Polyaromatic Hydrocarbon Degradation of Moss Endophytic Fungi Isolated from *Macromitrium* sp. in Sri Lanka

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Abstract: Polyaromatic hydrocarbons (PAHs), which are the major by-products of fossil fuel burning, are released to the environment with the immense growth of urbanization and industrialization. These pollutants are subsequently deposited on many substrates including plant surfaces. Due to their toxicity, mutagenicity, carcinogenicity and recalcitrant nature, they can result in many hazardous effects on human health. Application of endophytes in bioremediation has shown much promise in removing these PAHs from contaminated substrates. In the present study, an attempt was made to isolate and identify endophytic fungi from the moss plant *Macromitrium* sp. (frequently available) in Sapugaskanda (highly polluted) and Hettimulla (less polluted) areas in Sri Lanka. Subsequently, their potential in degrading PAHs (naphthalene and phenanthrene) was investigated. Endophytes from the moss were isolated following the surface sterilization method, and their physiological roles in degrading naphthalene and phenanthrene were carried out using plate assays, spectrophotometric and high-performance liquid chromatography (HPLC) analysis. Most of the endophytic fungi isolated from *Macromitrium* sp. were able to grow in Bacto Bushnell-Haas (BBH) medium incorporated with naphthalene and phenanthrene, separately, displaying colony diameters more than 30 mm. As per the results obtained from spectrophotometric and HPLC analysis, *Penicillium oxalicum*, *Nigrospora oryzae*, *Aspergillus oryzae*, *A. aculeatus*, *Penicillium* sp.1, *Penicillium* sp.5, *Eupenicillium* sp.2 and *Mortierella* sp.1 degraded both naphthalene and phenanthrene more than 85%. The findings of the present investigation provide some insight into how these endophytic fungi could be used for bioremediation of PAHs in environmental sites where contamination prevails, and also open avenues for future research in the relevant field.

Key words: Polyaromatic hydrocarbons, bioremediation, *Macromitrium* sp., endophytic fungi.

1. Introduction

Contamination of indoor and outdoor environment by any chemical, physical and biological agent resulting in changing the natural characteristics of the atmosphere is referred to as air pollution. In Sri Lanka, rapid air pollution is due to extensive growth of cities together with certain industries and transport systems [1]. Sapugaskanda in Sri Lanka is a residential area, which has been developed later into an industrial area where petroleum refinery, several industries and several thermal power plants are located [2].

Polyaromatic hydrocarbons (PAHs), which are prime pollutants emitted from many anthropogenic activities, are usually transported over a long distance

through air and precipitated as wet and dry depositions on soil, vegetation, sea or inland waters [3]. Phenanthrene and naphthalene are two of the most abundant PAHs in the environment [4]. Disorders, such as haemolytic anemia, lung cancer, oral squamous carcinoma, nausea, vomiting, diarrhea and blood in the urine [5] could be caused by inhaling naphthalene-contained air at the vicinity of heavy vehicle traffic, petrol stations and oil refineries [6]. Inhalation of phenanthrene in urbanized areas with high traffic conditions and industrial zones with fossil fuel combustion [4] could cause cancers in human [7].

Bioremediation is a technology that utilizes the metabolic ability of microorganisms to clean up hazardous material contaminated environment [8]. In the process of biodegradation, PAHs are bio-transformed

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through mineralization into unarmful inorganic materials, such as CO₂ and H₂O aerobically and CH₄ anaerobically [9, 10].

Endophytes are microorganisms that colonize internal parts of the plants without causing any adverse effects to the host [11]. Leaf endophytes frequently encounter problems caused by the deposition of pollutants, such as heavy metals, dust and PAHs. However, most of them take the advantage of these depositions, adapting to degrade them as their sole carbon [12] and energy source [13]. Most studies of the endophytes have been focused on vascular plants, but non-vascular plants, like mosses, also provide habitats for endophytes [14].

The genus *Macromitrium* belonging to the family Orthotrichaceae, is a large genus of up to 35 species of bryophytes (moss) that are widely distributed in tropical and sub-tropical regions of the world. In Sri Lanka, this moss is scattered in dry, intermediate and wet zones as dense green clumps or mats, often in damp or shady locations. As mosses lack a root system and a proper vascular system, but contain rhizoids, they are able to absorb heavy metals and PAHs in significant levels and accumulate within them [10, 11, 15, 16]. Apart from that, mosses have been used for indirect assessment of atmospheric air pollution. One such example is *Hylocomium splendens* being used in a road tunnel experiment in Vienna, Austria to analyze emissions from road traffic [17].

Due to these reasons, it was speculated that endophytic fungi within the moss *Macromitrium* sp. might have a satisfactory ability to degrade PAHs. Literature revealed no publication on moss endophytes and their potential in degrading PAHs. Therefore, the present investigation was carried out in an attempt to isolate and identify the endophytic fungi of the moss *Macromitrium* sp. in an urbanized and industrialized area (Sapugaskanda), Sri Lanka, where petroleum or PAH contaminations occur in significant levels. Their ability in degrading phenanthrene and naphthalene was also investigated, hypothesizing most of the

isolated moss endophytic fungi have the potential in degrading phenanthrene and naphthalene at significant levels.

2. Materials and Methods

2.1 Sampling Sites

Moss samples were randomly collected from Sapugaskanda area (highly polluted, urbanized and industrialized) in the Colombo district, Western province, as well as Hettimulla (rural area), in the Kegalle district, in the Sabaragamuwa province (less polluted), Sri Lanka, which was selected as the control site.

2.2 Sample Collection

Healthy *Macromitrium* sp. grown on the walls, bricks, stones and barks of the trees at road sides, were randomly collected from five replicative sites, which were close to the oil refinery center at Sapugaskanda area. The same moss species grown in the rural area at Hettimulla were also collected randomly in five replications from the five sub-sites. The samples from both areas were placed in clean polythene bags and transported to the laboratory at the University of Kelaniya and stored at 4 °C until used.

2.3 Microscopic Observations of Fungal Endophytes

Moss plants were washed thoroughly with tap water. To assess the presence of fungal endophytes in the moss plants, small fragments from the plants were mounted with a drop of cotton blue stain in lacto phenol and observed under a phase contrast microscope (CX46 Olympus trinocular research microscope with digital camera DP26) under high and mid powers (Fig. 1).

2.4 Percentage Frequency of Occurrence of Moss Endophytes

Percentage frequency of occurrence of moss endophytes was assessed to understand the distribution of fungal endophytes in the moss plant. The experiment

Fig. 1 Presence of endophytic fungal mycelia in mesophilic tissues of *Macromitrium* sp..

was carried out as indicated in Ref. [18]. Surface sterilized plants were cut into small pieces (2 mm × 2 mm), and 40 randomly selected moss pieces were plated on 2% malt extract agar (MEA) and incubated for 7 d at room temperature (27 ± 2 °C). Each fungal species that grew from each moss piece was isolated into pure cultures, and percentage frequency of occurrence of each of the endophytic fungus was then calculated using the following Eq. (1) [18-20]:

$$\text{Frequency of occurrence for each fungus (\%)} = \frac{\text{number of pieces colonized by the fungus}}{\text{total number of pieces plated}} \times 100 \quad (1)$$

2.5 Isolation and Identification of the Fungal Endophytes from the Moss *Macromitrium* sp.

Prior to the isolation of the fungal endophytes, the moss *Macromitrium* sp. was surface sterilized as described in Refs. [18, 21]. Moss plants were first washed with running tap water for 10 min. Then, they were immersed in 70% ethanol for 1 min, 0.025% aqueous sodium hypochlorite for 15 min and rinsed with 70% ethanol for 1 min. Moss plants were subsequently washed with double distilled water for

four serial washings for 5 min. The surface sterilized plants were dried under aseptic conditions. Moss plants were then cut into 2 mm × 2 mm pieces aseptically. Subsequently, randomly selected 40 moss pieces from each replicative sample were chosen and plated on 2% MEA medium incorporated with tetracycline (500 mg/L). The Petri dishes were then incubated at room temperature (27 ± 2 °C) for two weeks and checked frequently for developing endophytic fungal colonies. Fungi growing from each moss piece were then sub-cultured into water agar to prepare pure cultures.

Morphological characteristics of the isolated fungi were observed as indicated by Domsch et al. [22]. Microscopic characteristics, such as nature of hyphae, sporulating structures and spores, were observed under low, mid and high powers of a phase contrast microscope following sticky tape method [23]. Using the data obtained, isolated fungi were identified into genus level following online tools and identification keys [22]. Some of them were identified into species level by comparing them with the available identified fungal cultures [24] at the Department of Botany, University of Kelaniya.

2.6 Screening of PAHs Utilization Ability of the Endophytic Fungal Strains

Plate assays to determine endophytic fungal growth in the presence of PAHs were carried out as indicated by Undugoda et al. [24]. Bacto Bushnell-Haas medium (BBH: MgSO₄ 0.2 g/L, CaCl₂ 0.02 g/L, KH₂PO₄ 1.00 g/L, K₂HPO₄ 1.00 g/L, NH₄NO₃ 1.00 g/L, FeCl₃ 0.05 g/L) was prepared adding either phenanthrene or naphthalene (100 ppm), separately. Fungal disks of 10 mm diameter were obtained from pure colonies of fungal endophytes grown in 2% MEA medium using a sterilized cork borer. They were inoculated into the plates containing BBH medium incorporated with naphthalene (100 ppm) and phenanthrene (100 ppm), separately. Plates with fungal disks were then incubated for 10 d at room temperature (27 ± 2 °C), and the diameter of each of the fungal colonies was measured throughout the incubation period. At the same time, control plates were also prepared without incorporating PAHs into the BBH medium. This experiment was carried out with six replicates of the fungal isolates from both Sapugaskanda area and Hettimulla area, separately.

2.7 Screening of PAH Degradation Ability of the Endophytic Fungal Strains

As mentioned in Ref. [24], BBH broth was prepared with phenanthrene (100 ppm) and naphthalene (100 ppm), separately. The broth (25 mL) was poured into 100 mL Erlenmeyer flasks, and all the flasks were autoclaved. Autoclaved methylene blue (25 µL) was then added to each flask as an indicator, and fungal disks (10 mm) obtained from pure colonies of moss endophytes were introduced to the BBH broth in the flasks following six replications for each fungal endophyte. They were incubated at room temperature (27 ± 2 °C) with constant shaking at 180 revolutions/min for 10 d. At the same time, a control was prepared without inoculating any moss endophyte. Subsequently, the aliquots (4 mL) were checked frequently for any color change from deep

blue to colorless. After the incubation period, all the samples were filtered through filter paper, and the absorbance was measured by UV-Vis spectrophotometer at 609 nm wavelength. Percentage of biodegradation of naphthalene as well as phenanthrene was calculated using the following Eq. (2) for each moss endophyte [24, 25]:

$$\text{Biodegradation \%} = \left(1 - \frac{\text{absorbance of treated sample}}{\text{absorbance of the control}}\right) \times 100 \quad (2)$$

2.8 Analysis of PAH Degradation Ability of the Endophytic Fungal Strains

PAH degradation ability of isolated fungal strains was quantitatively analyzed using high-performance liquid chromatography (HPLC) method. Two agar plugs (1 cm² each) of a pure growth of each isolate, were inoculated into BBH broth (50 mL/250 mL Erlenmeyer flask) with sterile PAH (100 ppm). After 10 d incubation period at room temperature, the residual phenanthrene and naphthalene in the broth were extracted using acetone and hexane solvents and methanol solvent, respectively. Cleanup procedure was carried out using a chromatographic column (250 mm long, 4.6 mm diameter), which was packed with porous spherical C18 material (packed size, 5 µm). The extracts were analyzed by Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 1200 diode array detector. Then, 20 µL of each sample was injected into a Zorbax Eclipse Plus C18 column (Agilent Technologies, USA; 4.6 mm × 100 mm × 3.5 µm particle size). Acetonitrile-water mixture (75:25) was used as a mobile phase for PAHs at a flow rate of 1.0 mL/min. A standard curve of each PAH compound was plotted using the following concentrations 1, 10, 20, 40, 60, 80 and 100 ppm, respectively, to determine retention time and to study linearity of the detector. Then, PAH concentrations in the samples were calculated against the standard curves. Percentage of PAH degradation

was calculated by the following Eq. (3):

$$\text{PAH degradation\%} = 100 \times \frac{M_i - M_s}{M_i} \quad (3)$$

where, M_s is the concentration of PAH in each treatment and M_i is the initial PAH concentration present in the sample [24].

2.9 Statistical Analysis of Data

Colony diameters of endophytic fungi on BBH medium incorporated with PAHs and percentage degradation of PAHs by each fungus obtained from spectrophotometric (colorimetric) and HPLC analysis were subjected to one-way ANOVA, and the mean values were then compared using Tukey's pairwise comparison test. Statistical analysis was carried out using the Minitab 16 statistical package. The level of $P < 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1 Isolation of Endophytic Fungi

As indicated in Table 1, 54 fungal endophytes were isolated and identified from the moss *Macromitrium* sp. collected from Sapugaskanda (urbanized and industrialized) and Hettimulla (less polluted) areas. Among them, 35 were from Sapugaskanda and 25 were from Hettimulla. All isolates of Sapugaskanda belonged to 13 genera, such as *Penicillium*, *Aspergillus*,

Table 1 Frequency of occurrence of the moss (*Macromitrium* sp.) endophytic fungi isolated from Sapugaskanda and Hettimulla areas.

Endophytic fungus	Frequency of occurrence (%)	
	Sapugaskanda	Hettimulla
<i>Eupenicillium</i> sp.1	95.0 ± 2.00	20.0 ± 0.40
White sterile sp.7	80.0 ± 2.61	32.5 ± 0.21
<i>Nigrospora oryzae</i>	42.5 ± 0.75	-
<i>Eupenicillium</i> sp.2	32.5 ± 0.81	17.5 ± 0.38
White sterile sp.13	30.0 ± 0.25	-
<i>Acremonium</i> sp.2	25.0 ± 0.63	-
White sterile sp.5	25.0 ± 0.45	-
White sterile sp.1	22.5 ± 0.10	-
<i>Botryotrichum</i> sp.1	22.5 ± 0.58	-
<i>Aspergillus aculeatus</i>	22.5 ± 0.58	7.5 ± 0.30
<i>Aspergillus oryzae</i>	22.5 ± 0.49	-

(Table 1 continued)

Endophytic fungus	Frequency of occurrence (%)	
	Sapugaskanda	Hettimulla
<i>Rhizopus</i> sp.1	17.5 ± 0.51	7.5 ± 0.75
<i>Penicillium</i> sp.1	17.5 ± 0.40	-
White sterile sp.2	17.5 ± 0.25	-
<i>Penicillium oxalicum</i>	15.0 ± 0.58	-
<i>Aureobasidium</i> sp.2	15.0 ± 0.20	-
White sterile sp.6	12.5 ± 0.32	-
<i>Penicillium</i> sp.4	10.0 ± 0.38	2.5 ± 0.32
White sterile sp.9	10.0 ± 0.20	-
<i>Phoma</i> sp.1	7.7 ± 0.40	-
<i>Acremonium</i> sp.1	7.5 ± 0.25	-
<i>Cladosporium</i> sp.1	7.5 ± 0.25	-
White sterile sp.8	7.5 ± 0.25	-
<i>Humicola</i> sp.1	7.5 ± 0.25	-
White sterile sp.10	7.5 ± 0.25	-
<i>Gilmaniella</i> sp.1	5.0 ± 0.25	-
<i>Acremonium</i> sp.4	5.0 ± 0.25	-
<i>Aureobasidium</i> sp.1	5.0 ± 0.25	-
White sterile sp.4	5.0 ± 0.25	-
<i>Mortierella</i> sp.1	5.0 ± 0.25	-
<i>Penicillium</i> sp.3	5.0 ± 0.25	-
Grey sterile sp.1	5.0 ± 0.25	-
White sterile sp.12	5.0 ± 0.20	-
White sterile sp.3	2.5 ± 0.20	-
White sterile sp.11	2.5 ± 0.40	-
Grey sterile sp.3	-	32.5 ± 0.25
<i>Penicillium</i> sp.6	-	20.0 ± 0.25
<i>Penicillium</i> sp.5	-	17.5 ± 0.49
White sterile sp.17	-	17.5 ± 0.20
<i>Eupenicillium</i> sp.3	-	15.5 ± 0.23
<i>Aspergillus</i> sp.5	-	15.0 ± 0.45
White sterile sp.16	-	12.5 ± 0.75
White sterile sp.19	-	12.5 ± 0.25
<i>Aspergillus</i> sp.3	-	10.0 ± 0.25
<i>Aspergillus</i> sp.4	-	10.0 ± 0.25
<i>Penicillium</i> sp.7	-	7.5 ± 0.40
White sterile sp.18	-	7.5 ± 0.25
Dark sterile sp.1	-	5.0 ± 0.51
White sterile sp.14	-	5.0 ± 0.40
<i>Acremonium</i> sp.3	-	5.0 ± 0.40
Grey sterile sp.2	-	5.0 ± 0.40
<i>Chaetomium</i> sp.1	-	5.0 ± 0.20
White sterile sp.15	-	2.5 ± 0.00
Yellow and white sterile sp.1	-	2.5 ± 0.25

Values represent the mean frequency of occurrence of six samples ± standard deviation. - indicates the absence of the particular isolate.

Eupenicillium, *Acremonium*, *Nigrosporum*, *Aureobasidium*, *Humicola*, *Cladosporium*, *Rhizopus*, *Phoma*, *Mortierella*, *Gilmaniella*, *Botrytrichum* and unidentified white and grey sterile species. Under Hettimulla, five genera—*Penicillium*, *Aspergillus*, *Eupenicillium*, *Chaetomium*, *Acremonium* and unidentified white, dark and grey sterile species were recorded. As per the investigation carried out by Undugoda et al. [24], from the phyllosphere of ornamental plants of *Ixora chinensis*, *Ervatamia divartica*, *Hibiscus rosa-sinensis* and *Amaranthus cruentus*, fungi such as *A. oryzae*, *A. aculeatus*, *Penicillium oxalicum* and *Nigrospora oryzae* were isolated at higher frequencies from the same site. This reflects the presence of these fungi as endophytes as well as phyllosphere organisms in highly polluted areas especially in Sapugaskanda area where PAH contaminations prevail, suggesting their ability to survive in various plant parts in the presence of many pollutants including PAHs. As highlighted by Undugoda et al. [24], these fungal species could be one of the potential biodegraders of PAHs, which are present in significantly higher levels in ambient air in Sapugaskanda area.

3.2 PAH Utilization Ability of Endophytic Fungal Strains

All endophytic fungi tested were able to grow in BBH medium incorporated with PAHs separately. According to the colony diameter given in Table 2, they were categorized as high utilizers (> 30 mm), moderate utilizers (20-30 mm) and poor utilizers (< 20 mm). Among all the endophytic fungi isolated from Sapugaskanda, *N. oryzae* and *Penicillium* sp.5 had the highest colony diameter in naphthalene (85.2 mm, 80.3 mm) and phenanthrene (59.5 mm, 40.3 mm) incorporated medium, respectively. This suggests that these fungal strains either convert PAHs into non-toxic form to be used as a sole carbon source or absorb PAHs into fungal cells [24]. In almost all isolates, their growth on naphthalene was significantly

higher than that of phenanthrene. However, all isolates collected from Hettimulla had colony diameters lower than 20 mm (Table 3), implying that utilization of PAHs for their survival was poor and some strains showed no growth on PAH incorporated medium. Undugoda et al. [24] demonstrated that except 14

Table 2 Diameter of fungal colonies in PAH containing media from Sapugaskanda.

Name of the fungus	Diameter of fungal colonies (mm)	
	Naphthalene medium	Phenanthrene medium
<i>Nigrospora oryzae</i>	85.2 ± 5.34	59.5 ± 6.66
<i>Penicillium</i> sp.5	80.3 ± 8.09	40.3 ± 2.50
<i>Aspergillus aculeatus</i>	42.3 ± 3.01	27.7 ± 2.94
<i>Mortierella</i> sp.1	41.8 ± 1.60	40.0 ± 1.67
<i>Acremonium</i> sp.2	39.0 ± 1.26	19.8 ± 4.53
White sterile sp.1	36.0 ± 1.78	25.3 ± 4.17
<i>Eupenicillium</i> sp.2	35.5 ± 3.98	20.8 ± 2.22
<i>Penicillium oxalicum</i>	35.2 ± 4.30	21.5 ± 2.94
<i>Penicillium</i> sp.1	35.0 ± 7.15	27.0 ± 1.26
<i>Aspergillus oryzae</i>	33.3 ± 8.80	26.2 ± 6.70
White sterile sp.9	33.3 ± 3.07	17.5 ± 1.64
<i>Acremonium</i> sp.1	31.7 ± 2.48	15.0 ± 2.44
White sterile sp.6	29.2 ± 2.31	12.8 ± 0.75
White sterile sp.13	27.2 ± 1.16	12.7 ± 1.03
<i>Eupenicillium</i> sp.1	27.0 ± 1.09	19.0 ± 0.89
<i>Auerobasidium</i> sp.2	25.0 ± 4.27	16.2 ± 1.83
<i>Penicillium</i> sp.3	24.7 ± 4.71	19.7 ± 3.07
White sterile sp.2	23.5 ± 3.27	20.0 ± 1.63
White sterile sp.12	22.7 ± 9.54	12.7 ± 1.03
<i>Cladosporium</i> sp.1	19.3 ± 1.03	13.3 ± 1.03
<i>Gilmaniella</i> sp.1	18.2 ± 1.94	15.0 ± 4.28
Grey sterile sp.1	18.2 ± 2.56	13.0 ± 1.26
<i>Phoma</i> sp.1	17.7 ± 5.75	14.8 ± 4.87
<i>Acremonium</i> sp.3	17.2 ± 2.78	14.0 ± 2.44
<i>Penicillium</i> sp.4	17.0 ± 2.75	13.5 ± 1.37
White sterile sp.4	15.0 ± 2.42	14.5 ± 2.25
White sterile sp.7	15.0 ± 1.09	13.2 ± 1.47
White sterile sp.10	14.8 ± 0.98	13.2 ± 0.75
White sterile sp.3	14.7 ± 2.42	14.2 ± 0.16
<i>Rhizopus</i> sp.1	14.2 ± 1.32	12.3 ± 0.51
<i>Auerobasidium</i> sp.1	14.0 ± 1.26	12.2 ± 0.75
<i>Botrytrichum</i> sp.1	13.3 ± 2.06	13.2 ± 1.47
<i>Humicola</i> sp.1	13.0 ± 1.41	11.3 ± 0.51
White sterile sp.5	12.6 ± 1.03	11.0 ± 0.63
White sterile sp.8	12.3 ± 0.51	11.3 ± 1.21

Values represent the mean of six replicates ± standard deviation.

Table 3 Diameter of fungal colonies in PAH containing media from Hettimulla.

Name of the fungus	Diameter of fungal colonies (mm)	
	Naphthalene medium	Phenanthrene medium
White sterile sp.19	20.3 ± 1.03	12.8 ± 0.75
<i>Acremoiium</i> sp.3	20.2 ± 1.16	14.2 ± 0.98
White sterile sp.18	19.2 ± 1.32	12.7 ± 1.03
<i>Chaetomium</i> sp.1	19.2 ± 0.98	12.5 ± 1.04
<i>Penicillium</i> sp.7	19.2 ± 1.32	13.0 ± 0.63
Dark sterile sp.1	19.2 ± 2.56	15.7 ± 0.83
Grey sterile sp.3	14.0 ± 1.54	10.8 ± 0.75
White sterile sp.15	13.5 ± 3.20	10.3 ± 0.51
<i>Aspergillus</i> sp.3	13.2 ± 1.83	10.1 ± 0.40
<i>Rhizopus</i> sp.1	12.3 ± 2.50	10.0 ± 0.40
<i>Aspergillus</i> sp.4	12.2 ± 0.75	12.2 ± 0.83
White sterile sp.14	12.0 ± 1.03	11.1 ± 0.98
<i>Eupenicillium</i> sp.1	11.8 ± 0.75	10.3 ± 0.51
White sterile sp.17	11.7 ± 0.51	10.5 ± 0.54
<i>Aspergillus</i> sp.5	11.7 ± 0.81	10.0 ± 0.00
<i>Eupenicillium</i> sp.3	11.3 ± 0.83	10.2 ± 0.51
<i>Penicillium</i> sp.6	11.3 ± 0.51	10.8 ± 0.98
White sterile sp.16	11.2 ± 0.75	10.0 ± 0.00
<i>Eupenicillium</i> sp.2	11.0 ± 0.63	10.0 ± 0.00
<i>Aspergillus aculeatus</i>	11.0 ± 0.63	10.0 ± 0.00
<i>Penicillium</i> sp.4	11.0 ± 0.63	10.0 ± 0.00
Grey sterile sp.2	10.5 ± 0.54	10.0 ± 0.00
Yellow and white sterile sp.1	10.2 ± 0.40	10.0 ± 0.00
White sterile sp.7	10.0 ± 0.00	10.0 ± 0.00

Values represent the mean of six replicates ± standard deviation.

phyllosphere isolates, all others exhibited better growth in aromatic hydrocarbon incorporated medium with colony diameters at *Penicillium oxalicum* (34.66 mm, 26.22 mm), *Nigrospora oryzae* (28.73 mm, 24.81 mm), *A. oryzae* (26.58 mm, 18.42 mm), *A. aculeatus* (25.38 mm, 21.63 mm), *Aspergillus* sp.1 (23.81 mm, 17.74 mm) and *Trichoderma* sp.2 (21.69 mm, 19.53 mm) in naphthalene and phenanthrene incorporated media, respectively.

3.3 PAH Degradation Ability of Endophytic Fungal Strains

To determine the percentage degradation of PAHs by the endophytic fungal strains, spectrophotometric analysis was performed. As mentioned in the method

section in the experiment, a specially defined BBH broth with methylene blue was used as a color indicator. PAH degradation of inoculated fungal strains resulted in changing the color of methylene blue into colorless due to a redox reaction, indicating oxidation of PAHs [24, 25].

As shown in Fig. 2, all isolates isolated from Sapugaskanda were able to degrade PAHs at significantly different levels ($P < 0.05$). Out of 35 isolates, 12 species degraded PAHs more than 90% after 10 d incubation period. Almost all the endophytic fungi degraded PAHs more than 28% and majority of isolates demonstrated degradation of > 40%. Out of all fungal species tested, *Penicillium oxalicum* performed the highest degradation (98.6%) for naphthalene, while *Nigrospora oryzae* displayed the best phenanthrene degradation. Majority of the strains isolated from Hettimulla showed relatively poor degradation except one dark sterile species, highlighting that the fungal strains at less polluted area had lesser ability in degrading PAHs (Fig. 3). It may be due to the reason that they are not much exposed to PAH contaminants as with the ones isolated from Sapugaskanda. This is proved by Frenandez-Luqueno et al. [26], who emphasized that gene expression levels of microorganisms that code for degradative enzymes of PAHs, increase after exposure to hydrocarbon pollution. Those genes are active during adaptation to the new PAH-rich environment, increasing degradation of PAHs.

Current data are also supported by the findings of many of the previous research. *A. versicolor*, *A. niger* and *A. flavus* isolated from petroleum contaminated soil had the highest onset and fastest extent in biodegradation, more than 99% for phenanthrene and several other PAHs [25]. *Penicillium janthinellum* isolated from separate creosote and manufactured gas plant-contaminated soil degraded PAHs, like pyrene, chrysene and benzo(a)pyrene, into polar metabolites [27]. *Penicillium frequentans* and *A. niger* grown on sugarcane bagasse and added to a soil, with 400 ppm phenanthrene, achieved 54% removal of the pollutant

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Fig. 2 Percentage of PAH degradation by moss endophytic fungi from Sapugaskanda, determined by spectrophotometric method.
Each value represents mean of six replicates $\pm$ standard error.

Fig. 3 Percentage of PAH degradation by moss endophytic fungi isolated from Hettimulla, determined by spectrophotometric method.
Each value represents mean of six replicates $\pm$ standard error.

from the soil [28]. *Penicillium oxalicum*, isolated from different ornamental plants of urbanized areas in Sri Lanka was able to degrade PAHs, such as naphthalene and phenanthrene at significantly higher rates [24]. Although there are no reports on use of moss endophytic fungi to degrade PAHs, Dai et al. [13] reported that the endophytic fungus *Cerbasidium stevensii* (strain B6) isolated from *Bischofia polycarpam* was successful in degrading phenanthrene (89.51%).

According to HPLC results indicated in Figs. 4 and 5, naphthalene and phenanthrene degradation ability of endophytic fungi in Sapugaskanda has a noticeable difference than that in Hettimulla. As shown by colorimetric results, the HPLC data also reveal that out of 35 isolates, 12 species degraded PAHs more than 90%. *Penicillium oxalicum* showed the highest degradation of both naphthalene (96.1%) and phenanthrene (94.25%). All endophytic isolates from moss *Macromitrium* from Hettimulla except dark sterile species were significantly less efficient in degrading both naphthalene and phenanthrene.

In the present study, a positive correlation was evident between biodegradation and colony diameter as highlighted by Undugoda et al. [24]. The best biodegraders had significantly higher colony diameters in contrast to the relatively poor degraders with lower colony diameters in the PAH incorporated media. All the endophytes efficient in utilization of PAHs (> 30 mm diameter) showed more than 80% naphthalene degradation. However, phenanthrene degradation levels were low and diverse to some extent but not significant as supported by Kafilzadeh and Pour [29], who described that increased number of rings in PAHs could decline the rate of degradation. Moreover, microbial degradation of PAHs could be attributed to metabolism of PAHs by different array of enzymes, like lignin peroxidase, manganese peroxidase, laccase, cytochrome P450 and epoxide hydrolase [30].

3.4 Frequency of Occurrence

As indicated in Table 1, *Eupenicillium* sp.1 had the highest frequency of occurrence (95%) in Sapugaskanda. Other fungi, such as white sterile sp.7,

Fig. 4 Percentage of PAH degradation by moss endophytic fungi from Sapugaskanda, determined by HPLC method. Each value represents mean of six replicates $\pm$ standard error.

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Isolated from *Macromitrium* sp. in Sri Lanka**

Fig. 5 Percentage of PAH degradation by moss endophytic fungi isolated from Hettimulla, determined by HPLC method. Each value represents mean of six replicates $\pm$ standard error.

Nigrospora oryzae, *Eupenicillium* sp.2, white sterile sp.13, *Acremonium* sp.2 and white sterile sp.6 had next high frequencies of occurrence, such as 80%, 42.5%, 32.5%, 30%, 25% and 25%, respectively. According to Undugoda et al. [24], endophytic fungi *A. oryzae*, *A. aculeatus* and *Penicillium oxalicum* were found to be common and *Nigrospora oryzae* was occasional in Sapugaskanda. However, in agreement with Undugoda et al. [24], it was noteworthy that endophytic fungi found with higher frequency of occurrence from Sapugaskanda were much more efficient in degrading PAHs compared to that of Hettimulla area. White sterile sp.7 was dominantly found in Sapugaskanda with 80% frequency of occurrence and showed over 90% degradation of naphthalene and over 80% degradation of phenanthrene. The same species isolated from *Macromitrium* sp. from Hettimulla with 32.5% frequency of occurrence showed very low (4%) degradation of naphthalene and phenanthrene. This observation was common to *Eupenicillium* sp. isolated from both polluted and less polluted areas. This

suggests that continuous exposure to specific environmental conditions makes the fungal consortium more adaptive to a particular environment [13]. Early records revealed that there was higher accumulation of PAHs inside the moss *Hyophila involuta* [31] and surface of ornamental plants (*Ixora chinensis*, *Ervatamia divartica*, *Hibiscus rosa-sinensis* and *Amaranthus cruentus*) in Sapugaskanda area [24].

4. Conclusions

As per findings of the present investigation, it could be concluded that *Penicillium oxalicum*, *Penicillium* sp.5, *Penicillium* sp.3, *A. oryzae*, *A. aculeatus*, *Nigrospora oryzae*, *Mortierella* sp.1, *Aureobasidium* sp.2, *Eupenicillium* sp.2 and white sterile sp.7 are the best PAHs degraders, and they dominate inside the moss *Macromitrium* sp. as endophytic fungi, especially in highly polluted areas in Sri Lanka. Their efficiency in degrading PAHs would be useful in optimization of new bioremediation processes to remove PAHs and other pollutants in different environmental sites.

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