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How does the endophytic fungus *Mucor* sp. improve *Arabidopsis arenosa* vegetation in the degraded environment of a mine dump?

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ABSTRACT

The endophytic fungus *Mucor* sp. was isolated from *Arabidopsis arenosa* inhabiting post mining wastes lands. Its role in plant adaptation to toxic metal enriched environment was evaluated. Plants inoculated with the fungus yielded significantly more biomass. Their growth response was correlated with significant elongation of root hairs, an improved water and P status and a significant upregulation of the expression of genes associated with nutrient uptake. The mechanism of root hair elongation was investigated with auxin and ethylene insensitive *Arabidopsis thaliana* mutants. The results clearly indicate that the root hair elongation phenotype results from fungi induced alterations in ethylene metabolism. The upregulation of close to 50 genes associated with ethylene biosynthesis and signaling confirm these results. The accumulation of Zn and Fe was lower in endophyte inoculated plants. Additionally, root to shoot translocation of Fe, Cd and Zn was improved. The expression of metal transporters associated with metal influx, efflux and distribution within the plant corresponded with altered metal homeostasis. The results of this study clearly show that the endophytic fungus plays an important role in the adaptation of the non-mycorrhizal *A. arenosa* to metal toxicity.

1. Introduction

There is a growing number of evidence indicating the importance of endophytic fungi in plant growth, fitness and adaptation to the environment. According to the definition, fungal endosymbionts are a diverse group of microorganisms inhabiting plant tissues without causing any visible symptoms of disease (Rodriguez et al., 2009; Schulz and Boyle, 2005). Their existence, ubiquity and abundance have been long recognized, however, only recently, their ability to accelerate plant growth and to protect plants against a wide variety of stress factors has gained the attention of the scientific community (Van Der Heijden et al., 2008; Yuan et al., 2010; Kanchiswamy et al., 2015; Kauppinen et al., 2016; Rho et al., 2017).

Metal pollution has dramatically increased during the last centuries and it is expected to rise in the future. As a result, further losses in the complexity and bio-diversity of environments are expected. Contamination of the food chain due to the transfer of toxic metals from the soil to plants and to ground and drinking water reservoirs cause severe risks for human health and agriculture. In degraded site management plants play an essential, remediating role, thus several efforts have been made to improve plant toxic metal tolerance (Ali et al., 2013; Rozpądek et al., 2017).

An important but underestimated aspect of toxic metal tolerance is the ability of the plant to form mutual associations with symbiotic fungi. The best described and probably most common beneficial plant-fungi symbiosis is the association with mycorrhizal fungi (Martin and Kohler, 2013). The role of mycorrhiza in toxic metal stress tolerance and the mechanisms of mycorrhiza dependent trace metal management have been previously described. Numerous reports indicate that mycorrhiza greatly improves plant fitness in metalliferous soils (Orłowska et al., 2012; Rozpądek et al., 2014; Ruytinx et al., 2016; Turnau et al., 2010). Not all plants, however, have the ability to form functional symbiosis with mycorrhizal fungi, including numerous metallophytes and hyperaccumulators from the *Brassicaceae* family. Species from this family are one of the most highly represented plants inhabiting environments enriched in toxic metals including degraded, post-industrial

Abbreviations: ABA, abscisic acid; ABC, ATP-ase binding cassette; ET, ethylene; EtOH, ethanol; BR, brassinosteroids; CDF, cation diffusion facilitator; JA, jasmonic acid; MS, Murashige and Skoog; MSR, Strullu-Romand medium; NaOCl, sodium hypochlorite; NRAMP, natural resistance-associated macrophage protein; PGPR, plant growth promoting rhizobacteria; SA, salicylic acid; SL, strigolactones; ZIF, zinc induced facilitator; ZIP, zinc/iron-like protein

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Table 1
Element concentration in substratum from the "Bolesław" mine dump.

Element Concentration	Zn	Pb	Cd	Fe	K	P/P_{labile}
"Bolesław" [mg kg^{-1}]	12680 ± 380	5240 ± 160	79.0 ± 2,4	(5.83 ± 0,43) 10^4	22 ± 15	1200 ± 300/12.0 ± 6.4
Aro [mg kg^{-1}]	156 ± 33	–	–	(7.5 ± 1.8) 10^3	1750 ± 38	4900 ± 1500/78.0 ± 6.7
Literature averages [mg kg^{-1}]	3.0–2900, median 52 (Mertens and Smolders, 2013)	17 (Steinnes, 2013)	0.1–1 (Smolders and Mertens, 2013)	(2–4 10^4) (Cornell and Schwertmann, 2003)	(0.4 –30) 10^3 (Zörb et al., 2014)	502/30.5 (Deng et al., 2017)

habitats (van der Ent et al., 2013; Verbruggen et al., 2009). The role of fungal symbionts in *Brassicaceae* plants metal management has not been described yet.

Several reports indicate that *Arabidopsis thaliana* can benefit from symbiosis with root endophytes, basidiomycete *Piriformospora indica* and *Sebacina vermifera* (Lahrmann et al., 2015; Verma et al., 1998) as well as species from the *Trichoderma* genus (Brotman et al., 2013; Contreras-Cornejo et al., 2009). To this date reports indicating improved growth or fitness, drought stress and induced resistance against pathogens have been published (Oelmüller et al., 2009; Stein et al., 2008; Waller et al., 2005). Recently, *A. thaliana* was found to harbor a number of fungal endophytes, in both shoots and roots, however, their ecological significance is not fully recognized (García et al., 2013; Junker et al., 2012). According, to a most recent report, the *A. thaliana* root endophyte *Colletotrichum tofieldiae* improved plant growth and fitness by improving P solubilization and uptake from the substratum in P limiting conditions (Hiruma et al., 2016). The role of endophytes in facilitating vegetation (species other than *Arabidopsis*) in environments highly polluted with toxic metals has also been indicated (Li et al., 2011; Wężowicz et al., 2017; Deng et al., 2011), however, our knowledge of the mechanisms enabling plant-endophyte consortia to thrive under such conditions is scarce.

Plants exposed to stress exhibit a broad range of morphogenic changes. Adaptations in root architecture and activation of root hair elongation are probably the best described adaptations to suboptimal soil conditions including environments enriched in toxic metals or/and low nutrient availability (Keunen et al., 2016; Potters et al., 2007). Root hairs are tubular epidermal outgrowths which account for up to 91% of total root surface area (Gahoonia and Nielsen, 1998; Bates and Lynch 1996), but more importantly root hairs are responsible for the majority of water and nutrient uptake, particularly when exposed to nutrient deficiencies (Gahoonia and Nielsen, 1998; Grierson and Schiefelbein, 2002). Another important role of root hairs is modifying the rhizosphere by exudation of organic acids, enzymes, chelating agents and other secondary metabolites (Grierson and Schiefelbein, 2002).

Root hair growth is modulated by the action of plant hormones: ethylene (ET), auxin, strigolactones (SL), jasmonic acid (JA) and brassinosteroids (BR) (Koltai and Kapulnik, 2011; Lee and Cho, 2013; Muday et al., 2012; Zhu et al., 2006). The best described and probably most significant role play ET and auxin. In regard to root hair growth the functional relationship between ET and auxin is synergistic, both hormones play a positive role in this phenomenon (Muday et al., 2012). In addition to adaptation to abiotic factors, root hair elongation (and plant growth acceleration in general) was shown to be induced by PGPR (plant growth promoting rhizobacteria) *via* altering the homeostasis of these two hormones (Poupin et al., 2016). *Arabidopsis* growth and root architecture were also improved by inoculation with *Trichoderma virens* and *T. atroviride*. The response was suggested to be associated with stimulating auxin signaling/auxin production by the fungus (Contreras-Cornejo et al., 2009).

Effective trace metal (Cu, Fe, Zn) management (uptake, compartmentation and detoxification) is required for sufficient supply to different plant organs and in prevention of nonessential metals (Cd, Co, Pb) from inducing deleterious effects on plant cells. Once metals enter the root, the plant deals with it either by storing it in vacuoles of root cells or by transporting it radially through the root to be loaded into the xylem for transport to the shoot (Milner and Kochian, 2008). Metal uptake and distribution is regulated by a complex network of metal carriers. Protein transporters from various families such as the CDF (cation diffusion facilitator), NRAMP (natural resistance-associated macrophage protein), ABC (ATP-ase binding cassette), ZIP (zinc/iron-like protein) and ZIF (zinc induced facilitator) were shown to play a significant role in trace metal homeostasis maintenance (Mirzahossini et al., 2015). A tightly regulated mechanism of adapting carrier quantity/exposition to current metal availability and thus, controlling metal flux from the rhizosphere into the plant and distribution within its tissues determines metal tolerance and supply (DalCorso et al., 2013; Verbruggen et al., 2009).

The aim of this study was to investigate the role of *Mucor sp*. in adaptation of *Arabidopsis arenosa* to a highly polluted environment of a mining waste dump. *Mucor sp*. is a member of the *Mucormycetes*, recently shown to associate with extant, basal land plants, such as liverworts, hornworts and lycopods, in a symbiosis whose mutualistic nature is suspected (Plett and Martin, 2015). The fungus was isolated from plants inhabiting the "Bolesław" Zn, Cd and Pb mine dump, located in S Poland (50°16′58″N 19°32′9″E), in close proximity to the "Śląsk/Silesia" mining region. A detailed description of the climatic and physical-chemical properties of the soil can be found in Orłowska et al. (2005).

2. Materials and methods

2.1. Plant cultivation

Following harvest plants were cultivated in pot culture in a green house in commercial soil (ARO, PL for description see Table 1) to obtain seeds. Seeds were surface sterilized with 8% NaOCl for 5 min, 96% EtOH and 75% EtOH for 1 min, rinsed in distilled water 3 times, sown to petri dishes with 1/4 MS medium and placed in darkness for 48 h. Subsequently, seeds were transferred to a growth chamber (Panasonic ML*R*-352H-PE, JPN) with a 16 h photoperiod, 21/17 °C day/night temperature and 50% humidity. After 10 days seedlings were moved to MSR medium with no sugar (10 plants per petri dish) to facilitate fungi colonization and inoculated with the fungus. Inoculation was performed by placing 2 × 2 mm pieces of mycelium in 5 mm distance from the root tip. Inoculated plants were described E+, not inoculated E-. After 10 days of growth, plants were transferred to 80 ml multipots (1 plant per pot) filled with sterile substratum from the mine dump 'Bolesław' mixed with sterile sand in a 2:1 (v/v) ratio or a mixture of ARO soil with sand in a 2:1 (v/v) ratio for control. The substrate from the mine dump contained elevated quantities of Pb, Zn and Cd, the abundance of essential macronutrients (N, P, K) was very low (Table 1). Multipots were placed in 23 × 45 × 18 cm plexiglass chambers (45 pots per chamber) covered with polyamide gauze to avoid contamination. Plants were grown in the growth chamber for 25 days. Alternatively, 5-day old seedlings of *A. arenosa* were inoculated with *Mucor* sp. (NCBI accession number KU234656) by adding 3.2 × 10^5 spores (in suspension) to a single pot. Three independent chambers per treatment