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The diversity of endophytic fungi in *Verbascum lychnitis* from industrial areas

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Abstract The microbial diversity of *Verbascum lychnitis* community from industrial areas was investigated. The plants harbor a variety of endophytic fungi most of which belong to the *Ascomycetes*. The isolated fungal endophytes were identified according to ITS1-5.8S-ITS2 rDNA sequence similarity and were found to belong to 22 genera and 12 orders. The most frequently isolated genera were *Diaporthe* spp., *Alternaria* spp. and *Pichia* spp. An unidentified species from the *Xylaria* genus was isolated from *V. lychnitis*, which is a novel finding since most *Xylaria* species were reported to be solely wood-inhabiting fungi. The composition of fungal endophytes from the tailings site showed higher diversity, particularly in leaf tissues, than in non-tailings sites.

Keywords Fungal endophytes · *Verbascum lychnitis* · Tailings · *Xylaria*

1 Introduction

In natural and agricultural environments, plants are host to a multitude of microorganisms forming unique, integrated plant - microbial communities. A large variety of microorganisms colonizes the inner and outer surfaces of plant tissues. One of these groups are plant endophytes. The term "endophyte" was first used in XIX century to describe any microorganism colonizing internal plant tissues (cited from Petrini 1991). Since then the term has been evolving (Bayman 2007). Currently, one of widely accepted definitions describes endophytes as bacterial or fungal microorganisms that colonize symptomlessly the living, internal tissues of their host, even though they may, after an incubation or latency period, cause disease (Petrini 1991).

It has been hypothesized that the interaction between fungal endophytes and host plants is characterized by a well-established equilibrium between fungal virulence and plant defense, in which any shift in balance results in the development of plant disease. Nevertheless, endophytic fungi are thought to interact mutualistically with their host plant. The presence of the fungal endophyte may improve water and nutrient uptake and confer protection against both biotic and abiotic environmental stresses (Schulz and Boyle 2005) including drought (Ravel et al. 1997; Cheplick et al. 2000,) and high temperature (Redman et al. 2002), insect pests and herbivores (Siegel and Bush 1996; Schardl and Phillips 1997), low pH (Lewis 2004), high salinity (Waller et al. 2005) and metal toxicity (Monnet et al. 2001). The importance of endophytes is also emphasized by their ability to produce phytohormones, vitamins and other plant growth-promoting substances (Sirrenberg et al. 2007; Dai et al. 2008).

Species from the *Verbascum* genus, commonly known as 'mulleins', are the largest group of the *Scrophulariaceae* family, encompassing approximately 2500 species (Kahraman et al. 2011). *Verbascum* plants are widespread from the temperate to the subtropical zones and have modest soil requirements (Zielińska-Pisklak et al. 2013). They occur spontaneously and grow well at the slope of the tailings near Kraków (Poland), where growing conditions are extremely difficult (Turnau et al. 2010; Turnau et al. 2012).

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Due to their medicinal properties, mulleins have been used in folk medicine since ancient times (Sezik et al. 2001; Leporatti and Ivancheva 2003; Turker and Gurel 2005; Sher 2011). *Verbascum* species are abundant in a number of bioactive substances such as saponins (Turker et al. 2004), iridoid and phenylethanoid glycosides (Akdemir et al. 2004; Akkol et al. 2007) and flavonoids (Akdemir et al. 2003) which confer tolerance to difficult environmental conditions of post-industrial wastes. Mulleins are also being utilized by the pharmaceutical industry. *Verbascum* species show potential for bioremediation of polluted soils accumulating toxic metals such as As, Pb, Zn (Turnau et al. 2010), Ba (Kowalska et al. 2012) Mo, Cu and Ag (Sagiroglu et al. 2006).

Due to a high concentration of toxic elements and the poor water holding capacity in the substratum, tailings are highly unfavorable for plant growth and the development of the necessary soil biota. The first 40–50 years of natural succession are characterized by the presence of pioneer species such as *Fragaria vesca*, *Hippophae rhamnoides*, *Arrhenatherum elatius*, *Trifolium repens*, *Viola tricolor* (Turnau et al. 2001) and grasses, particularly from the family *Poaceae* (Ryszka and Turnau 2007). Plants, besides their unquestionable impact on physical-chemical properties of the soil (Hinsinger 2013), are an important niche for beneficial microorganisms.

Verbascum species commonly occur at the tailings near Chrzanów, calcareous grasslands in Poland (Turnau et al. 2008) and in other post-industrial wastes in Europe (Góralska 2014). The species of *Verbascum* with the greatest ability to colonise degraded areas is *V. thapsus*. In the experiment designed by Turnau et al. (2008) *V. thapsus* with other plants from calcareous grassland were introduced onto the tailings site. According to the results *V. thapsus* was among a few plant species which survived, produced seeds and spread out on the tailings (Turnau et al. 2008). Survival under extreme conditions is also possible thanks to the ability of *Verbascum* seeds to survive up to 100 years (Turnau et al. 2012).

In our study we decided to focus on a species that spontaneously occur in large numbers on post-industrial sites - *V. lychnitis*. The plant cover in the area of sampling was restricted to grass species, with the exception of only a few dicots, including *V. lychnitis.* As mentioned previously, plants are a necessary niche for microorganisms engaged in detoxifying polluted soil. This relationship is mutual; for many plants the presence of fungal microorganisms is obligatory for survival (Redman et al. 2002). For others the presence of endophytic fungi is required for growth and reproduction (Clay 1984). With that being said, we can assume that the biodiversity of plants may correspond with the diversity of microorganisms present in the ecosystem. Thus, identyfing the diversity of plant associated endophytic fungi will be a step towards understanding the processes that allow plants to survive in extreme environments. In recent years plant-microbe consortia have been gaining interest for their potential use in phytoremediation. Pioneer plants are a potential reservoir for beneficial microorganisms, thus their identification can be an asset for phytoremediation.

The purpose of present study was to 1) investigate the diversity of microbial community of *V. lychnitis* growing at the slope of the tailings and non-tailings sites and 2) isolate and characterize the fungal endophytes from these plants.

2 Materials and methods

2.1 Research area

V. lychnitis plants were collected from the following locations: meadows located near Olkusz (N 50°18′26″ E 19°33′48″), wastelands located in the vicinity of Klucze (N 50°19′47″ E 19°33′32″) and three plots from the tailings of the ZG Trzebionka Mining Company located near Chrzanów (N 50°09′19″ E 19°25′10″). All of these sites are situated in an industrial region, 30–50 km west of Kraków, Poland where the toxic metal (Cd, Pb and Zn particularly) concentration in the soil is elevated (http://www.jura.eko.org.pl/doc/srodowisko/Gmina_Klucze.pdf, http://www.jura.eko.org.pl/doc/srodowisko/Gmina_Olkusz.pdf). In contrast to the Klucze and Olkusz sites the substratum from the tailings contained almost no organic matter and was P- and N- deficient. It was slightly alkaline (pH 7.4), and contained up to 108.15 $\mu g\ g^{-1}$ of Cd, 2372.03 $\mu g\ g^{-1}$ of Pb and 12067.0 $\mu g\ g^{-1}$ of Zn, although the bioavailability of these elements is not particularly high (Orłowska et al. 2005a; Orłowska et al. 2005b).

2.2 Plant sampling

From the tailings site, Olkusz and Klucze, 16, 9 and 6 plants respectively were harvested. Juvenile (1 year) and mature (2 year old) plants were collected and left in distilled water overnight. The *V. lychnitis* samples from field sites were then separated into roots, shoots (stems, leaves) and seeds.

2.3 Endophyte isolation

Fungi isolation was performed twice from plants from each site. For isolation, plant tissues lacking visible injury were used. In order to eliminate epiphytic communities, the outer surface of plant tissues were initially surface sterilized.

Aerial parts were treated with 70 % ethanol (5 min), immersed in 1 % sodium hypochlorite solution (1 min) and incubated in 0.01 % Tween 20 solution (1 min). Samples were rinsed three times in sterile distilled water. Subsequently, plant tissues were dried on sterile filter paper, cut into 0.5×0.5 cm pieces and placed on potato dextrose agar (PDA) supplemented with oxytetracycline (50 mg/1) to prevent bacterial growth. Parafilm (Bemis, USA) - sealed petri dishes were incubated for 4–7 days in darkness at 25 °C. Plates were evaluated for hyphae of emerging fungi on a daily basis and transferred to fresh medium. In order to check the effectiveness of the surface sterilization procedure, aliquots of water from the last rinsing were smeared on a PDA plate (Santamaria and Bayman 2005 [modified]); Hallmann et al. 2006; Verma et al. 2007 [modified]; Compant et al. 2011).

The roots of *Verbascum* were rinsed in running tap water and surface sterilized with 99 % ethanol (1 min), incubated in 3 % hydrogen peroxide solution (5 min) and immersed in 75 % ethanol (5 min) (Compant et al. 2011 [modified]). Subsequently, roots were air-dried on sterile filter paper. The younger roots were cut to 1 cm segments and placed on PDA medium supplemented with oxytetracycline (50 mg/l).

Surface sterilization of the seeds was performed by immersion in 1 % sodium hypochlorite solution with 1 droplet (200 μl) of Tween 80 in 100 ml of solution (30 min). The samples were subsequently rinsed in sterile distilled water three times. The sterility of the seeds was verified by spreading aliquots of water from the last rinsing onto PDA medium. The endophytes were isolated from seeds by pressing in a sterile mortar after addition of 10 mM $MgSO_4$ solution (Mastretta et al. 2009 [modified]). After incubation of the PDA plates supplemented with oxytetracycline (50 mg/l), the hyphae of growing fungi were transferred to fresh medium.

2.4 Endophyte identification

Isolated endophytic fungi were identified according to their macroscopic and microscopic characteristics and using molecular methods.

Genomic DNA was extracted from mycelium of fungi growing on PDA by cutting the mycelium from the youngest part of the agar culture (7–8 days old) and transferring to test tubes filled with two tungsten beads each. Subsequently, samples were homogenized in a tissue lyser (LT, Qiagen, Germany).

DNA was isolated with 2 % cetyltrimethyl ammonium bromide (CTAB) buffer according to the protocol (Gardes and Bruns 1993). Reaction mixtures (25 μl) contained 12.5 ul Dream Taq Green PCR Master Mix (Thermo Scientific) and primers (Genomed) ITS1F (5′-CTTGGTCATTTAGAGGAA GTAA-3′) (Gardes and Bruns 1993) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) (White et al. 1990).

Amplification was carried out as follows: a preliminary denaturation step at 95 °C for 3 min was followed by 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 72 °C, PCR products were separated by electrophoresis in 1.5 % agarose gel. For visualization gels were stained with Gel Red Nucleic Acid Stain (BIOTIUM).

PCR products were purified with 3 M sodium acetate solution and isopropanol (http://openwetware.org/wiki/Isopropanol_Precipitation_for_PCR_Purification), cycle sequenced with ABI BigDye Terminator ver.3.1 (Applied Biosystems, USA) and purified according to the manufacture's protocol with 125 mM EDTA and 96 % ethanol. Sequences were analyzed in a sequenator 3130xl Genetic Analyzer (Applied Biosystems).

Finally, DNA sequences from each culture were edited using BioEdit (Hall 1999) and Chromas (www.technelysium.com.au) software. Using the BLASTn (Altschul et al.1997), sequences were compared to those published in the NCBI (www.ncbi.nlm.nih.gov) databases. A positive identification of species was confirmed if they shared≥98 % ITS region sequence identity with the most similar (reference) sequence from NCBI databases. The obtained sequences within 2 % nucleotide difference were categorized into a single operational unit and assigned an identical name. The correctness of Latin names was adopted from www.indexfungorum.org. Sequences were submitted to GenBank and assigned accession numbers from KP174665 to KP174711

2.5 Biodiversity measurements

Shannon-Wiever index (H') of biodiversity was applied to the fungal species community from the tailings and non-tailings sites.

3 Results

3.1 Isolation of endophytic fungi from *V. lychnitis*

In this study, a total of 263 fungal cultures were isolated from 31 *V. lychnitis* plants collected from the tailings site, Olkusz and Klucze (Table 1).

From fungal cultures, 47 isolates were identified based on ITS1-5.8S-ITS2 rDNA sequencing. 68 and 32 % of the identified taxa were isolated from juvenile and matured plants respectively.

Table 1 Number of fungal cultures isolated from *Verbascum lychnitis* plants from the Z.G."Trzebionka" tailings and Klucze and Olkusz sites

Locality	*V. lychnitis* tissue		
	roots	shoot	seeds
Tailings	33	101	4
Klucze	27	43	5
Olkusz	13	33	4

3.2 Molecular identification of endophytic fungi

The isolated fungal endophytes belonged to 22 genera and 12 orders (Table 2). The most frequent genus was *Diaporthe* with 14.89 % of identified endophytes belonging to this genus, followed by *Alternaria* (12.76 %) and *Pichia* (8.51 %). Other genera were represented by one to three identified isolates. The *Pleosporales* occurred as the most prevalent order with 36.17 %, followed by *Diaporthales* and *Hypocreales* (14.89 % each). The identity percentage among obtained sequences compared with those available in GenBank ranged from 96 to 100 %.

3.3 Endophyte distribution in plant organs

Pyrenophora and *Xylaria* species were isolated from *Verbascum* leaves, while *Alternaria* and *Chaetomium* species were isolated from the shoots of the plants. *Cochliobolus*, *Gibberella, Phoma, Pichia* and *Trichoderma* species were isolated from all parts of *Verbascum* plants. *Diaporthe* spp. were isolated from leaves apart from one strain which inhabited plant roots. *Diaporthe sp.* was also the only genus which occurred in plants from all examined sites. *Cadophora*, *Cochliobolus Pyrenophora* and *Xylaria* species were isolated only from the tailings site.

3.4 Biodiversity and species richness

The Shannon-Weiner index was significantly higher (3.044) in plants from the tailings site than in plants from non-tailings sites (2.294).

The most abundant in fungal taxa were leaves (12 species) and roots (9 species) of plants growing on the tailings site (Table 2). In Klucze and Olkusz, the frequency of endophytes isolated from different parts of plants ranged from 1 to 4 species.

4 Discussion

The association of *Verbascum* with vesicular mycorrhizal fungi has been shown several times previously (Pendleton and Smith 1983; Harley and Harley 1987; Góralska 2014), however, the presence and identity of other, important classes of microbial organisms from mulleins have not so far been studied.

In this study the plants were found to harbor a variety of endophytic fungi most of which (97.87 %) belong to *Ascomycetes*. The most frequent genera were *Diaporthe* (*Phomopsis*), *Alternaria* and *Pichia.*

Fungi from the genus *Phomopsis* have been described as deleterious plant pathogens. Species such as *P. viticola* cause cane and leaf spots in grapevine, and its occurrence leads to significant yield loss (Scheper et al. 2000). *P. amygdali* infections result in twig canker and blight of almonds and peach (Tuset and Portilla 1989 cited from Udayanga et al. 2011). *P. helianthi*, reported by Heller and Gierth (2001), causes a stem canker of sunflower. Some species such as *P. leptostromiformis* which infects lupines (*Lupinus* sp.) may also be dangerous to animals causing lupinosis, a type of mycotoxicosis in sheep (Wood and Sivasithamparam 1989). However, species of *Phomopsis* are also commonly isolated as endophytes from leaves of different species of dicot plants, and in many cases these fungi dominate the endophyte assemblage of a host. Sun et al. (2011) showed that the *Phomopsis* sp. was one of the most dominant endophyte species in *Acer truncatum*. An unidentified fungus from this genus conferred tolerance against the plant pathogen *Microcyclus ulei* (Rocha et al. 2011). According to Suryanarayanan et al. (2002) *Phomopsis* sp., C*olletotrichum* sp. and *Phyllosticta* sp. contributes 49–70 % to a trees' endophyte assemblage irrespective of the season and forest type, indicating that they are well adapted to survive as endophytes and elude different types of host defences.

Another highly represented fungus was *Alternaria alternata,* a common pathogen causing a variety of diseases of plants (Maiti et al. 2007; Abkhoo and Sabbagh 2013) including marketable fruits (Lee et al. 2013). It also belongs to a group of only eight species of this genus which have been identified as human pathogens (Farina et al. 2007). Fungi from this genus are also commonly isolated as endophytes from medicinal plants (Aly et al. 2008; Bhagat et al. 2012), *Malus domestica* (Camatti-Sartori et al. 2005), *Coffea arabica* (Fernandes et al. 2009), evergreen rhododendrons (Purmale et al. 2012) and *Nicotiana tabacum* (Spurr and Welty 1975). *A. alternata* has been routinely isolated as an endophyte of leaves from wheat (Larran et al. 2002). As in the case of *Phomopsis* sp*., Alternaria* sp. fungi are reported as common endophytes (Suryanarayanan et al. 2011). Since *A. alternata* is an opportunistic pathogen for humans, it should not be considered for phytoremediation.

The third most frequent endophyte species isolated from *V. lychnitis* was *Pichia guilliermondi*. Buzzini et al. (2003) isolated these ascomycetous yeasts from three rain forests, where *Pichia* sp. comprised 56 % of the total strains. This genus is reported as a biocontroller of pathogenic fungal growth in plants (Petersson and Schnürer 1995), and a source of antimicrobial metabolites (Zhao et al. 2010). However, Gai et al. (2009) showed that the supernatant from a culture of *P. guilliermondii* increased the *in vitro* growth of the pathogen, suggesting that the yeast could assist in the establishment of this pathogen in its host plant, therefore contributing to the development of disease symptoms.

The only isolated fungus belonging to *Basidiomycetes* was *Rhizoctonia solani*. This fungus has gained a reputation of a widespread and deleterious plant pathogen with unlimited

Table 2 Molecular identification of fungal endophytes isolated from *Verbascum lychnitis* plants from the Z.G. „Trzebionka"(T) tailings and Klucze (K) and Olkusz (O) sites

Isolate	Accession number	Site, Plant tissue	BLAST Search			
			Most probable taxonomic matches	Accession number	Identity	Order
1	KP174666	T, R	*Trichoderma hamatum*	KC690895	99 %	*Hypocreales*
2	KP174669	T, R	*Cadophora* sp.	JN859262	99 %	*Helotiales*
3	KP174683	T, R	*Paraphoma radicina*	KJ188707	99 %	*Pleosporales*
4	KP174685	T, R	*Fusarium* sp.	KF752620	99 %	*Hypocreales*
5	KP174687	T, R	*Cladosporium sphaerospermum*	EU570258	99 %	*Capnodiales*
6	KP174681	T, R	*Pichia guilliermondii*	EF191048	98 %	*Saccharomycetales*
7	KP174690	T, R	*Phomopsis* sp.	EF432281	99 %	*Diaporthales*
8	KP174699	T, R	*Cochliobolus sativus*	JQ936203	98 %	*Pleosporales*
9	KP174705	T, R	*Pichia guilliermondii*	EF191048	97 %	*Saccharomycetales*
10	KP174708	T, R	*Phialophora mustea*	JN123359	99 %	*Chaetothyriales*
11	KP174671	T, S	*Aureobasidium* sp.	FJ196616	97 %	*Dothideales*
12	KP174670	T, S	*Alternaria alternata*	KJ957793	99 %	*Pleosporales*
13	KP174676	T, S	*Chaetomium globosum*	KF156299	99 %	*Sordariales*
14	KP174682	T, S	*Cochliobolus sativus*	JQ936203	98 %	*Pleosporales*
15	KP174707	T, S	*Alternaria alternata*	KJ957793	99 %	*Pleosporales*
16	KP174710	T, L	*Phoma exigua* var. *exigua*	EU343168	99 %	*Pleosporales*
17	KP174701	T, L	*Pyrenophora leucospermi*	JN712475	98 %	*Pleosporales*
18	KP174711	T, L	*Alternaria alternata*	KJ957793	98 %	*Pleosporales*
19	KP174665	T, L	*Pyrenophora leucospermi*	JN712475	99 %	*Pleosporales*
20	KP174702	T, L	*Pyrenophora leucospermi*	JN712475	99 %	*Pleosporales*
21	KP174667	T, L	*Fusarium* sp.	FJ210604	99 %	*Hypocreales*
22	KP174668	T, L	*Trichoderma* sp.	JX014378	99 %	*Hypocreales*
23	KP174677	T, L	*Cadophora luteo-olivacea*	KF156297	98 %	*Helotiales*
24	KP174678	T, L	*Diaporthe* sp.	AB245446	96 %	*Diaporthales*
25	KP174679	T, L	*Chaetosphaeronema hispidulum*	KF251148	99 %	*Pleosporales*
26	KP174686	T, L	*Myrothecium* sp.	JN859396	99 %	*Hypocreales*
27	KP174688	T, L	*Phomopsis* sp.	GU462148	99 %	*Diaporthales*
28	KP174689	T, L	*Pichia guilliermondii*	EF191048	99 %	*Saccharomycetales*
29	KP174691	T, L	*Xylaria* sp.	DQ780448	99 %	*Xylariales*
30	KP174695	T, L	*Alternaria alternata*	AY433814	99 %	*Pleosporales*
31	KP174696	T, L	*Bipolaris sorokiniana*	KC662104	98 %	*Pleosporales*
32	KP174697	T, L	*Xylaria* sp.	DQ780448	98 %	*Xylariales*
33	KP174700	T, SE	*Cladosporium cladosporioides*	AJ300335	98 %	*Capnodiales*
34	KP174709	K, R	*Rhizoctonia solani*	FJ492158	99 %	*Cantharellales*
35	KP174675	K, S	*Epicoccum nigrum*	JF440590	98 %	*Pleosporales*
36	KP174672	K, L	*Fusarium avenacea*	HM991285	96 %	*Hypocreales*
37	KP174673	K, L	*Diaporthe* sp.	JX624294	97 %	*Diaporthales*
38	KP174674	K, L	*Phomopsis* sp.	GU462144	99 %	*Diaporthales*
39	KP174685	K, L	*Pichia guilliermondii*	EF191048	99 %	*Saccharomycetales*
40	KP174694	K, SE	*Phoma herbarum*	JF340263	100 %	*Pleosporales*
41	KP174703	K, SE	*Alternaria alternata*	JF440581	99 %	*Pleosporales*
42	KP174704	K, SE	*Trichoderma gamsii*	HM176570	99 %	*Hypocreales*
43	KP174706	K, SE	*Alternaria alternata*	KJ957793	99 %	*Pleosporales*
44	KP174680	O, L	*Diaporthe* sp.	JX624294	96 %	*Diaporthales*
45	KP174692	O, L	*Diaporthe neoarctii*	NR_111854	98 %	*Diaporthales*
46	KP174693	O, L	*Chaetomium globosum*	JX981455	98 %	*Sordariales*

Table 2 (continued)

Isolate	Accession number	Site, Plant tissue	BLAST Search			
			Most probable taxonomic matches	Accession number	Identity	Order
47	KP174698	O, L	*Colletotrichum truncatum*	KC464339	98 %	*Glomerellales*

Plant tissue: R-roots, L-leaves, S-stem, SE-seeds

host ranges (Parmeter 1970; Ohkura et al. 2009). However, it has also been reported as an orchid symbiont (Shimura et al. 2009) including orchids that occur on the industrial wastes examined in this study (Wojtczak 2013).

Seven of the endophyte genera/species found in the present study were represented by a single isolation. According to Sánchez Márquez et al. (2012) species that are isolated only once in an endophyte survey are considered rare in the host plant. The interactions between plants and rare species may represent unstable associations that possibly only occur when a given plant and fungal genus encounter one another.

Among grasses and perennial plants, the most common endophytic taxa belong to Ascomycota (Stone et al. 2004). The vast majority of endophytes isolated from *V. lychnitis* were ascomycetous. In *V. lychnitis, Pleosporales* was the most prevalent order followed by *Diaporthales* and *Hypocreales*. Sieber (2007) noticed that representatives of the orders *Dothideales, Pleosporales, Mycosphaerellales* and the *Xylariales* can be dominant in endophytic communities from angiosperms and gymnosperms. Stone et al. (2004) pointed out that *Diaporthales* and *Hypocreales* were among the most frequent isolates from foliage and woody perennials. The majority of the isolated species come from juvenile plants. Fungi isolation from young tissues is more efficient than from older tissues. The latter often contain many slow growing species from which isolation is difficult (Bacon and White 1994).

The H' values for isolates from the tailings were higher in comparison to non-tailings sites, which indicates that the fungal community from plants growing on the tailings was more diverse, particularly in leaf tissues, in comparison with non-tailings sites. These data are inconsistent with published literature (Jurc et al. 1996; Lappalainen et al. 1999). In this study, higher H' values for isolates from the tailings line reflect the diversity of fungal community in one particular species - *V. lychnitis,* but not the biodiversity of microorganisms at the site of sampling. In order to gain a comprehensive understanding of the effect of toxic metals on the diversity of microorganisms inhabiting the plants, a much more thorough study is necessary.

The most frequent species isolated from *V. lychnitis* leaves from the tailings site were *Pyrenophora leucospermi* (3 isolates), *Alternaria alternata*, *Diaporthe* sp. and *Xylaria* sp. (2 isolates of each). The most frequent species isolated from *V. lychnitis* leaves from non-tailings sites were *Diaporthe* sp. (3 isolates), *Diaporthe neoartcii* (1 isolate) and *Alternaria alternata* (2 isolates). *P. leucospermi* is a novel species isolated from plants belonging to the *Proteaceae* family also known as a pathogen causing severe blight in current-season leaves, stems, and flower heads (Crous et al. 2011). *Alternaria* sp., *Diaporthe* sp. and *Xylaria* sp. are all common endophytes but the isolation of *Xylaria* from *V. lychnitis* is particulary interesting, since most *Xylaria* species have been reported as wood-inhabiting fungi occurring commonly on decaying trees (Wang et al. 2014). The results may indicate that the most abundant endophytes have adapted to the long-term toxic metal stress conditions and the leaf tissue was their niche. Since *A. alternata* is an opportunistic pathogen for humans (Anaissie et al. 1989), only *Diaporthe* sp. and *Xylaria* sp. should be considered as potential candidates for phytoremediation. Endophytes from the *Xylaria* genus have not been reported in herbaceous species. In this study this fungi was found inhabiting the leaves of plants growing in the tailings site, thus we speculate that the presence of toxic metal in the substratum impacts the interaction between the plant and the fungi rendering it possible for the fungi to colonize *V. lychnitis*.

Some endophytic ascomycetous fungi are known to be very defensive against saprobic basidiomycetes. In the current study *Phomopsis* sp. and *Xylaria* sp. were one of the most frequent inhabitants in leaf tissue of plants from the tailings, which potentially indicate their defensive role against secondary colonizers. Similar results have been presented by Fukasawa et al. (2009), in which a *Xylaria* sp. in particular was highly combative against the secondary colonizer *Phanerochaete filamentosa.*

Another important factor possibly determining the fungal community is the life cycle of the fungi. Xylariaceous fungi are endophytes in trees; however, they remain dormant until triggered to grow and sporulate by natural leaf senescence, abscission or damage (Promputtha et al. 2010). Xylariaceous fungi have the advantage of occupying tree tissues earlier than other fungi that usually colonize plants from the outside via airborne or soil-borne spores or hyphae. This is probably the reason why small leaves and phyllome tissues are dominated by endophytic Xylariaceous species in their early stages of decomposition. It may be possible for endophytic fungi to become saprobes following the senescence of host tissue

(Wong and Hyde 2001; Ghimire and Hyde 2004). According to Promputtha et al. (2010) saprobes may have evolved from endophytic fungi as a result of their ability to colonize leaves and produce specific enzymes during fungal succession. Indeed some endophytes may cause decay of the host tissue and then persist as saprobes after senescence (Ghimire and Hyde 2004; Oses et al. 2008).

5 Conclusions

The endophyte diversity of grass and tree species from different regions has been extensively studied and, to our knowledge, this is the first comprehensive report of the fungal endophyte diversity of *V. lychnitis*. This mullein species was earlier investigated for the presence of bioactive compounds (Alipieva et al. 2014) or in studies on succession (Rebele 2008). The most frequent genera isolated from *V. lychnitis* were typical endophytes inhabiting other monocotyledonous and dicotyledonous plants. The isolation of *Xylaria* sp. from the leaves of *Verbascum,* a biannual dicotyledon, is a novel finding. The composition of fungal endophytes at the tailings site showed higher diversity than at non-tailings sites.

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