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Citation style: Malicka Monika, Magurno Franco, Posta Katalin, Chmura Damian, Piotrowska-Seget Zofia. (2021). Differences in the effects of single and mixed species of AMF on the growth and oxidative stress defense in Lolium perenne exposed to hydrocarbons. “Ecotoxicology and Environmental Safety” (Vol. 217 (2021), art. no. 112252, s. 1-10), DOI:10.1016/j.ecoenv.2021.112252

Ecotoxicology and Environmental Safety 217 (2021) 112252

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Differences in the effects of single and mixed species of AMF on the growth and oxidative stress defense in *Lolium perenne* exposed to hydrocarbons

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ARTICLE INFO

Edited by Dr R Pereira

Keywords:
Claroideoglomus walkeri
Diversispora varaderana
Funneliformis caledonium
Oxidative stress
Phenol
Polynuclear aromatic hydrocarbons

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are ubiquitous mutualistic plant symbionts that promote plant growth and protect them from abiotic stresses. Studies on AMF-assisted phytoremediation have shown that AMF can increase plant tolerance to the presence of hydrocarbon contaminants by improving plant nutrition status and mitigating oxidative stress. This work aimed to evaluate the impact of single and mixed-species AMF inocula (*Funneliformis caledonium*, *Diversispora varaderana*, *Claroideoglomus walkeri*), obtained from a contaminated environment, on the growth, oxidative stress (DNA oxidation and lipid peroxidation), and activity of antioxidative enzymes (superoxide dismutase, catalase, peroxidase) in *Lolium perenne* growing on a substrate contaminated with 0/0–30/120 mg phenol/polynuclear aromatic hydrocarbons (PAHs) kg^{-1}. The assessment of AMF tolerance to the presence of contaminants was based on mycorrhizal root colonization, spore production, the level of oxidative stress, and antioxidative activity in AMF spores. In contrast to the mixed-species AMF inoculum, single AMF species significantly enhanced the growth of host plants cultured on the contaminated substrate. The effect of inoculation on the level of oxidative stress and the activity of antioxidative enzymes in plant tissues differed between the AMF species. Changes in the level of oxidative stress and the activity of antioxidative enzymes in AMF spores in response to contamination also depended on AMF species. Although the concentration of phenol and PAHs had a negative effect on the production of AMF spores, low (5/20 mg phenol/PAHs kg^{-1}) and medium (15/60 mg phenol/PAHs kg^{-1}) substrate contamination stimulated the mycorrhizal colonization of roots. Among the studied AMF species, *F. caledonium* was the most tolerant to phenol and PAHs and showed the highest potential in plant growth promotion. The results presented in this study might contribute to the development of functionally customized AMF-assisted phytoremediation strategies with indigenous AMF, more effective than commercial AMF inocula, as a result of their selection by the presence of contaminants.

1. Introduction

Arbuscular mycorrhizal fungi (AMF, *Glomeromycota*) form mutualistic associations with approximately 79% of terrestrial plant species (Brundrett and Tedersoo, 2018). AMF develop an extensive hyphal network that provides plants access to water and nutrients beyond the depletion zone formed around roots (Wipf et al., 2019). AMF systemically induce biochemical, morphological, and physiological changes in plants, protecting them from abiotic stresses, which include drought, flood, and contamination with xenobiotics (Cameron et al., 2013; Calvo-Polanco et al., 2014; Ferrol et al., 2016; Rajtor and Piotrowska-Seget, 2016; Duc et al., 2018). Soil pollution with organic contaminants like pesticides, hydrocarbons, and phenols constitutes one of the major environmental problems, which affects the biodiversity of plants as well as soil, rhizospheric and endophytic microorganisms (Arellano et al., 2017; Gałązka et al., 2018; Nowak and Mrozik, 2018).

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AMF, arbuscular mycorrhizal fungi; CAT, catalase; HC, high-level contaminated; LC, low-level contaminated; LM, linear model; MDA, malondialdehyde; NC, non-contaminated; PAHs, polynuclear aromatic hydrocarbons; POX, peroxidase; SOD, superoxide dismutase.

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https://doi.org/10.1016/j.ecoenv.2021.112252
Received 25 January 2021; Received in revised form 24 March 2021; Accepted 11 April 2021
Available online 27 April 2021

Phytoremediation, supported by microorganisms, including AMF, represents a promising method that can be perspectively used to decontaminate polluted environments on a large scale. It has been found that soil contaminants drive a selection of AMF that are more effective in transferring nutrients to plants (Hassan et al., 2014; Millar and Bennett, 2016; Lee et al., 2020). Moreover, AMF can increase plant tolerance to the presence of contaminants by mitigating oxidative stress (Criquet et al., 2000; Verdin et al., 2006; Debiane et al., 2009). Some studies revealed increased activity of antioxidative enzymes in plant tissues in response to mycorrhizal colonization. Mycorrhizal roots intensively release oxidoreductases which can significantly improve the phytoremediation of organic contaminants (Criquet et al., 2000; Debiane et al., 2009; Yu et al., 2011). For example, higher peroxidase activity in the rhizosphere of wheat colonized by *Rhizophagus irregularis* and higher PAH degradation rate, in comparison to non-inoculated control, was reported by Lenoir et al. (2017). AMF-assisted phytoremediation requires the functional selection of mycorrhizal symbionts. Its effectiveness depends on numerous environmental and experimental factors, including soil physicochemical parameters and the concentration and molecular structure of contaminants (Zhou et al., 2009; Yu et al., 2011; Aranda et al., 2013). The highest efficacy of AMF-assisted phytoremediation was reported in experiments with AMF strains isolated from contaminated sites, adapted to the presence of hydrocarbons. Nonetheless, most of the studies were conducted with AMF belonging to the family *Glomeraceae,* that are a common component of commercially available AMF inoculants (Cabello, 1999; Binet et al., 2000; Alarcón et al., 2008; Rajtor and Piotrowska-Seget, 2016).

In pot experiments carried out under laboratory conditions, single species AMF inocula are most commonly used. However, this approach might not be suitable for phytoremediation purposes, considering that single inoculation does not allow to consider potentially synergic effect of mixed inocula. It has been suggested that root co-colonization by two or even more AMF species could grant a broader spectrum of advantages and be more beneficial to plants (Jansa et al., 2008; Zhou et al., 2020). The positive effect of AMF symbiosis on plant growth varied with host and AMF species as well as abiotic environmental features. On the other hand, the results of some studies suggest that the application of single AMF species might promote the removal of contaminants from soil and be more effective than mixed-species AMF inocula (Liu and Dalpé, 2009; Crossay et al., 2019). Moreover, it has been recognized that AMF-plant interactions strongly depend on the relatedness among AMF (Roger et al., 2013; Crossay et al., 2019). Depending on the experimental conditions, inoculation with distantly related AMF may result in functional complementarity or disequilibrium between them (Yang et al., 2017; Crossay et al., 2019).

Following these findings, phytoremediation strategies should be customized to particular environmental conditions where plants are inoculated with cooperative, autochthonous AMF tolerant to the presence of contaminants. This study aimed to evaluate the impact of single and mixed-species AMF inoculation on the growth of *Lolium perenne* on phenol and PAH-contaminated substrate. Additionally, the effect of mycorrhizal inoculation on the level of oxidative stress and activity of antioxidative enzymes in *L. perenne* tissues was determined. Moreover, the tolerance to phenol and PAHs of AMF obtained from a contaminated environment was assessed by the evaluation of mycorrhizal root colonization, spore production, and the level of oxidative stress and antioxidative activity in AMF spores. To the best of our knowledge, this is the first report on the level of oxidative stress and the activities of antioxidative enzymes in AMF exposed to phenol and PAH stress.

2. Materials and methods

2.1. Fungal isolates

AMF used in this study were isolated from bulk soil associated with the roots of *Poa trivialis* L. Plants were collected from the shore of Kalina pond located at Świętochłowice in the Upper Silesian Industrial Region, Poland (50°16′49″N, 18°55′38″E). The sampling area has been contaminated with leakages from an adjacent toxic waste dump where sludge rich in phenol and PAHs was deposited. The concentrations of phenol and PAHs and the physicochemical features of the soil samples are provided in Malicka et al. (2020). Soil samples and root pieces were mixed with river sand and LECA (1:4, *v/v*), and used to establish trap cultures with a mixture of plant species (*Plantago lanceolata* L., *Trifolium pratense* L., *Medicago sativa* L., *Hordeum vulgare* L.) (photoperiod: 16/8 h day/night at 20 °C, 8000 lux, 50–70% relative humidity). After six months, AMF spores were isolated from the substrate and used to establish single spore cultures with *P. lanceolata* in a mixture of sterile river sand and bentonite clay (9:1, *v/v*) (photoperiod: 16/8 h day/night at 20°C, 8000 lux, 50–70% relative humidity). After six months, the cultures were screened for the presence of mycorrhizal structures. 25–100 spores (depending on the size) from positive cultures were collected and used for molecular characterization. Spores were crushed with a micropestle and suspended in 25 μl of 2,5x DreamTaq Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The spore suspension was immediately incubated at 99 °C for 15 min and centrifuged (5 min, 16,000×*g*). The supernatant was used as a template for PCR with primers SSUmAf and LSUmAr (Krüger et al., 2009). PCR with DreamTaq polymerase (Thermo Fisher Scientific) was performed in C1000 Touch™ thermal cycler (Bio-Rad, Hercules, California, USA), according to the manufacturer's protocol. Additionally, 3 mM $MgCl_2$ and 0.02 mg BSA per 50 μl of the master mix were included in the reaction. The thermal cycling conditions were as follows: 95 °C for 5 min; 34 cycles: 95 °C for 20 s, 51 °C for 30 s, 72 °C for 1 min 45 s; 72 °C for 5 min. Positive amplicons were cloned with pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin, USA) and sequenced at Genomed S.A. (Warsaw, Poland). Sequence editing was conducted manually using Chromas Lite 2.01 (Technelysium Pty Ltd, Brisbane, Queensland, Australia) and Mega 7.0 (Kumar et al., 2016). AMF species were identified, using Blastn, as *Funneliformis caledonium*, *Diversispora* cf. *varaderana* and *Claroideoglomus walkeri* with at least 98% level of sequence identity. The results of molecular identification were confirmed by morphological characterization performed by Prof. Janusz Błaszkowski (data not shown). The sequences were deposited in GenBank with accession numbers: MH590064.1, MH590063.1, MH590062.1.

2.2. Experimental design

Pot cultures were prepared in 0.5 L pots filled with sterile river sand and bentonite (9:1, v/v). The substrate was contaminated with different concentration of an equimolar solution of PAHs (naphthalene, phenanthrene, fluoranthene, pyrene) in acetone and an aqueous solution of phenol, resulting in the following treatments: non-contaminated (NC), low-level contaminated (LC) with 5/20 mg phenol/PAHs kg^{-1}, medium-level contaminated with 15/60 mg phenol/PAHs kg^{-1}, and high-level contaminated (HC) with 30/120 mg phenol/PAHs kg^{-1}. After the addition of PAH solution, but before the addition of phenol, the substrate was incubated at 100 °C for 30 min to remove the acetone. Pots were sown with *L. perenne* L. cv. Rela seeds, previously surface sterilized with 70% ethanol for 5 min and 2.5% NaOCl for 10 min. Seven days after germination, the seedlings were thinned to three per pot. Subsequently, NC, LC, MC, and HC pots were inoculated with the spores of *F. caledonium, D. varaderana*, or *C. walkeri*. AMF were introduced to pots as single species (40 spores for species) and mixed-species inocula (15 spores of each AMF species). Additionally, non-AMF cultures were established as a negative control. Plants were growing for six months in a growth chamber (16/8 h day/night, 20 °C, 8000 lux, 50–70% air humidity), watered twice a week with distilled water and modified Long-Ashton solution with low P concentration (Supplementary Table 1). The experiment was carried out in four replicates.

2.3. Assessment of plant growth parameters

To assess the impact of AMF and pollutants on plant growth, shoot length, shoot dry weight, and root dry weight were measured. Plant roots were separated from the growth substrate by manual shaking and washing in tap water. Shoot length was measured as the length of the longest shoot of each plant in a pot. Shoot and root dry weight were measured after drying plant tissues at 70 °C for 48 h. Shoot dry weight was measured for each plant separately, while root dry weight was measured for the total root system in a pot.

2.4. Estimation of the mycorrhizal colonization of plant roots

Mycorrhizal colonization was estimated for 1–1.5 cm root pieces detached from the root system after washing. Root fragments preserved in 70% ethanol were washed with tap water and depigmented in 10% KOH. Highly pigmented roots were additionally bleached with alkaline H_2O_2. Depigmented roots were acidified with 3–4% HCl and stained using ink and vinegar solution, according to Vierheilig et al. (1998). The level of mycorrhizal colonization and arbuscule abundance was evaluated under the microscope for one hundred root pieces per pot, according to Trouvelot et al. (1986). Values obtained from microscopic observations were introduced to Mycocalc software (https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) to calculate the following parameters: the frequency (F%) and intensity (M%) of mycorrhizal colonization in the root system, arbuscule abundance in the root system (A%) and arbuscule abundance in mycorrhizal parts of root fragments (a%).

2.5. Estimation of the number of AMF spores in the growth substrate

AMF spores were isolated from 50 g of growth substrate using the wet sieving and decanting method described by Gerdemann and Nicolson (1963). Spores were purified by centrifugation in 50% saccharose solution according to Daniels and Skipper (1982) and counted under a dissecting microscope.

2.6. Evaluation of the level of oxidative stress and the activity of antioxidative enzymes in plant tissues

To assess the influence of AMF and pollutants on oxidative stress in plants the level of lipid peroxidation and DNA oxidation were determined, based on the concentrations of malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in plant tissues. The antioxidative activity in plants was evaluated by measuring the activities of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) in plant tissues. For this purpose, 0.5 g of fresh *L. perenne* tissues (roots, shoots) were ground in a mortar with sterile quartz sand and 2.5 ml PBS. Tissue homogenates were centrifuged (5 min, 8000×*g*), and the supernatant was stored at − 80 °C before further analysis. MDA and 8-OHdG assays were conducted with the use of OxiSelect™ TBARS Assay Kit (Cell Biolabs, San Diego, California, USA) and DNA Damage Competitive ELISA Kit (Invitrogen, Carlsbad, California, USA). Assessment of the activities of SOD, CAT, and POX were performed with the use of Superoxide Dismutase Assay Kit (Sigma-Aldrich, Saint Louis, Montana, USA), Amplex® Red Catalase Assay Kit (Invitrogen), and Amplex® Red Peroxidase Assay Kit (Invitrogen). Colorimetric assays were performed in two technical repetitions on 96-well microtiter plates using the Biolog MicroStation (Biolog, Hayward, California, USA).

2.7. Evaluation of the level of oxidative stress and the activity of antioxidative enzymes in AMF spores

Spores isolated from the growth substrate were crushed in a 1.5 ml microtube with a micropestle. Spore homogenate was suspended in PBS to reach OD600 = 0.4. MDA and 8-OHdG concentrations, and SOD, CAT, and POX activities were assayed using the commercial kits described above.

2.8. Statistical analysis

All statistical analyses of data (deposited in Malicka et al. (2021)) were performed in R (R Core Team 2020). The impact of mycorrhizal inoculation and the concentrations of phenol and PAHs on plant growth parameters, the parameters of mycorrhizal root colonization, the number of AMF spores, oxidative stress markers, and the enzymatic activities in plant tissues was evaluated using the linear model (LM) since mycorrhizal inoculation is a factor variable (five variants), whereas the concentrations of phenol and PAHs are interval variables (four levels). As the concentration of PAHs was correlated with the concentration of phenol, only the latter was treated as a proxy of contamination. In total, 20 LMs, with the interaction between AMF and phenol, were performed using the function lm() from the package 'stats'. The significance of variables was examined by the Wald test using the function Anova() from the package 'car'. The post-hoc tests after LMs were performed with the Tukey's HSD test. Before this test, one-way ANOVA was conducted where a grouping variable resulted from the interaction between the contamination and the mycorrhizal inoculation (n = 20 groups). In case of non-significant results, post-hoc tests were performed only among groups associated with the main effects. One-way ANOVA, followed by Tukey's HSD test, was performed for each type of mycorrhizal inoculation to assess the impact of contamination on the oxidative stress markers and the enzymatic activities in AMF spores. The normality of distribution was checked by the Shapiro-Wilk test ($p > 0.05$) and the homogeneity of variance was checked by the Levene test ($p > 0.05$).

3. Results

3.1. Plant growth parameters

Statistical analysis showed a significant interaction between the mycorrhizal inoculation and phenol and PAH concentration, which substantially influenced the growth parameters of *L. perenne* (Supplementary Table 2).

Although the presence of contaminants decreased the length and dry weight of *L. perenne* shoots in all experimental variants, mycorrhizal inoculation significantly alleviated this adverse effect. The highest length and biomass of shoots were observed in *L. perenne* inoculated with *F. caledonium*, followed by *D. varaderana* and *C. walkeri*. The lowest shoot growth values were observed in plants inoculated with mixed-species AMF inoculum and non-AMF plants (Table 1, Supplementary Fig. 1). Moreover, mycorrhizal inoculation affected the morphology of above-ground plant organs. Plants inoculated with *D. varaderana* and *F. caledonium* produced longer, wider, and more numerous leaf blades than non-AMF plants. Plants inoculated with *C. walkeri* had numerous leaves; however, they were thin and prematurely withered (Supplementary Fig. 1).

A similar detrimental effect was observed in the roots where phenol and PAH contamination decreased the root biomass (Supplementary Table 2). In comparison to non-AMF plants, inoculation with *F. caledonium* increased the weight of *L. perenne* roots, but it was statistically significant only in NC cultures. Inoculation with *D. varaderana* did not affect root weight, while inoculation with *C. walkeri* and mixed AMF species significantly decreased root weight, regardless of the concentration of contaminants (Table 1). In non-AMF plants, the root/shoot weight ratio was much higher, especially in the contaminated cultures. The presence of mycorrhiza reversed this trend by stimulating shoot growth and reducing root weight (Table 1).

3.2. Root colonization and spore production by AMF

Both the mycorrhizal inoculation and the contamination had a

Table 1
The growth parameters of *L. perenne* cultured on a substrate contaminated with phenol and PAHs and inoculated with AMF.

AMF inoculation	Phenol/PAH [mg/kg]	Shoot length [cm]	Shoot weight [mg DW/plant]	Root weight [mg DW/pot]	Root/shoot ratio
Non-AMF	0/0	11.81 ± 0.74	22.02 ± 0.77	20.81 ± 1.03	0.95 ± 0.03
	5/20	11.79 ± 0.40	21.44 ± 0.31	21.45 ± 0.99	0.99 ± 0.06
	15/60	9.31 ± 1.07[b]	15.83 ± 1.11[b]	18.50 ± 0.58	1.17 ± 0.10[b]
	30/120	8.63 ± 0.70[b]	14.29 ± 0.48[b]	16.67 ± 0.47[b]	1.17 ± 0.04[b]
F. caledonium	0/0	15.04 ± 1.23[a]	25.87 ± 0.95[a]	26.47 ± 0.79[a]	1.02 ± 0.06
	5/20	14.81 ± 0.89[a]	25.11 ± 0.39[a]	23.25 ± 0.47	0.93 ± 0.03
	15/60	13.47 ± 1.15[a]	20.79 ± 0.63[ab]	19.42 ± 0.39[b]	0.93 ± 0.03[a]
	30/120	12.35 ± 1.13[a]	18.63 ± 0.57[ab]	16.69 ± 0.93[b]	0.90 ± 0.03[a]
D. varaderana	0/0	14.02 ± 1.28	25.42 ± 0.91[a]	21.97 ± 3.62	0.86 ± 0.05
	5/20	13.34 ± 1.98	22.91 ± 0.80	20.55 ± 1.61	0.90 ± 0.05
	15/60	11.94 ± 1.27[a]	19.18 ± 0.60[ab]	17.57 ± 0.83[b]	0.92 ± 0.03[a]
	30/120	11.86 ± 1.19[a]	19.17 ± 1.48[ab]	15.61 ± 1.74[b]	0.81 ± 0.03[a]
C. walkeri	0/0	15.13 ± 2.24[a]	24.20 ± 1.41	16.44 ± 1.17[a]	0.68 ± 0.04[a]
	5/20	11.70 ± 0.69[b]	19.69 ± 1.69[b]	12.22 ± 1.56[ab]	0.62 ± 0.04[a]
	15/60	11.11 ± 0.83[b]	17.18 ± 0.52[b]	8.79 ± 0.50[ab]	0.51 ± 0.04[ab]
	30/120	9.38 ± 0.87[b]	15.57 ± 0.94[b]	7.20 ± 0.75[ab]	0.46 ± 0.03[ab]
Mixed AMF	0/0	11.61 ± 1.26	20.89 ± 0.68	15.22 ± 0.85[a]	0.73 ± 0.03[a]
	5/20	10.51 ± 0.97	18.32 ± 2.29[a]	14.33 ± 0.45[a]	0.79 ± 0.07[a]
	15/60	11.58 ± 0.40	20.18 ± 0.89[a]	14.50 ± 0.58[a]	0.72 ± 0.02[a]
	30/120	10.04 ± 0.48	16.58 ± 1.02[ab]	14.44 ± 0.39	0.87 ± 0.03[ab]

Abbreviations: DW – dry weight.
[a]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the AMF and non-AMF cultures.
[b]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the contaminated and NC cultures.

significant effect on the intensity of mycorrhizal colonization and arbuscule abundance in *L. perenne* roots. Interaction between the contamination and mycorrhizal inoculation was significant (Supplementary Table 2). In LC and MC cultures inoculated with *F. caledonium* and *C. walkeri*, root colonization parameters (F%, M%, A% and a%) were higher, whereas in HC cultures, these parameters were lower compared to NC cultures. In MC and HC cultures inoculated with *D. varaderana* and mixed AMF species, root colonization parameters (particularly A%, a%) were significantly lower than in NC cultures (Table 2).

The number of spores produced by AMF strongly depended on AMF species and the level of phenol and PAH contamination (Supplementary Table 2). *C. walkeri* produced significantly more spores than *F. caledonium* and *D. varaderana*, especially in mixed-species cultures. In the cultures inoculated with single AMF species, the number of spores produced by *D. varaderana* and *C. walkeri* was significantly lower in the contaminated substrate, while the number of *F. caledonium* spores was not affected by phenol and PAH concentration. In the contaminated cultures inoculated with mixed AMF species, the number of spores produced by *C. walkeri* and *F. caledonium* was significantly reduced compared to non-contaminated cultures (Table 3).

Table 2
The parameters of mycorrhizal colonization of *L. perenne* roots in the cultures contaminated with phenol and PAHs and inoculated with AMF.

AMF inoculation	Phenol/PAH [mg/kg]	F%	M%	A%	a%
F. caledonium	0/0	49.32 ± 9.46	18.09 ± 3.30	6.06 ± 1.52	30.47 ± 8.33
	5/20	74.37 ± 5.15[a]	29.06 ± 3.50	12.60 ± 2.86	41.19 ± 10.60
	15/60	66.90 ± 7.70[a]	23.94 ± 1.84	12.82 ± 1.44	43.81 ± 8.76
	30/120	53.96 ± 9.60	5.92 ± 2.90	1.68 ± 0.68[a]	30.30 ± 5.55
D. varaderana	0/0	71.05 ± 7.06	10.92 ± 2.04	3.40 ± 1.07	32.10 ± 7.89
	5/20	81.39 ± 12.56	16.73 ± 2.60	1.65 ± 0.54	13.59 ± 2.82[a]
	15/60	66.92 ± 7.82	10.11 ± 2.98	1.24 ± 0.50[a]	15.82 ± 6.95[a]
	30/120	66.42 ± 4.79	7.01 ± 2.62	0.76 ± 0.42[a]	10.38 ± 2.48[a]
C. walkeri	0/0	69.21 ± 12.23	13.52 ± 2.61	5.25 ± 1.29	39.17 ± 7.42
	5/20	90.87 ± 1.67[a]	31.42 ± 5.72[a]	11.99 ± 1.11[a]	39.71 ± 8.19
	15/60	88.51 ± 3.03	30.82 ± 6.85[a]	12.20 ± 2.31[a]	39.90 ± 3.93
	30/120	90.68 ± 5.45	24.05 ± 6.33	6.47 ± 1.77	27.15 ± 4.41
Mixed AMF	0/0	90.43 ± 6.22	26.32 ± 5.98	6.34 ± 2.49	28.49 ± 8.33
	5/20	81.58 ± 5.84	16.36 ± 3.13	2.95 ± 0.83	20.70 ± 3.21
	15/60	86.53 ± 7.44	12.98 ± 2.01[a]	1.82 ± 0.70[a]	14.62 ± 4.23[a]
	30/120	70.76 ± 4.84	13.01 ± 4.80[a]	2.31 ± 1.92[a]	17.79 ± 6.64

[a]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the contaminated and NC cultures.

Table 3
The number of spores produced by AMF in 50 g of substrate for *L. perenne* pot cultures contaminated with phenol and PAHs.

phenol/PAH [mg kg^{-1}]	AMF species		
	F. caledonium	*D. varaderana*	*C. walkeri*
	Single species AMF inoculum [number of spores g^{-1}, mean ± SD]		
0/0	52 ± 15	125 ± 42	551 ± 103
5/20	67 ± 11	33 ± 8[a]	322 ± 105
15/60	73 ± 18	38 ± 6[a]	184 ± 57[a]
30/120	62 ± 9	39 ± 10[a]	186 ± 43[a]
	Mixed species AMF inoculum [number of spores g^{-1}, mean ± SD]		
0/0	106 ± 40	0	1479 ± 708
5/20	23 ± 13	37 ± 28[a]	484 ± 84
15/60	12 ± 3[a]	0	45 ± 26[a]
30/120	16 ± 4[a]	8 ± 11	8 ± 7[a]

[a] Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the contaminated and NC cultures.

3.3. The level of oxidative stress and the activity of antioxidative enzymes in plant tissues

Phenol and PAH contamination and mycorrhizal inoculation significantly affected MDA and 8-OHdG concentrations in *L. perenne* shoots (Supplementary Table 2). MDA concentration was strongly increased in plant shoots in non-AMF MC and HC cultures. The level of lipid peroxidation in shoots in the contaminated cultures was decreased in the presence of AMF, especially *F. caledonium* and the mixed-species inoculum (Table 4). In contrast to MDA concentration, 8-OHdG concentration in shoots was significantly higher in the cultures inoculated with *F. caledonium* than in non-AMF plants. 8-OHdG concentration in shoots was the lowest in the cultures inoculated with mixed AMF species and not affected in the cultures inoculated with *D. varaderana* or *C. walkeri* (Table 4). The presence of contaminants did not affect MDA concentration in roots, but it increased 8-OHdG concentration in roots. Mycorrhizal inoculation had little influence on MDA and 8-OHdG concentrations in root tissues (Supplementary Table 2 and 3).

Both substrate contamination and AMF inoculation had a significant effect on the activity of antioxidative enzymes in *L. perenne* shoots (Supplementary Table 2). In the plants non-inoculated with AMF, SOD,

Table 4
The parameters of oxidative stress and the activity of antioxidative enzymes in the shoots of L. *perenne* plants cultured on a substrate contaminated with phenol and PAHs and inoculated with AMF.

AMF inoculation	Phenol/PAH [mg/kg]	MDA shoots [nM/g fresh weight]	8-OHdG shoots [μg/g fresh weight]	SOD shoots [% activity IR]	CAT shoots [kU/g fresh weight]	POX shoots [U/g fresh weight]
Non-AMF	0/0	69.44 ± 16.71	1.980 ± 0.319	71.28 ± 2.05	11.92 ± 1.81	256.79 ± 64.76
	5/20	126.56 ± 22.27[b]	2.336 ± 0.098	70.77 ± 2.23	13.66 ± 0.86	239.51 ± 56.52
	15/60	141.67 ± 13.61[b]	2.570 ± 0.159	83.66 ± 1.26[b]	13.70 ± 1.34	455.56 ± 68.47[b]
	30/120	166.67 ± 17.01[b]	2.517 ± 0.177	85.27 ± 2.48[b]	20.16 ± 2.39[b]	635.80 ± 4.27[b]
F. caledonium	0/0	99.31 ± 16.18	2.906 ± 0.589[a]	82.42 ± 2.27[a]	15.57 ± 1.07	592.59 ± 45.08[a]
	5/20	104.17 ± 18.40	3.377 ± 0.325[a]	83.18 ± 2.31[a]	9.98 ± 2.00[b]	417.28 ± 37.50[a]
	15/60	88.02 ± 16.53[a]	3.878 ± 0.480[a]	74.61 ± 2.96[ab]	5.30 ± 0.80[ab]	280.25 ± 74.50[ab]
	30/120	101.56 ± 16.17[a]	3.738 ± 0.356[a]	63.23 ± 4.51[ab]	6.26 ± 1.90[ab]	146.91 ± 42.17[ab]
D. varaderana	0/0	65.10 ± 16.79	2.232 ± 0.109	81.63 ± 2.81[a]	16.05 ± 1.62	430.86 ± 68.35[a]
	5/20	127.60 ± 16.53[b]	2.224 ± 0.236	78.56 ± 4.05[a]	20.51 ± 1.11[a]	555.56 ± 60.14[a]
	15/60	121.35 ± 20.44[b]	2.448 ± 0.333	83.20 ± 2.26	18.68 ± 2.68	382.72 ± 50.12
	30/120	104.69 ± 16.44[ab]	2.505 ± 0.400	89.03 ± 1.57	19.90 ± 2.64	517.28 ± 63.29
C. walkeri	0/0	62.50 ± 14.73	2.527 ± 0.189	78.49 ± 2.39[a]	16.13 ± 2.32	435.80 ± 64.94[a]
	5/20	81.94 ± 30.36	2.688 ± 0.306	77.54 ± 2.26	13.93 ± 1.04	339.51 ± 101.40
	15/60	119.44 ± 16.71[b]	2.887 ± 0.439	84.55 ± 1.34	19.90 ± 2.10[a]	498.77 ± 68.78
	30/120	135.42 ± 33.81[b]	3.200 ± 0.370	68.74 ± 2.35[ab]	13.30 ± 2.12[a]	460.49 ± 79.66
Mixed AMF	0/0	75.71 ± 5.86	1.397 ± 0.186[a]	74.45 ± 2.50	19.18 ± 1.00[a]	435.66 ± 28.17[a]
	5/20	76.70 ± 3.91	1.436 ± 0.174[a]	83.42 ± 3.47[ab]	18.13 ± 1.20	426.47 ± 12.88[a]
	15/60	81.25 ± 7.59[a]	1.528 ± 0.100[a]	83.12 ± 3.73[b]	17.43 ± 1.76	413.60 ± 34.52
	30/120	89.20 ± 17.24[a]	1.811 ± 0.355[a]	83.00 ± 2.09[b]	17.33 ± 0.55	391.91 ± 38.38[a]

Abbreviations: 8-OHdG – 8-hydroxy-2′-deoxyguanosine, CAT – catalase, IR – inhibition rate, MDA – malondialdehyde, POX – peroxidase, SOD – superoxide dismutase.
[a]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the AMF and non-AMF cultures.
[b]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the contaminated and NC cultures.

CAT, and POX activities in shoots were increasing with the concentrations of phenol and PAHs in the growth substrate. In the presence of mycorrhizal inoculum, enzymatic activities in shoots increased in NC and LC cultures and decreased in HC cultures, compared to non-AMF cultures. The lowest enzymatic activities in shoots were observed in HC cultures inoculated with *F. caledonium* (Table 4). Moreover, the contaminants and mycorrhizal inoculation affected SOD and POX, but not CAT activity in roots. Like in the shoots, SOD and POX activities in roots were increasing with the concentrations of contaminants. In the mycorrhizal plants, SOD and POX activities in roots increased in NC cultures and decreased in HC compared to non-AMF cultures (Supplementary Table 2 and 3).

3.4. The level of oxidative stress and the activity of antioxidative enzymes in AMF spores

Phenol and PAH contamination significantly increased the level of lipid peroxidation and DNA oxidation in *F. caledonium* and *C. walkeri* spores ($P < 0.001$, one-way ANOVA) with no effect on the level of DNA oxidation in *D. varaderana* spores ($P < 0.149$, one-way ANOVA) (Table 5). MDA assay for *D. varaderana* was not performed due to an insufficient amount of spore homogenate. Changes in the activity of antioxidative enzymes in AMF spores in response to phenol and PAH contamination were statistically significant ($P < 0.001$, one-way ANOVA) but different for each AMF species. SOD, CAT, and POX activities increased in *F. caledonium* spores and decreased in *D. varaderana*

Table 5
The activity of antioxidative enzymes in AMF spores produced in *L. perenne* pot cultures contaminated with phenol and PAHs.

AMF inoculation	Phenol/PAH [mg/kg]	MDA [nM/l SH]	8-OHdG [ng/ml SH]	SOD [% activity IR]	CAT [U/ml SH]	POX [μU/ml SH]
F. caledonium	0/0	2.17 ± 0.13	3.96 ± 3.10	7.16 ± 0.71	289.53 ± 2.25	6.43 ± 2.29
	5/20	2.64 ± 0.10	3.56 ± 1.26	13.19 ± 1.42[a]	291.93 ± 16.10	20.04 ± 1.88[a]
	15/60	3.41 ± 0.46[a]	2.09 ± 1.62	17.82 ± 0.49[a]	422.93 ± 9.53[a]	17.08 ± 1.76[a]
	30/120	4.11 ± 0.19[a]	15.15 ± 1.05[a]	21.84 ± 0.31[a]	421.66 ± 19.29[a]	16.71 ± 1.39[a]
D. varaderana	0/0	ND	2.36 ± 0.67	19.45 ± 4.11	518.35 ± 34.10	35.21 ± 1.84
	5/20	ND	1.51 ± 0.89	11.31 ± 0.69[a]	465.56 ± 17.10	18.50 ± 2.58[a]
	15/60	ND	1.04 ± 0.79	12.01 ± 3.35[a]	182.80 ± 27.07[a]	16.51 ± 1.52[a]
	30/120	ND	1.88 ± 0.30	7.15 ± 0.26[a]	274.74 ± 25.50[a]	12.81 ± 2.30[a]
C. walkeri	0/0	3.06 ± 0.23	27.36 ± 1.58	10.12 ± 2.84	593.06 ± 16.68	45.19 ± 2.63
	5/20	3.11 ± 0.26	37.43 ± 1.89[a]	5.21 ± 4.07	573.45 ± 28.53	45.27 ± 0.93
	15/60	2.86 ± 0.12	37.04 ± 0.59[a]	11.44 ± 1.87	545.59 ± 26.94	55.27 ± 0.96[a]
	30/120	3.55 ± 0.13[a]	63.22 ± 3.46[a]	6.20 ± 2.96	530.60 ± 36.58[a]	49.82 ± 2.94[a]

Abbreviations: 8-OHdG – 8-hydroxy-2′-deoxyguanosine, CAT – catalase, IR – inhibition rate, MDA – malondialdehyde, ND – no data, POX – peroxidase, SH – spore homogenate, SOD – superoxide dismutase.
[a]Significant differences with $P < 0.05$ (one-way ANOVA, Tukey's test) between the contaminated and NC cultures.

spores. CAT and POX activities in *C. walkeri* spores decreased and increased, respectively, while the contamination had no significant effect on SOD activity (Table 5).

4. Discussion

The AMF isolates obtained from the environment contaminated with phenol and PAHs showed a high potential to enhance the growth of host plants cultured on the contaminated substrate; however, their effect on the level of oxidative stress and the activity of antioxidative enzymes in plant tissues depended on the type of AMF inoculum. The AMF had different effects on a host plant and were characterized by a high functional diversity and low relatedness. *C. walkeri* and *F. caledonium* belong to the families *Claroideoglomeraceae* and *Glomeraceae,* respectively, in the order *Glomerales. Glomerales* are described as common AMF species with low ecological requirements, producing high biomass and colonizing plant roots rapidly and intensively. Mycorrhiza formed by *Glomerales* can develop from numerous spores and mycorrhizal root fragments. The third species used in the experiment, *D. varaderana*, belongs to the family *Diversisporaceae*, in the order *Diversisporales*, whose members grow slowly and have low intra- and extraradical biomass. *Glomerales* and *Diversisporales* also utilize different strategies for avoiding stress factors. *Glomerales* allocate biomass to intraradical mycelium (IRM). Inside the root, they are not directly exposed to soil stress factors. *Diversisporales* produce extraradical mycelium (ERM) of high regenerative properties and viability (Chagnon et al., 2013; Varela-Cervero et al., 2016; Aguilar-Trigueros et al., 2019).

F. caledonium, *C. walkeri*, and *D. varaderana* were introduced to the pots with *L. perenne* as single or three-species inoculum. Inoculation with mixed AMF species had a different effect on plant height and biomass than inoculation with single AMF species. Generally, single species inoculation had a beneficial effect on plant growth, regardless of the concentration of contaminants, while three-species inoculation did not enhance but even reduced the growth of *L. perenne* seedlings in NC and LC cultures. Supposedly, plant growth impairment was caused by excessive development of *C. walkeri* which was observed in pots inoculated with mixed AMF cultures. The intensive sporulation of *C. walkeri* could have required a considerable carbon delivery which resulted in weak plant growth (Smith and Smith, 2013; Friede et al., 2016). Studies on interactions between different AMF species have shown that AMF inhabiting the same root system can compete or cooperate. Interactions among different AMF species and between AMF and a host plant may depend on AMF species composition and their succession in a community, the density of AMF inoculum, a host plant species, and environmental conditions (Jansa et al., 2008; Janoušková et al., 2009; Thonar et al., 2014). For example, the dominance of *F. mosseae* over *Rhizophagus intraradices* and *Claroideoglomus claroideum* was observed in *Medicago truncatula* cultures (Jansa et al., 2008). Likewise, in this experiment, the dominance of *C. walkeri* might have resulted from competitive interactions between the AMF species. Allegedly, rapidly growing *C. walkeri* occupied the available ecological niche limited by a small pot and took over most of the plant-derived carbon, slowing down the growth of the other AMF species (Werner and Kiers, 2015). The dominance of *C. walkeri* over *F. caledonium* was overthrown in MC and HC

cultures by the toxic effect of phenol and PAHs on AMF development., which resulted in significantly better plant performance.

Significant growth stimulation of *L. perenne* shoots by inoculation with *C. walkeri* was reported only in NC cultures. Moreover, *C. walkeri* highly decreased root weight, which was also observed in the cultures inoculated with three-species AMF inoculum, dominated by *C. walkeri*. Being the nutrient uptake through AMF hyphae more effective than the direct uptake through roots, mycorrhizal plants would not be required to invest a great deal of resources into forming an extensive root system (Veresoglou et al., 2011; Dreyer et al., 2014). *C. walkeri*, introduced as a single species, favored the allocation of plant biomass to shoots. At the same time, its excessive growth in the mixed AMF cultures caused a substantial decrease in root and shoot weight, probably due to a disturbed carbon economy in plants or less efficient P transfer (Janoušková et al., 2009; Thonar et al., 2014). Similarly, *F. caledonium* and *D. varaderana* enhanced the total plant biomass, but with its preferential allocation to shoots.

Among the AMF species employed in the study, *F. caledonium* was the most effective in plant growth promotion. High efficiency of *F. mosseae*, a species closely related to *F. caledonium*, in the growth enhancement of plants exposed to PAHs was previously reported. Inoculation of *Corchotus capsularis* exposed to anthracene (150 mg kg^{-1}) with *F. mosseae* significantly increased the plant biomass compared to non-inoculated plants. Similarly, Rabie (2005) reported that inoculation with *F. mosseae* caused a 93%, 52%, and 21% increase in the biomass of *Triticum aestivum*, *Vigna radiata,* and *Solanum melongea* cultured in soil contaminated with a mixture of PAHs (500 mg kg^{-1}), respectively. *L. perenne* inoculated with *F. mosseae* was used in phytoremediation of PAH-contaminated substrate, where a positive effect of *F. mosseae* on plant growth and the activity of PAH-degrading bacteria has been reported (Binet et al., 2000; Joner et al., 2001; Joner and Leyval, 2001, 2003).

The growth of *L. perenne* in both phenol and PAHs contaminated and non-contaminated cultures was also stimulated by inoculation with *D. varaderana*. The occurrence of *Diversisporaceae* in the soil contaminated with hydrocarbons has been previously reported (de la Providencia et al., 2015). However, the potential of *Diversisporaceae* to promote plant growth on a contaminated substrate has never been studied.

The effect of phenol and PAHs on AMF development also depended on AMF species. *Glomerales* colonized roots with higher intensity than *Diversisporales* that usually grow slower (Chagnon et al., 2013; Treseder, 2013; López-García et al., 2014). Moreover, *F. caledonium* and *C. walkeri* colonized plant roots with higher intensity and produced more arbuscules in LC and MC cultures than in NC and HC cultures. The stimulation of mycorrhizal colonization by moderate stress, like the low concentration of contaminants, might result from the high adaptability of those AMF strains to develop in the presence of phenol and PAHs. Depending on their lifestyle, AMF may allocate their biomass to IRM or ERM in response to stress. In this experiment, a decreased spore production and increased intensity of mycorrhizal colonization were observed, which might be associated with allocating carbon and energy resources to IRM. An intensified IRM development has been observed in AMF exposed to different abiotic stresses: drought, high temperature, flood, and arsenic contamination (Staddon et al., 2003; Bona et al., 2011; Wu et al., 2013; Lenoir et al., 2016a, 2016b). Moreover, mycorrhizal colonization in stress conditions might be stimulated by a plant host, which produces more strigolactones after exposure to stress factors (Mostofa et al., 2018).

Besides better nutrition, plants get other benefits from mycorrhizal associations, like the induction of systemic resistance and stimulation of antioxidative mechanisms (Pozo and Azcón-Aguilar, 2007; Latef et al., 2016). Higher antioxidative enzymatic activity and lower oxidative stress have been reported in plants exposed to hydrocarbons colonized by *R. irregularis* and *F. mosseae* (Criquet et al., 2000; Debiane et al., 2009; Lenoir et al., 2016a, 2016b). A similar pattern was observed in our experiment when the inoculant was *F. caledonium*. The higher antioxidative activity in *F. caledonium* spores was associated with the lower activity of antioxidative enzymes in plant tissues, especially in the cultures contaminated with phenol and PAHs. On the other hand, we reported a higher antioxidative activity in plants colonized by *D. varaderana* and lower SOD, CAT, and POX activities in *D. varaderana* spores. Finally, the activity of antioxidative enzymes in *C. walkeri* spores and plants colonized by this species did not depend neither on the phenol and PAHs concentrations or the status of mycorrhizal colonization. Nonetheless, plants and AMF may interact with each other in the regulation of antioxidative mechanisms. Those interactions might be complex and depend on the functional traits of mycorrhizal symbionts. High activities of antioxidative enzymes in AMF may support a plant in the scavenging of reactive oxygen species. However, the performed experiment has not proven that higher SOD, CAT, and POX activities in plant tissues or AMF spores contributed to significant alleviation of oxidative stress in plants and AMF.

In this experiment, the primary attention has been drawn to interactions between different AMF species. However, competitive and cooperative associations of AMF with other fungal and bacterial members of mycorrhizosphere microbial communities might be equally important. Mycorrhiza act as a mediator between plant and soil microbial communities. AMF deliver photosynthetically derived carbon to the soil, which may increase the activity and biodiversity of plant growth-promoting, nutrient solubilizing microorganisms and those which degrade xenobiotic contaminants. Conversely, AMF transfer inorganic and organic nutrients solubilized by those microorganisms to the plant host (Miransari, 2011; Hao et al., 2021). Such synergy between organisms is essential for the effectiveness of AMF-assisted phytoremediation, but it is also reliant on the delicate balance of their interactions. For example, in a low-nutrient environment, AMF are forced to compete for carbon, resulting in a lower nutrient supply for microorganisms (Zhang et al., 2021). In recent studies, where agricultural or garden soil was used as a substrate, AMF had a stimulatory effect on functionally vital groups of microorganisms (Chen et al., 2020; Hao et al., 2021). In the sandy soil, without additional organic amendment, the effect of AMF inoculation on microbial diversity was suppressive (Zhang et al., 2021). Therefore, beside selecting plant and AMF symbionts, future experiments on phytoremediation strategies should consider the effect of AMF on other microbial communities.

5. Conclusions

This study revealed a different influence of single species and mixed-species AMF inocula, obtained from a contaminated environment, on the growth and oxidative stress in *L. perenne* cultured on a phenol and PAH-contaminated substrate. Single species AMF inocula positively affected plant growth, regardless of the level of contamination, while mixed-species AMF inoculum had no effect or even decreased plant height and biomass. Among the studied AMF species, *F. caledonium* showed the highest tolerance to phenol and PAHs and the highest potential in plant growth promotion, which have made this species a right candidate for a future experiment on AMF-assisted phytoremediation. Adverse effects of mixed AMF inoculum on *L. perenne* growth might have resulted from competitive interactions between the AMF species.

The effect of mycorrhizal inoculation on the antioxidative activity in plants was different for each mycorrhizal treatment; however, a general pattern was observed, with an increase in the activity of antioxidative enzymes in AMF spores associated with a reduction in the enzymatic activity in the plant tissues. Our findings suggest that the fungal partner might regulate the antioxidative activity in the host plant. However, the nature and mechanisms of this putative regulation are still not clear, especially considering that the intensification of the antioxidative activity was not followed by a pronounced reduction of the oxidative stress in the plant tissues. Even though this aspect requires further investigation, our results might contribute to developing customized AMF-

assisted phytoremediation strategies with indigenous AMF outperforming commercial AMF inocula in the presence of contaminants.

CRediT authorship contribution statement

Monika Malicka: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft. **Franco Magurno:** Formal analysis, Writing - review & editing. **Katalin Posta:** Formal analysis, Writing - review & editing. **Damian Chmura:** Formal analysis, Writing - review & editing. **Zofia Piotrowska-Seget:** Funding acquisition, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We wish to thank Prof. Janusz Błaszkowski, West Pomeranian University of Technology, for the morphological analysis of AMF species. Funding: This work was supported by the The National Science Centre in Poland [Grant number 2017/25/N/NZ8/01676].

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112252.

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