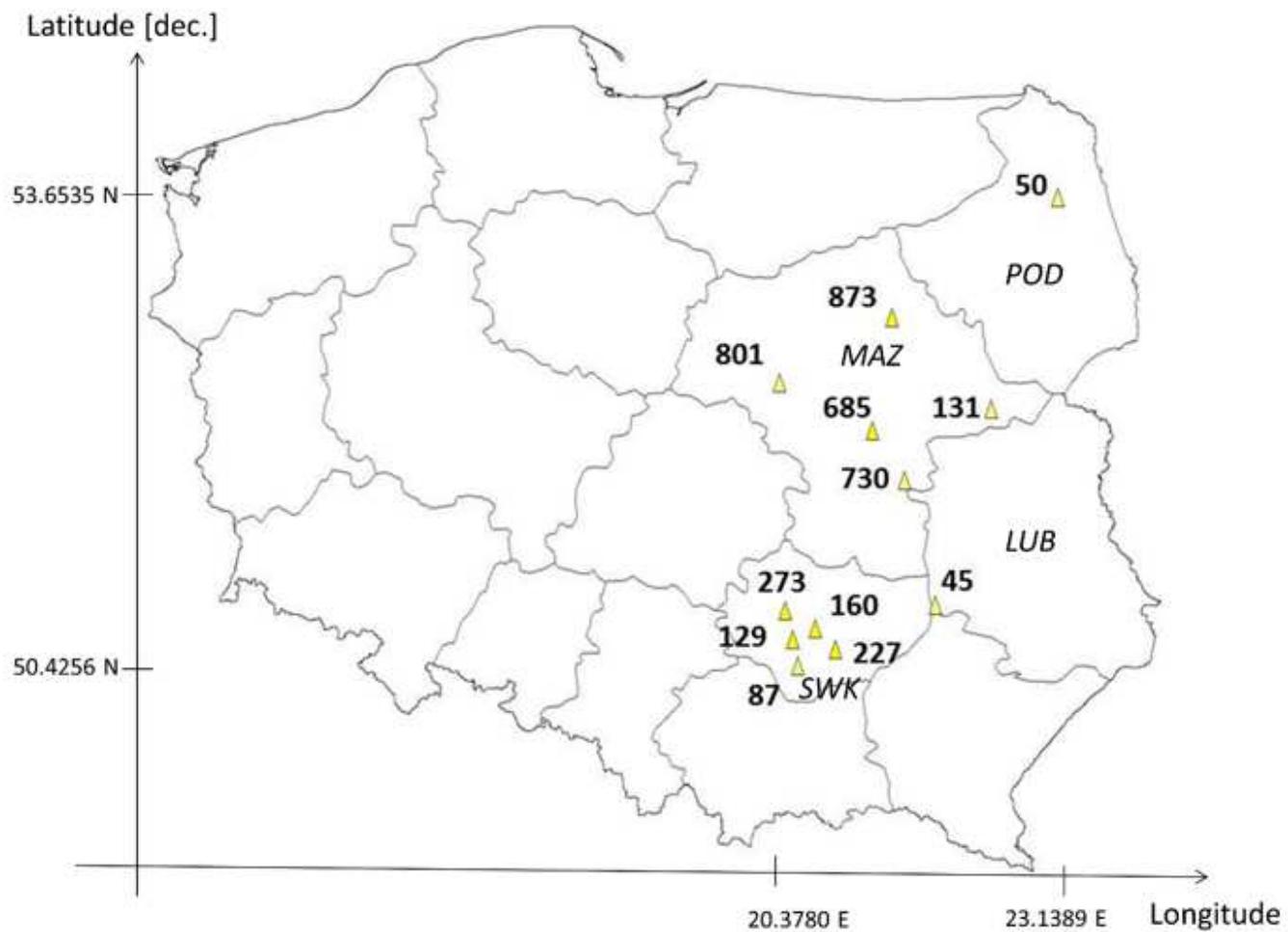


Figure 1

Map of the distribution of ecotype collection sites in regions of Poland: POD (Podlaskie), MAZ (Mazowieckie), 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 software (<http://www.diva-gis.org>)

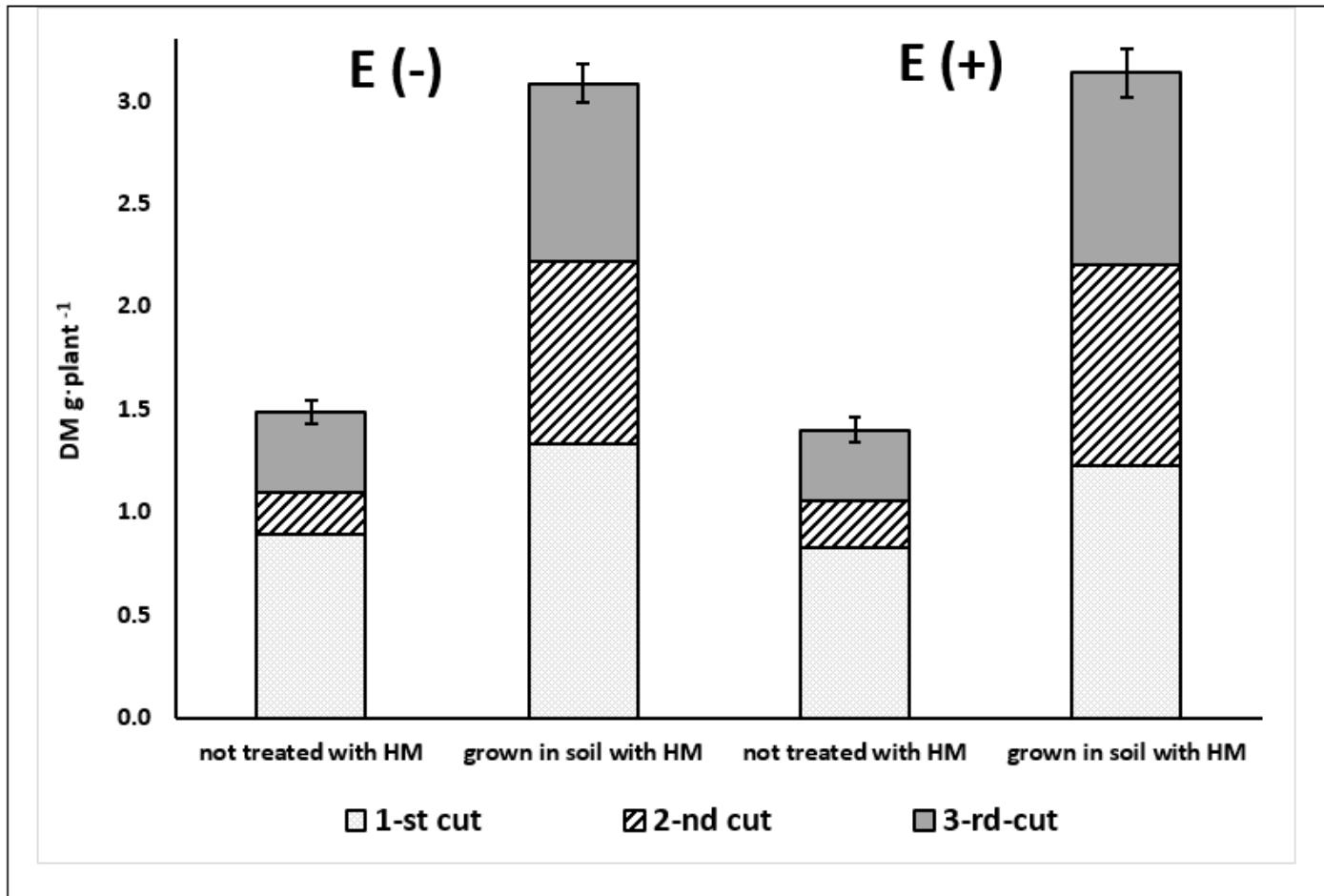
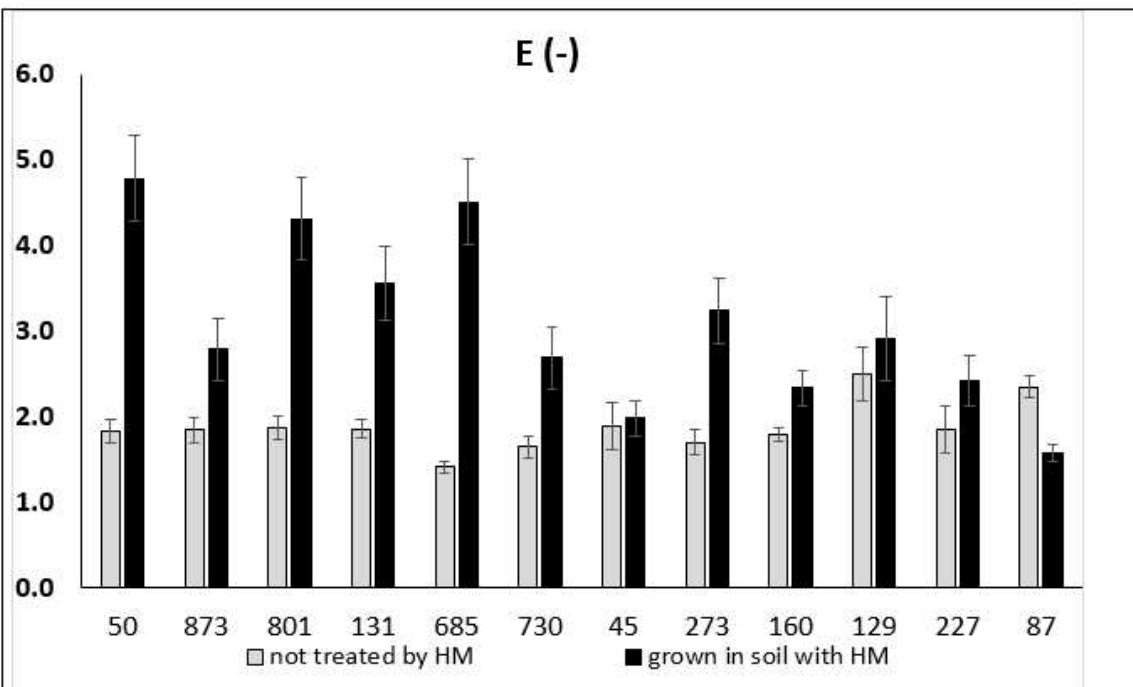
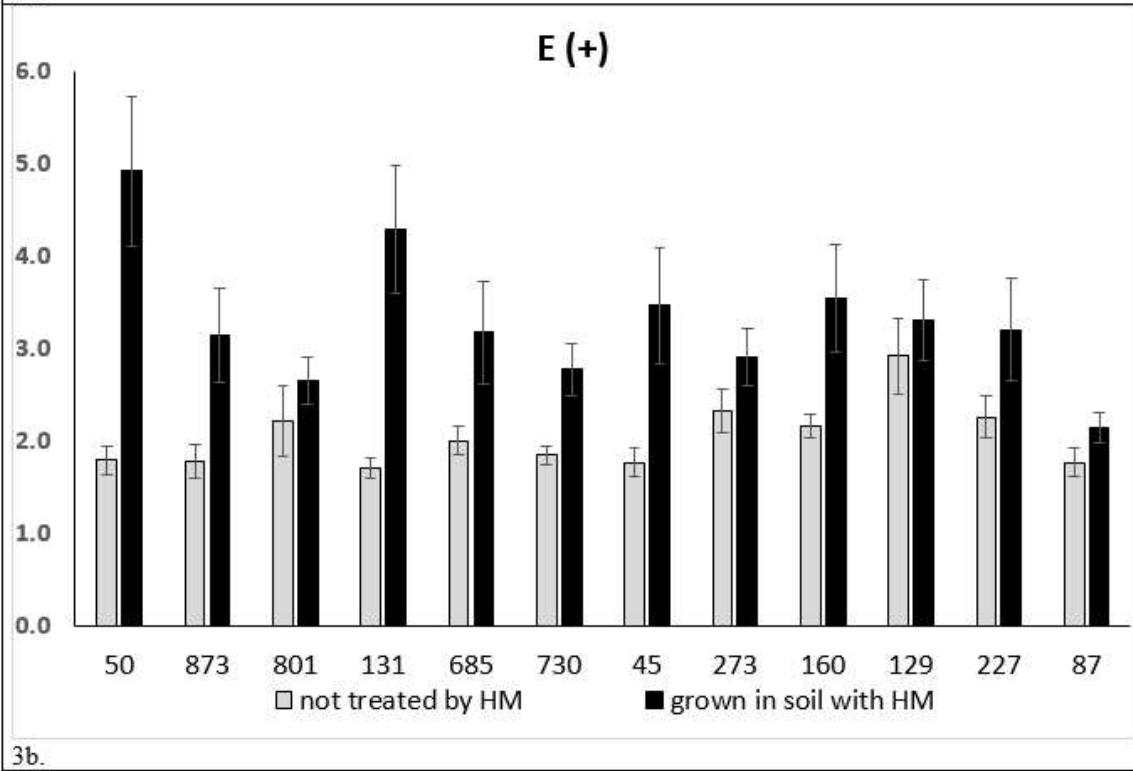


Figure 2

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 symbionts (E+) grown in control conditions (left bar for each ecotype) and in the presence of HM ions (right bar for each ecotype)

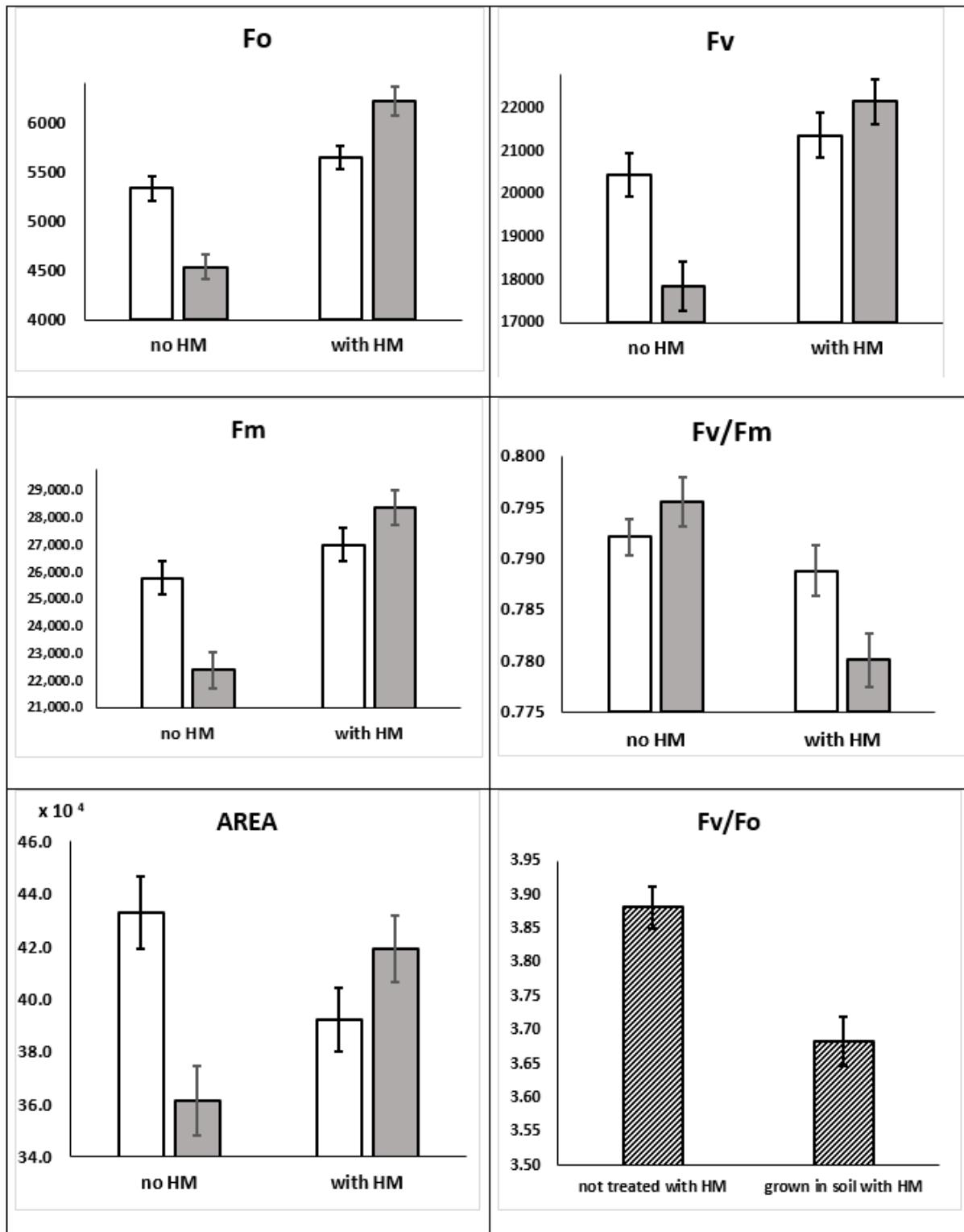


Figure 4

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.

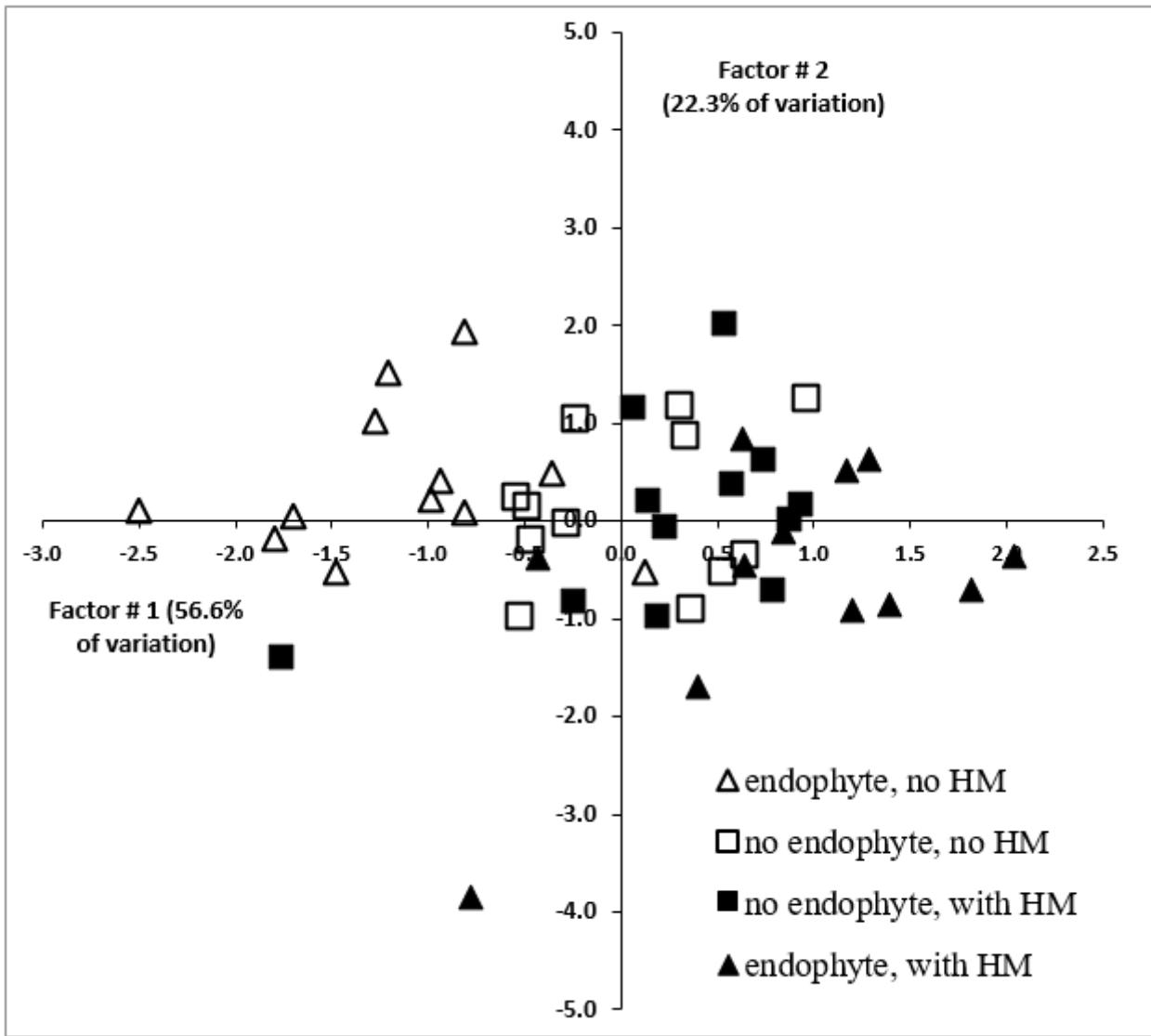


Figure 5

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

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