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Endophytes from *Phragmites* for metal removal: evaluating their metal tolerance, adaptive tolerance behaviour and biosorption efficacy

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ABSTRACT

This study determined the potential of fungal endophytes as novel group of biosorbents for metal removal. The endophytic fungi were first isolated from *Phragmites*, a plant typically used to treat wastewater or leachate, and screened for tolerance and biosorption potential towards various metals. Results revealed that all 21 endophytes demonstrated tolerance to metals tested (Cd^{2+}, Cu^{2+}, Cr^{3+}, Pb^{2+} and Zn^{2+}), with three isolates (*Trichoderma asperellum* Iso11, *Phomopsis* sp. Iso9 and *Saccharicola bicolour* Iso22) showing the most potential. Of the three, *T. asperellum* demonstrated better tolerance and adaptive tolerance behaviour to various metals compared to *Phomopsis* sp. and *S. bicolour* which were unable to adapt to increasing metal concentrations (up to 2,000 mg L^{-1}). All three isolates showed similar efficacy in removing metals in single-metal solutions. On the contrary, in multi-metal solutions, *T. asperellum* and *S. bicolour* showed higher affinity to adsorb Cu^{2+}, followed by Cr^{3+} and Pb^{2+}, while *Phomopsis* sp. had affinity towards metals in the following trend: $Cu^{2+} > Pb^{2+} > Cr^{3+}$. This study is the first to document the metal tolerance and sorption efficacy of endophytes from *Phragmites*.

Keywords: Adaptive behaviour; Biosorption; Endophytes; Metal tolerance; *Phragmites*

1. Introduction

Endophytic fungi, commonly known as endophytes, are defined as fungi living in tissues of plants without showing profound symptoms [1]. They acquire nutrients and protection from their hosts, and in return, produced secondary metabolites that aid host plants in tolerating both pathogens and abiotic stresses [2]. Endophytes have been isolated from various host plants; from grasses to herbs and to flowering plants [3]. The role of endophytes in removing metals is poorly understood, although they have been closely associated with hyperaccumulators and wetland plants. One such plant is the *Phragmites*, which are used to remove metal in natural bioremediation systems. *Phragmites* have wide geographical distribution, are easy to grow, and have shown tolerance to heavy metals and herbicides [4,5]. As such, they have been used in phytoremediation to bioremediate metal-laden humus to produce toxic-free humus [6]. *Phragmites* are also able to remove organic matters such as phenol, phosphorus and nitrogen via the nitrification–denitrification process, hence has been incorporated in efforts to treat wastewater [7,8].

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Although *Phragmites* is extensively studied, the endophytes associated with these plants are poorly understood, particularly the metal tolerance and biosorption potential of these endophytes.

In this study, we isolated endophytes residing in *Phragmites*, and investigated their tolerance to various metals and concentrations, and determined their biosorption efficacy. Hypothetically, *Phragmites* as host plants are ideal as the constant exposure to high metal concentrations in wastewaters may induce metal tolerance traits in the endophytes they harbour [9]. Thus, by establishing and documenting their tolerance and biosorption characteristics, these endophytes from *Phragmites* are introduced as novel biosorbents with potential for further development. The biosorption efficacies of the selected endophytes in single- and multi-metal solutions were also compared to reveal the metal uptake preference and postulate biosorption behaviour of endophytes when used to treat natural wastewaters (multi-metal solutions). In this paper, we report our findings on the species of metal-tolerant endophytes isolated from *Phragmites*, their metal tolerance and adaptive tolerance behaviour to various metals and concentrations, and their biosorption potential in both single-metal and multi-metal solutions.

2. Materials and methods

2.1. Isolation of endophytes and identification of selected isolates

Leaf samples were collected from a single *Phragmites* plant growing in a leachate treatment site in a landfill (3˚29´34´´N, 101˚29´1´´E). The leaves were first washed under running tap water for 10 min, cut into smaller pieces (2 × 2 cm) and surface sterilized (95% ethanol for 1 min, 3.25% sodium hypochlorite for 10 min, 95% ethanol for 30 s and finally rinsed thrice in sterile distilled water) [10]. The surface-sterilised tissues were then blot-dried using sterile paper towel, cut to expose the inner tissues and plated onto potato dextrose agar (PDA, Merck). Plates were incubated at room temperature (25 ± 2˚C) and fungal mycelia which grew from the plant tissues were subcultured onto fresh PDA plates. To validate the effectiveness of the surface sterilisation procedure, the final rinsing water was plated onto PDA and the plates were incubated. Absence of fungal growth indicates fungal growth from plant tissues were endophytes. Isolates selected for identification were first cultured in 100 mL potato dextrose broth (PDB, Merck). After 5 d of incubation (120 rpm, 25 ± 2˚C), the mycelia was filtered (filter paper) and the genomic DNA was extracted using the DNA extraction kit (GF-1 Nucleic Acid Extraction Kit, Vivantis) according to manufacturer's guidelines. This was followed by amplification initiated by preparing the PCR (Polymerase Chain Reaction) mixture consisting of 25 μL GoTaq Green Master Mix, 5 μL of ITS1 (TCC GTA GGT GAA CCT GCG G), 5 μL of ITS4 (TCC TCC GCT TAT TGA TAT GC), 5 μL of DNA template and 10 μL of nuclease free water. The amplification conditions were performed as follows: initial denaturation step at 95˚C for 1 min, followed by 35 repeated cycles of denaturation at 95˚C for 30 s, annealing at 52˚C for 40 s, extension at 72˚C for 1 min and 30 s and a final extension at 72˚C for 5 min (MJ Mini Personal Thermal Cycler). The amplicons were subsequently purified using DNA Purification Kit (MEGAquick-spin Total, iNtRon Biotechnology), validated by electrophoresis on 0.7% agarose gel, and gradually outsourced to First Base Laboratories and NHK Bioscience for DNA sequencing. Both forward and reverse DNA sequences obtained were then aligned using the BioEdit software and used for identification purposes via NCBI (BLAST). Total query cover, percentage of similarities and *E*-values (Expect-values) were noted.

2.2. Screening for metal tolerance

Metal tolerance was detected by inoculating fungal mycelial plugs (0.5 cm diameter) on PDA supplemented with 2,000 mg L^{-1} of metal salts. The metal salts used were: $Al_2(SO_4)_3{\cdot}16H_2O$ (R&M Chemicals), $Zn(NO_3)_2{\cdot}6H_2O$ (R&M Chemicals), $CuSO_4{\cdot}5H_2O$ (R&M Chemicals), $Pb(NO_3)_2$ (R&M Chemicals), $3CdSO_4{\cdot}8H_2O$ (R&M Chemicals) and $Cr(NO_2)_3{\cdot}9H_2O$ (R&M Chemicals); to induce response to Al^{3+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Cr^{3+}, respectively [11]. Concentrations of some metal salts were subsequently adjusted to 2,500 or 1,000 mg L^{-1}, for isolates which either showed excellent tolerance or non-tolerance to 2,000 mg L^{-1}, respectively. Inoculated plates were incubated (25 ± 2˚C) for 5 d where radial growth was measured on the 5th day. Controls were prepared by inoculating mycelial plugs on PDA without metal salts. Isolates which showed the most tolerance to various metals (preferably at higher concentrations) were then selected for subsequent tests to understand their adaptive tolerance behaviour and establish their biosorption potential.

2.3. Adaptive tolerance behaviour of selected endophytes

Selected endophytes were tested for their adaptive tolerance behaviour to evaluate their tolerance and adaptability to increasing concentrations of the various metals [11]. In this test, mycelial plugs (0.5 cm

diameter) were first inoculated onto PDA supplemented with 500 mg L^{-1} of metal salts. Isolates showing growth on the 500 mg L^{-1} plates were subsequently transferred to fresh PDA plates supplemented with 1,000 mg L^{-1} of metal salts. This process was gradually repeated for isolates with positive growth detected in previous concentrations up to 1,500 and 2,000 mg L^{-1} [11]. Incubation was carried out at 25 ± 2˚C for 9–18 d. Control plates were prepared by inoculating fungal mycelial plugs on PDA without the metal salts. The radial growth of fungal colonies was then measured every 5 d after inoculation and compared to the control plates. Tolerance index of the isolates towards each metal was then calculated based on the following equation:

$$\text{Tolerance Index} = \frac{\text{Radius of colony in metals}}{\text{Radius of colony in control}} \quad (1)$$

2.4. Biosorption potential of selected endophytes

The biosorption test was conducted in single- and multi-metal solutions. The biomass of the selected endophytes was first established by inoculating 8 mycelial plugs into 400 mL of PDB (Merck). The culture was incubated (120 rpm, 25 ± 2˚C) for 5 d. The biomass was then sieved (Whatman No. 1 filter paper) and autoclaved (121˚C, 20 min) to obtain non-viable cells for the biosorption test [12]. These non-viable cells (0.1 g) were added into 30 mL of 100 mg L^{-1} metal-laden solutions. For single metal analysis, metal solutions were diluted from 1,000 mg L^{-1} Zn^{2+}, Cu^{2+}, Pb^{2+} and Cd^{2+} stock solutions, and the initial pH adjusted to pH 5. The inoculated metal solutions were incubated with agitation (200 rpm, 25 ± 2˚C, 4 h), after which the solution was centrifuged (8,000 rpm, 15 min) and the supernatant collected for analysis using Atomic Absorption Spectrometry (AAS). For multi-metal solutions, the 30 mL solution consist of a mixture of 100 mg L^{-1} each of Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Cr^{3+} [13]. The biosorption potential in multi-metal solutions was determined as previously described. The values obtained from AAS were compared to standard curves and the biosorption capacity (Q) of isolates calculated based on the following Eq. (12):

$$Q = \frac{(C_o - C_e)V}{m} \quad (2)$$

where C_o: initial metal concentrations (mg L^{-1}); C_e: final metal concentrations (mg L^{-1}); V: volume of solution (L); m: mass of the biosorbents (g).

2.5. Statistical analysis

All experiments were conducted in triplicates. Data obtained were analysed using ANOVA and the means compared with Tukey Test ($HSD_{(0.05)}$) using the Social Science (SPSS) software (version 20.0).

3. Results and discussion

3.1. Identification of selected endophytes

A total of 21 endophytic fungal isolates were isolated from *Phragmites*. Of this, DNA sequencing was only performed for the three isolates which showed good potential for metal tolerance and removal. From the BLAST results, Iso11 was identified as a probable species of *Trichoderma asperellum* similar to *Trichoderma asperellum strain TR696* Accession number: KC993073.1 (based on 100% similarity, 0.0 *E*-value, 100% total query cover). For Iso9, this isolate was a probable species of *Phomopsis* sp. based on similarities to *Phomopsis* sp. *STAM 55* Accession number: FJ785447.1 (with 99% similarity, 0.0 *E*-value, 100% total query cover). As for Iso22, it was identified as a probable species of *Saccharicola bicolour* with similarities to *Saccharicola bicolour isolate wb557* Accession number: AF455415.1 (99% similarity, 0.0 *E*-value, 99% query cover). To date, this is the first documentation of these endophytic species isolated from *Phragmites*.

3.2. Metal tolerance of endophytes from Phragmites

Results revealed that all 21 endophytes were able to grow in 2,000 mg L^{-1} of Pb^{2+}, but only three isolates tolerated Al^{3+}, Zn^{2+} and Cr^{3+}, and one isolate tolerated Cd^{2+}, respectively, at the same concentration (data not shown). Isolates were mostly inhibited by Cu^{2+}, with only 2 and 16 isolates showing growth in reduced Cu^{2+} concentrations of 1,000 and 500 mg L^{-1}, respectively (data not shown). Poor growth in Cu^{2+} and Cd^{2+} was expected as these metals are known to kill spores and damage DNA [14–16]. Fig. 1 captures eight of the more tolerant isolates (Iso5, 8, 9, 11, 12, 13, 22, 23) and their growths in response to metals (1,000 or 2,000 mg L^{-1}). *T. asperellum* (Iso11) was observed to tolerate all metals tested (2,000 mg L^{-1} Al^{3+}, Zn^{2+}, Pb^{2+} and Cr^{3+}) and was the least affected by high concentrations of the metals. Radial growths of Iso11 were one- to three-folds higher (mean of >4 cm) than other

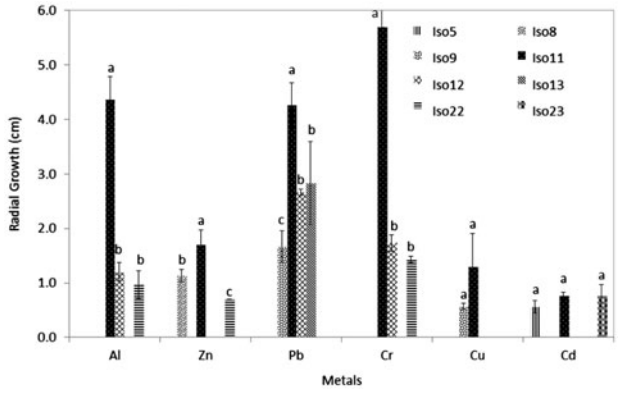

Fig. 1. The radial growth of endophytes on PDA supplemented with 2,000 mg L^{-1} of Al^{3+}, Zn^{2+}, Pb^{2+} and Cr^{3+}, and 1,000 mg L^{-1} of Cu^{2+} and Cd^{2+}. Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

isolates (mean of <3 cm) on PDA supplemented with Al^{3+}, Pb^{2+} and Cr^{3+} (Fig. 1). Similarly, isolate *S. bicolour* (Iso22) also showed tolerance to several metals (2,000 mg L^{-1} of Al^{3+}, Zn^{2+} and Cr^{3+}). The remaining isolates (Iso5, 8, 9, 13, 23) demonstrated tolerance to only one type of metal (Fig. 1). Three isolates were further selected for subsequent tests; *T. asperellum* (Iso11) and *S. bicolour* (Iso22) for their growth in all (if not most) metals, and isolate *Phomopsis* sp. (Iso9) for tolerance to Cu^{2+}. The metal tolerance characteristics of endophytic *T. asperellum* (Iso11) and *Phomopsis* sp. (Iso9) in this study correspond to the metal tolerance nature of their relevant non-endophytic species [17–22]. For *S. bicolour*, this is the first report of the species as an endophyte and its metal tolerance traits.

3.3. Adaptive tolerance behaviour to metals in selected endophytes

T. asperellum (Iso11) demonstrated excellent adaptability to increasing concentrations of Al^{3+} (tolerance index 1), Cd^{2+} (tolerance index 0.2–0.5) and Cr^{3+} (tolerance index 1), with tolerance index values not significantly different from 500–2,000 mg L^{-1} (Fig. 2). However, growth was implicated in increasing concentrations of Zn^{2+}, Pb^{2+} and Cu^{2+}. Exposure to Cu^{2+} was the most detrimental with a significantly lower tolerance index obtained upon transfer from 500 to 1,000 mg L^{-1} that is from 0.9 to 0.2. The isolate *Phomopsis* sp. (Iso9) was revealed to be the least adaptable to the increasing concentrations of metals with poor tolerance index values recorded for 500 mg L^{-1} of Al^{3+} (0.4) and Cu^{2+} (0.6), and moderate indexes for Pb^{2+} and Cr^{3+}. This isolate failed to grow in Zn^{2+} and Cd^{3+} (Fig. 3). Comparison with *Phomopsis* sp. (Iso9) revealed *S. bicolour* (Iso22) showed better adaptability with growth detected in 500–2,000 mg L^{-1} of Cr^{3+}, Al^{3+}, Zn^{2+} and Cd^{2+} (Fig. 4). *S. bicolour* (Iso22) was, however, unable to adapt to 2,000 mg L^{-1} Pb^{2+} and was severely inhibited by Cu^{2+} concentrations higher than 500 mg L^{-1} (Figs. 3 and 4).

Results here are, therefore, suggestive that endophytes from *Phragmites* are tolerant to metals (Fig. 1) and have the ability to adapt to increasing concentrations of metal (Figs. 2–4). This adaptability may be a contributing factor or a consequence of the endophyte-*Phragmites* interaction and use in wastewater and leachate treatment. Adaptability to increasing concentrations of metals that are otherwise toxic to the isolates is a useful mechanism for bioleaching and bioremediation [23]. Although the mechanisms of tolerance are not further investigated here, we postulate that metal complexation may have occurred in our isolates. Metal complexation often leads to detectable changes in pigmentation and sporulation of fungal isolates [24,25]. In this study, *Phomopsis* sp. (Iso9), *T. asperellum* (Iso11) and *S. bicolour* (Iso22) were observed to produce colonies with less dense mycelium compared to controls. Lighter pigmentation was also observed for Iso9 and 11, while darker colony pigmentation for *S. bicolour* (Iso22) was observed when cultured on PDA supplemented with Cd^{2+}. The poor mycelial growth and sporulation may be attributed to the presence of metals, most notably Zn^{2+}, Cd^{2+} and

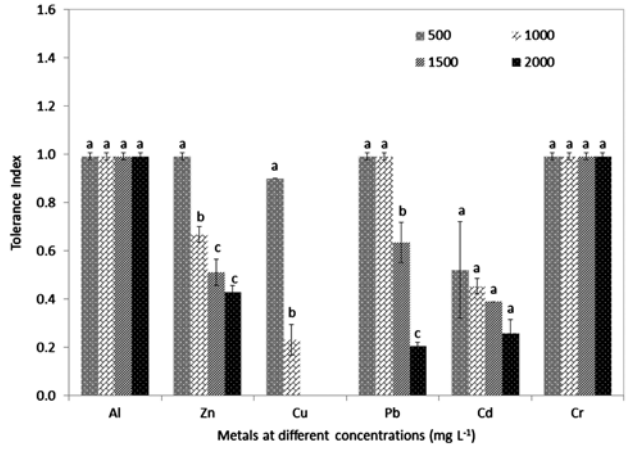

Fig. 2. Tolerance index for *T. asperellum* (Iso11) under the influence of increasing concentrations (500–2,000 mg L^{-1}) of various metals tested. Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

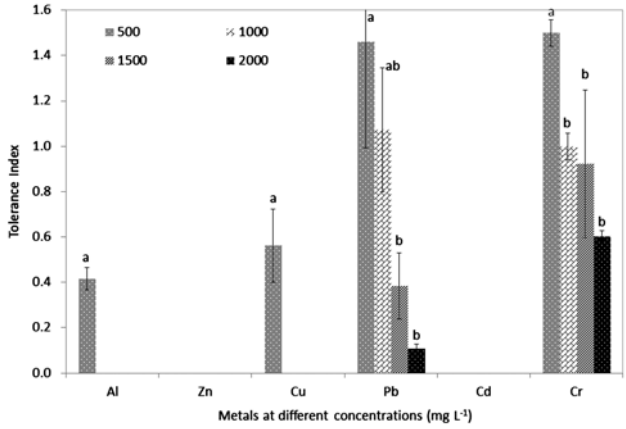

Fig. 3. Tolerance index for *Phomopsis* spp. (Iso9) under the influence of increasing concentrations (500–2,000 mg L^{-1}) of various metals tested. Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

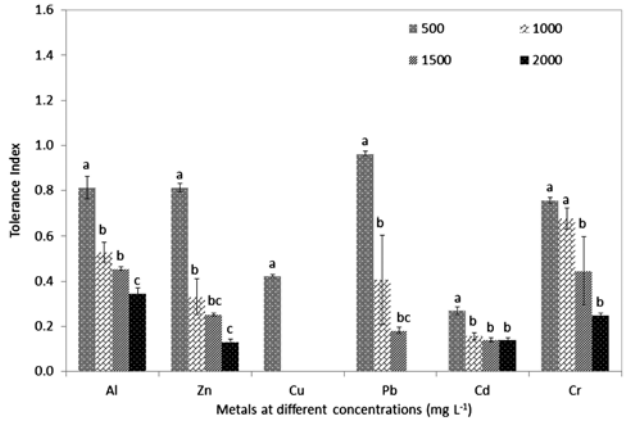

Fig. 4. Tolerance index for *S. bicolour* (Iso22) under the influence of increasing concentration (500–2,000 mg L^{-1}) of various metals tested. Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

Cu^{2+}, that reportedly cause atypical branching and septation of hyphae, thereby decreasing mycelial length [25–27].

3.4. Biosorption efficacy of selected endophytes

In single-metal solutions, the biosorption potential of *T. asperellum* (Iso11), *Phomopsis* sp. (Iso9) and *S. bicolour* (Iso22) were similar for all metals tested (Fig. 5). The fungal endophytes generally removed more Cd^{2+} with biosorption potential of 19.8, 20.1 and 19.6 mg g^{-1} for *T. asperellum* (Iso11), *Phomopsis* sp.

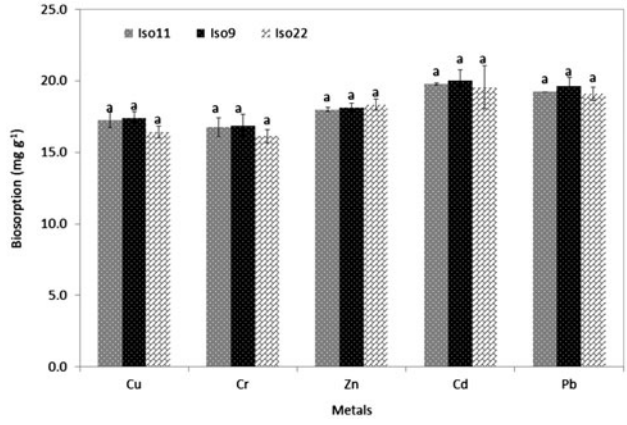

Fig. 5. Biosorption of various single metals (100 mg L^{-1}) by *T. asperellum* (Iso11), *Phomopsis* spp. (Iso9) and *S. bicolour* (Iso22). Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

(Iso9) and *S. bicolour* (Iso22), respectively. All three isolates removed less Cr^{3+}, with only 16.8, 16.9 and 16.1 mg g^{-1}, respectively (Fig. 5). The biosorption of metals was in the following order: Cd^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+} > Cr^{3+}. On the contrary, this trend was not repeated by the isolates in the multi-metal solution. Cu^{2+} appeared to be easily removed by all three isolates, followed by Cr^{3+} and Pb^{2+}. The removal of Zn^{2+} and Cd^{2+} by all three isolates was almost nil. Comparisons among isolates showed that *Phomopsis* sp. (Iso9) have higher affinity towards Cu^{2+} than towards Cr^{3+} and Pb^{2+} with 1.9, 0.1 and 0.2 mg g^{-1}, respectively (Fig. 6). *T. asperellum* (Iso11) adsorbed the most Cr^{3+} (1.6 mg g^{-1}), while *S. bicolour* (Iso22) preferred Cu^{2+}, followed by Cr^{3+} and Pb^{2+}(Fig. 6).

A quick comparison with literatures suggested that endophytes have comparable biosorption efficacy to non-endophytic isolates in removing metals. Results here recorded that *T. asperellum* (Iso11) have higher Cu^{2+}, Zn^{2+} and Cd^{2+} sorption compared to *T. asperellum* from freshwater ecosystem and sewage sludge [18] (Table 1). Comparisons with other species of *Trichoderma* revealed *T. asperellum* (Iso11) having similar Zn^{2+} and Cu^{2+} biosorption efficacy to *T. atroviride* isolated from polluted sediments [28], but were inferior to *T. longibrachium* from river samples for the removal of Pb^{2+} [29] (Table 1). For *Phomopsis* sp. (Iso9), this endophytic isolate showed higher biosorption capacity for Zn^{2+}, but were inferior for Cu^{2+}, Pb^{2+} and Cd^{2+} when compared to the non-endophytic *Phomopsis* sp. [22] (Table 1). Nevertheless, the *Phomopsis* sp. in [22] had been pre-treated with sodium hydroxide (NaOH) which may have led to more metals removed than the

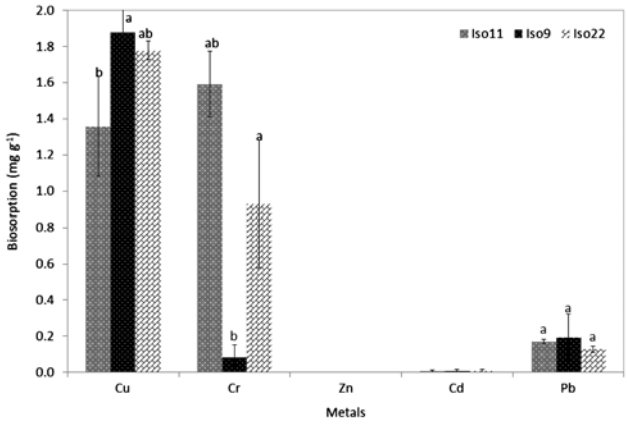

Fig. 6. Biosorption of a mixture of various metals (100 mg L^{-1} of each metal) in a solution by *T. asperellum* (Iso11), *Phomopsis* spp. (Iso9) and *S. bicolour* (Iso22). Values are mean of triplicates. Means with the same letters within metals are not significantly different ($HSD_{(0.05)}$). Error bars indicate standard deviations of means.

endophytic *Phomopsis* sp. (Iso9) used in this study. As for *S. bicolour* (Iso22), the biosorption potential of this isolate is documented here for the first time, thus comparisons could not be made. In multi-metal solutions, *S. bicolour* (Iso22) had higher preference to remove Cu^{2+} and Cr^{3+} more than Pb^{2+}, a trend similar to *T. asperellum* (Iso11) (Fig. 6). This suggested that endophytic isolates have comparable metal biosorption potential as non-endophytic isolates, attributed primarily to their cell wall composition (chitin, polysaccharides, amino and carboxyl groups with N^-, O^-, P^-, S^-, $RCOO^-$ and OH^-) which is consistent in both endophytes and non-endophytes [30,31]. It is also important to note that the endophytes in this study were isolated from *Phragmites* used in treating leachate, and not from wild *Phragmites*. It is, therefore, interesting to further pursue the two possible postulations that arise from this study. Firstly, that this inherent metal tolerance trait in endophytes may contribute to efficient metal uptake by *Phragmites*; and secondly, that the expressions of metal tolerance in endophytes were partly influenced by the host–endophyte–metal association developed through continuous exposure to metals.

The biosorption capacities of the isolates were generally lower in multi-metal solution compared to single-metal solutions. In multi-metal solution, the presence of various metal cations induces a

Table 1
Comparison of biosorption potentials of various *Trichoderma* sp. and *Phomopsis* sp. from this study (Iso11, 9) with other studies in non-competitive states (single-metal solutions) and competitive state (mixture of metal solutions)

Fungus	Types of metals	Biosorption (non-competitive) (mg g^{-1})	Biosorption (competitive) (mg g^{-1})	References
Iso11 (*Trichoderma asperellum*)	Zn^{2+}	18.0	0.0	This study
	Cu^{2+}	17.3	1.4	
	Pb^{2+}	19.2	0.2	
	Cd^{2+}	19.8	0.0	
	Cr^{3+}	16.8	1.6	
Trichoderma asperellum	Cu^{2+}	5.2	(NA)	[18]
	Pb^{2+}	20.8	(NA)	
Trichoderma atroviride	Zn^{2+}	18.1–26.7	(NA)	[28]
	Cu^{2+}	17.3	(NA)	
Trichoderma longibrachiatum	Pb^{2+}	58.8	(NA)	[29]
Trichoderma viride	Cu^{2+}	1.3	2.0	[21]
	Zn^{2+}	3.4	1.0	
	Cd^{2+}	4.7	3.5	
Iso9 (*Phomopsis* sp.)	Zn^{2+}	18.1	0.0	This study
	Cu^{2+}	17.4	1.9	
	Pb^{2+}	19.6	0.2	
	Cd^{2+}	20.1	0.0	
	Cr^{3+}	16.9	0.1	
Phomopsis spp.	Zn^{2+}	9.8	(NA)	[22]
	Cu^{2+}	24.8	(NA)	
	Pb^{2+}	180.3	(NA)	
	Cd^{2+}	25.9	(NA)	

Note: NA—not applicable.

competitive state to bind to the limited functional groups present on the fungal cell wall, resulting in possible selective accumulation of metal cations [13,21]. Hence, while endophytic isolates demonstrated good potential for metal biosorption, this may be limited to single metal biosorption and their application in multi-metal solutions requires further investigations. Further studies may include optimising biosorption processes through manipulation of pH, temperature, contact time, biomass and metal concentrations [32–34].

4. Conclusion

This study is the first to report the metal tolerance, adaptive tolerance behaviour and biosorption properties of endophytes from *Phragmites*. Of the 21 isolates, three (*T. asperellum* Iso11, *Phomopsis* sp. Iso9, *S. bicolour* Iso22) showed the most potential for use in metal removal, with efficacies comparable to non-endophytes from environmental samples. Metal tolerance and biosorption were attributed to adaptive tolerance and was most efficiently demonstrated by *T. asperellum* (Iso11), for both single- and multi-metal solutions. This study revealed the potential of using endophytes from *Phragmites* as biosorbents for metal removal.

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