DIVERSITY AND SEASONAL VARIATION OF ENDOPHYTIC FUNGI ASSOCIATED WITH BARK, STEM AND LEAF OF AGAR PLANT (*Aquilaria malaccensis* Lam.)

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Abstract

The diversity of endophytic fungi was isolated from the bark, stem and leaf of agar plant in monsoon and winter seasons at different locations of Dhaka and Habiganj districts. A total of 25 fungal isolates were found in monsoon and 22 in winter season. The highest diversity value was found in the bark of location 2 in monsoon according to Shannon diversity index and in the stem of location 1 in winter according to Simpson diversity index. The lowest value was found in the leaf and stem of location 1 and 4, respectively. The evenness of endophytic fungi was highest in the stem of location 1 and lowest in leaf in monsoon based on Shannon evenness index. The maximum species richness was recorded in the bark of location 2 (Margalef's index) and in the stem of location 1 in monsoon (Menhinick's index), and the minimum value recorded in the bark of location 4 in monsoon season.

Key words: Endophytic fungi; Monsoon; Winter; Species diversity; Species richness.

INTRODUCTION

Any organism that develops inside plant tissues for their entire lives or for a specific period of time during their life cycles is an endophyte, but they are now more specifically classified according to their types and relationships (Petrini *et al.* 1991, Cabral *et al.* 1993, Hallmann *et al.* 2011, Rosenblueth and Martínez-Romero 2006). Endophytes are a significant source of bioactive secondary metabolites, such as antibiotics, alkaloids, enzymes, and other compounds that enable them to flourish in unfavorable environments (Li *et al.* 2000, Strobel 2002). They are known to promote host's growth and nutrient uptake as well as increase the capacity to withstand different abiotic and biotic stresses. Numerous studies on the diversity and isolation of endophytic fungi have shown that these organisms play a significant role in biodiversity and have a quantitative impact on the diversity and composition of plant communities (Porras-Alfaro and Bayman 2011, Potshangbam *et al.* 2017).

Aquilaria malaccensis Lam. is a tropical tree belonging to the family Thymelaeaceae. It is commonly known as agarwood (aguru in Bengali), sandalwood, eaglewood, aloeswood, and gaharuwood (Bouverie 1885) for producing dark, fragrant and resinous heartwood. The aromatic agarwood production is a result of the plant's natural defense mechanism against damage caused by wounds and infection of specific pathogens (Lukman *et al.* 2023). Many virulent pathogenic fungi, which are primarily endophytic in nature, are responsible for pathogenic infections on various areas of the agar tree that gives rise to the scent of the agarwood (Hartono *et al.* 2019). A large number of bio-active secondary metabolites have been isolated and characterized from endophytic fungi since the discovery of endophytes (Strobel *et al.* 2004). The secondary metabolites isolated from endophytic fungi of agar plant have high potential in perfumes, incense, soap and shampoo industries (Islam and Chowdhary 2017). They are also used in traditional medicine in East Asia and the creations of Chinese medicine as well as pharmaceutical tinctures (Barden *et al.* 2000).

Due to the high commercial value of agar plant products, researchers from different countries have worked on various parts of agar plant in quest of isolating and identifying these diverse group

of endophytic fungi (Hartono *et al.* 2019), their biosynthetic activities (Du *et al.* 2022), chemical compounds isolated from endophytic fungi of agarwood (Zhang *et al.* 2022), fungal diversity (Mohamed *et al.* 2010, Chhipa and Kaushik 2017), antimicrobial properties (Mochahari *et al.* 2020), antioxidant and antifungal activity of endophytic fungi (Hidayat *et al.* 2019). From the literature regarding agar plant it is found that there is no report published on taxonomy of the endophytic fungi of the agar plant in Bangladesh. For this reason it was decided to isolate the endophytic fungi associated with different parts of agar plant at different locations of Dhaka and Habiganj, and report their diversity and seasonal variation. The systemic research will provide well documented information for future work.

MATERIAL AND METHODS

Plant materials

Mature and healthy leaf, stem and bark samples of agar plant were collected from four different locations, *i.e.* location 1 (Jagadishpur tea estate, Madhabpur, Habiganj), location 2 (Chundeechara tea estate, Chunarughat, Habiganj), location 3 (Botanical Garden, Curzon Hall Campus, University of Dhaka) and location 4 (Botanical Garden, Mirpur, Dhaka). The collected samples were placed in sterile polythene bags and were preserved in sterile brown paper bags and kept at 4°C in refrigerator.

Isolation of endophytic fungi

The endophytic fungi were isolated from healthy bark, stem and leaf tissues of agar plant. The conserved agar plant parts were cleaned under running water to remove the dust and debris. The plant parts were cut into tiny pieces of 5 mm² by 5 mm² with the help of sterilized scissors and immersed in a 2 - 4% aqueous Clorox solution for 1 to 1.5 minutes. Then the samples were rinsed out with sterile distilled water and surface dried on sterilized filter papers inside petri plates under aseptic condition. The surface sterilized plant segments were then placed on sterilized petri plates containing PDA medium. After inoculation, the Petri plates were incubated at a temperature of 28°C in an incubation chamber. Five days later, these plates were used to obtain pure cultures. A total of 27 inocula (with replications R1, R2, and R3) were used for each sample (9 petri plates for each part). Four isolations were done for bark, stem and leaf for each location during monsoon and winter season.

Morphological identification of fungi

Following standard literatures, morphological investigations of the isolated fungi were conducted to identify the fungi mostly based on the colony characteristics and microscopic investigation (Thom and Raper 1945, Booth 1971, Ellis 1971, 1976, Barnett and Hunter 1972, Benoit and Mathur 1970).

Data analysis and diversity indices

The relative percentage frequency of endophytic fungi was calculated following the standard formula of Lv *et al.* (2010). Species diversity, species evenness and species richness were estimated following Ludwick and Reynolds (1998).

RESULTS AND DISCUSSION

A total of twenty six fungal isolates *i.e.*, *Alternaria alternata*, *A. palandui*, *A. tenuissima*, *Aspergillus flavus* type 1, *A. flavus* type 2, *A. niger* type 1, *A. niger* type 2, *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Curvularia lunata*, *Diaporthe hongkongensis*, *D. perseae*, *Fusarium*

sporotrichioides, *Harknessia* sp., *Lasiodiplodia theobromae*, *L. pseudotheobromae*, *Penicillum digitatum*, *P. commune*, *P. italicum*, *Penicillium* sp. 1, *Penicillium* sp. 2, *Penicillium* sp. 3, *Penicillium* sp. 4, *Eupenicillium* sp. 1, *Eupenicillium* sp. 2 and *Sphaeropsis* sp. were isolated from different parts of agar plant (Fig. 1).

Determination of percentage frequency of endophytic fungi

From Habiganj district, maximum frequency was found in location 2, in *Lasiodiplodia pseudotheobromae* from stem in winter (42.5%) and minimum in location 1, in *Alternaria alternata*, *Aspergillus niger* type 1 and *A. niger* type 2 from bark in monsoon (1.47%). Maximum number of endophytic fungi was isolated from bark of Habiganj in monsoon season (Table 1).

Table 1. Percent frequency of endophytic fungi associated with agar plants of tea estates of Habiganj district in different seasons.

Locations	Name of fungi	Frequency (%) of fungi					
		Bark	Stem	Leaf	Bark	Stem	Leaf
Location 1 (Jagadishpur tea estate, Madhabpur, Habiganj)	*Alternaria alternata*	1.47	8.34	-	-	-	-
	Aspergillus flavus type 1	-	18.34	15.00	-	-	9.17
	A. niger type 1	1.47	-	7.50	3.45	-	1.67
	A. niger type 2	1.47	20.00	-	5.00	1.67	-
	Curvularia lunata	-	-	10.00	-	3.34	-
	Diaporthe hongkongensis	10.00	-	-	-	-	-
	Eupenicillium sp. 2	-	-	-	-	16.67	-
	Harknessia sp.	14.98	18.34	23.00	9.23	15.00	16.67
	Lasiodiplodia pseudotheobromae	13.88	-	-	9.40	-	-
	L. theobromae	25.88	26.67	40.50	17.07	7.50	10.00
	Penicillium commune	4.41	-	-	-	11.67	15.00
	P. digitatum	8.83	8.34	4.00	-	-	-
	P. italicum	8.83	-	-	17.07	6.67	13.34
	Penicillium sp. 2	2.94	-	-	-	-	-
	Penicillium sp. 3	2.94	-	-	38.80	20.83	16.67
	Penicillium sp. 4	2.94	-	-	-	16.67	17.50
Location 2 (Chundeechara tea estate, Chunarughat, Habiganj)	*Alternaria palandui*	-	-	-	-	-	10.00
	A. tenuissima	-	12.50	-	6.70	7.50	16.75
	Aspergillus flavus type 1	4.55	-	8.34	13.45	-	16.90
	A. niger type 1	6.11	6.25	-	15.17	9.00	7.50
	A. niger type 2	1.56	3.33	-	10.35	12.50	3.13
	Aspergillus sp. 1	3.13	-	-	-	-	10.00
	Aspergillus sp. 2	-	-	12.50	-	-	-
	Curvularia lunata	-	6.25	8.34	-	-	-
	Diaporthe hongkongensis	-	-	8.34	-	-	-
	Eupenicillium sp. 1	-	-	-	-	5.00	-
	Harknessia sp.	25.99	-	8.34	23.80	10.00	15.63
	Lasiodiplodia pseudotheobromae	9.23	35.84	27.78	13.60	42.50	11.25
	L. theobromae	23.01	35.84	26.39	13.70	13.50	9.38
	Penicillium commune	9.38	-	-	-	-	-
	P. digitatum	3.13	-	-	-	-	-
	P. italicum	1.56	-	-	-	-	-
	Penicillium sp. 2	3.13	-	-	-	-	-
	Penicillium sp. 4	4.69	-	-	-	-	-
	Sphaeropsis sp.	4.55	-	-	3.13	-	-

'–' represents the absence of respective fungi

From Dhaka district, maximum frequency was found in location 4, in *Penicillium* sp. 4 from stem in winter (43.33%) and minimum in location 3, in *Aspergillus niger* type 2 from leaf in winter (2.5%) and in location 4, in *Alternaria alternata* from bark in winter (2.5%). Maximum number of endophytic fungi was isolated from the bark of Dhaka in winter season (Table 2).

Table 2. Percent frequency of endophytic fungi associated with agar plants of the botanical gardens of Dhaka district in different seasons.

Locations	Name of fungi	Frequency (%) of fungi					
		Bark	Stem	Leaf	Bark	Stem	Leaf
Location 3 (Botanical Garden, Curzon Hall Campus, University of Dhaka)	*Alternaria tenuissima*	4.55	-	12.50	-	-	10.00
	Aspergillus flavus type 1	5.86	26.56	26.47	1.67	5.00	11.79
	A. niger type 1	-	25.00	-	11.67	16.38	11.07
	A. niger type 2	-	-	-	19.34	11.52	2.50
	Aspergillus sp. 1	-	-	12.50	-	-	-
	Eupenicillium sp. 1	4.55	-	-	-	-	-
	Harknessia sp.	33.28	-	-	11.34	10.88	-
	Lasiodiplodia pseudotheobromae	-	-	-	6.00	9.86	-
	L. theobromae	13.64	-	-	10.67	-	-
	Penicillium commune	21.06	12.50	7.36	-	-	-
	P. digitatum	3.95	15.63	11.77	-	-	-
	P. italicum	-	-	8.83	19.34	39.86	24.65
	Penicillium sp. 1	-	-	4.41	20.00	6.52	40.00
	Penicillium sp. 3	13.16	12.5	11.77	-	-	-
	Penicillium sp. 4	-	7.81	5.89	-	-	-
Location 4 (Botanical Garden, Mirpur, Dhaka)	*Alternaria alternata*	5.00	-	-	2.50	-	-
	A. palandui	-	-	15.00	3.57	5.00	-
	Aspergillus flavus type 1	-	5.00	-	-	10.00	-
	A. flavus type 2	-	-	23.75	-	-	9.67
	A. niger type 1	-	-	5.00	27.86	-	9.34
	A. niger type 2	7.50	-	-	12.86	3.34	10.67
	Aspergillus sp. 1	-	-	-	-	-	7.00
	Diaporthe hongkongensis	-	-	-	-	7.92	-
	D. perseae	-	-	-	-	10.00	-
	Eupenicillium sp. 2	10.00	-	-	7.50	-	-
	Fusarium sporotrichioides	-	10.00	-	-	-	-
	Harknessia sp.	26.79	40.00	20.00	6.79	5.42	5.34
	Lasiodiplodia pseudotheobromae	11.43	10.00	-	-	-	-
	L. theobromae	39.29	35.00	21.25	12.86	9.17	12.00
	Penicillium sp. 3	-	-	-	10.36	-	4.00
	Penicillium sp. 4	-	-	-	-	43.33	18.34
	P. italicum	-	-	15.00	15.70	5.83	26.00

'–' represents the absence of respective fungi

Analysis of diversity Ic

The highest value of Shannon diversity index was found in bark in location 2 during monsoon season (2.43), which indicates high species diversity and the lowest was in leaf in location 1 during monsoon season (1.28), which indicates low species diversity of endophytic fungi (Table 3). The maximum value of Simpson diversity index was found in stem in location 1 during winter season (0.89), which indicates high abundance of endophytic fungal species and minimum in stem in location 4 during monsoon season (0.70), which indicates low abundance of endophytic fungal species (Table 3).

The highest value of Shannon evenness index was found in stem in location 1 in monsoon season (1.0), which indicates that the number of endophytic fungi associating with the plant was fairly constant and the lowest value found in leaf in location 1 in monsoon season (0.71) which indicates the number of endophytic fungi associating with the plant was not constant (Table 3).

Table 3. Shannon diversity index, Simpson diversity index and Shannon evenness index of endophytic fungi associated with agar plant at different locations in monsoon and winter seasons.

Name of locations	Parts of plant	Season	Shannon diversity index (H)	Simpson diversity index (D)	Shannon evenness index (EH)
Location 1 (Jagadishpur tea estate, Madhabpur, Habiganj)	Bark	Monsoon	2.20	0.87	0.85
		Winter	1.68	0.78	0.86
	Stem	Monsoon	1.99	0.82	1.0
		Winter	2.03	0.89	0.92
	Leaf	Monsoon	1.28	0.80	0.71
		Winter	1.99	0.86	0.96
Location 2 (Chundeechara tea estate, Chunarughat, Habiganj)	Bark	Monsoon	2.43	0.86	0.95
		Winter	1.97	0.86	0.95
	Stem	Monsoon	1.46	0.73	0.81
		Winter	1.71	0.77	0.88
	Leaf	Monsoon	1.81	0.82	0.93
		Winter	2.13	0.88	0.97
Location 3 (Botanical Garden, Curzon Hall Campus, University of Dhaka)	Bark	Monsoon	1.85	0.81	0.89
		Winter	1.93	0.85	0.93
	Stem	Monsoon	1.73	0.81	0.97
		Winter	1.72	0.79	0.88
	Leaf	Monsoon	2.09	0.86	0.95
		Winter	1.54	0.75	0.86
Location 4 (Botanical Garden, Mirpur, Dhaka)	Bark	Monsoon	1.55	0.75	0.87
		Winter	2.0	0.78	0.91
	Stem	Monsoon	1.34	0.70	0.83
		Winter	1.83	0.78	0.83
	Leaf	Monsoon	1.72	0.82	0.96
		Winter	2.08	0.85	0.95

The maximum species richness of endophytic fungi was recorded in bark in location 2 during monsoon in Margalef's index (3.01) and in stem in location 1 during monsoon season in Menhinick's index (1.81), which indicate an increase in the biodiversity of endophytic fungi. The minimum value of species richness was recorded in bark in location 4 during monsoon season in both Margalef's index (1.25) and Menhinik's index (0.81), which indicates a decrease in the biodiversity of endophytic fungi (Table 4).

Agar plant (*Aquilaria malaccensis* Lam.) being commercially important and a rich source of bioactive secondary metabolites lead the researchers of different countries to report their findings on this particular field. Chhipa and Kaushik (2017) reported on the fungal diversity of *Aquilaria malaccensis* stem and soil in India where high value was reported in stem following Shannon diversity index, Simpson diversity index and Menhinick's index. Several endophytic fungi, such as *Alternaria* sp., *Curvularia* sp., *Fusarium* sp., *Sterilia* sp., *Cladosporium* sp., *Rhizopus* and *Penicillium* sp. were reported by Mochahari *et al.* (2020) as endophytic fungi of juvenile *Aquilaria malaccensis* in India. Premalatha *et al.* (2013) reported *Alternaria* sp., *Cladosporium* sp., *Curvularia* sp., *Davidiella* sp., *Fusarium* sp., *Hypocrea* sp., *Massarina* sp., *Phaeoacremonium* sp., *Pichia* sp. from the mature wood of agar plant in India. Hartono *et al.* (2019) isolated *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Tricoderma* sp., *Curvularia* sp. and *Peniophora* sp. as endophytic fungi in Indonesia. Lisdayani *et al.* (2015) reported *Acremonium* sp., *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., *Mucor* sp., *Nigrospora* sp., *Scopulariopsis* sp. and *Scytalidium* sp. from the stem of *Aquilaria malaccensis* in Indonesia. Mohamed *et al.* (2010) studied the fungal diversity in the wounded stem of agar plant in West Malaysia and reported *Fusarium* sp., *Lasiodiplodia* sp., *Cunninghamella* sp. and *Curvularia* sp.

Table 4. Species richness of endophytic fungi associated with agar plant at different locations in monsoon and winter season.

Locations	Plant parts used	Species richness	Monsoon season	Winter season
Location 1 (Jagadishpur tea estate, Madhabpur, Habiganj)	Bark	R1	2.94	1.54
		R2	1.69	1.00
	Stem	R1	2.09	2.01
		R2	1.81	1.22
	Leaf	R1	1.31	1.79
		R2	0.89	1.13
Location 2 (Chundeechara tea estate, Chunarughat, Habiganj)	Bark	R1	3.01	1.85
		R2	1.77	1.21
	Stem	R1	1.60	1.58
		R2	1.25	1.04
	Leaf	R1	1.72	2.23
		R2	1.22	1.50
Location 3 (Botanical Garden, Curzon Hall Campus, University of Dhaka)	Bark	R1	1.71	1.75
		R2	1.03	1.08
	Stem	R1	1.42	1.51
		R2	1.03	0.96
	Leaf	R1	2.20	1.29
		R2	1.46	0.87
Location 4 (Botanical Garden, Mirpur, Dhaka)	Bark	R1	1.25	2.07
		R2	0.81	1.30
	Stem	R1	1.67	2.01
		R2	1.51	1.22
	Leaf	R1	1.50	1.98
		R2	1.13	1.19

R1 = Margalef's index and R2 = Menhinick's index

Though the agar plant production and the endophytic fungi associated with agar plant are of high interest, not many reports have been published from Bangladesh regarding this field. Shoeb *et al.* (2010) isolated endophytic fungal strains from *Aquilaria malaccensis* Lamk. and studied their antibacterial activity. Akter and Tanbir (2013) reported agarwood production as multidisciplinary field to be explored in Bangladesh. A mathematical model for estimating total stem volume was reported by Islam and Chowdhury (2017) and the management practices of agar plant was reported by Ali and Kashem (2019).

Prior to this research, there was no report published on the taxonomy of endophytic fungi associated with the agar plant of Bangladesh. For this reason attempt was taken to isolate endophytic fungi from bark, stem and leaves of agar plant and report on their diversity as well as seasonal variation. From this research it was found that the species of endophytic fungi at different locations of Dhaka and Habiganj districts belong to *Aspergillus* sp., *Alternaria* sp., *Penicillium* sp., *Eupenicillium* sp., *Harknessia* sp., *Diaporthe* sp., *Sphaeropsis* sp., *Curvularia* sp., *Fusarium* sp. and *Lasiodiplodia* sp. Among them only *Diaporthe perseae* was not found in monsoon season and *Aspergillus* sp. 2, *Fusarium sporotrichioides*, *Penicillium* sp. 2 and *P. digitatum* were not found in winter season. The highest number of endophytic fungi was isolated from location 2. Bark was richer in endophytic fungi than stem and leaf of agar plant. The diversity and richness of endophytic fungi was found comparatively high in Habiganj (locations 1 and 2) than that of Dhaka (locations 3 and 4).

As the endophytic fungi associated with agar plant pose high industrial potential and is quite a new field of research for Bangladesh, further research is needed on the taxonomic identification, diversity and abundance of endophytic fungi of agar plant from other regions of Bangladesh.

Analysis on the essential secondary compounds isolated from endophytic fungi of agar plant is also an important field that needs to be explored to find the most suitable endophytic fungi that can be commercially used for producing agarwood in Bangladesh.

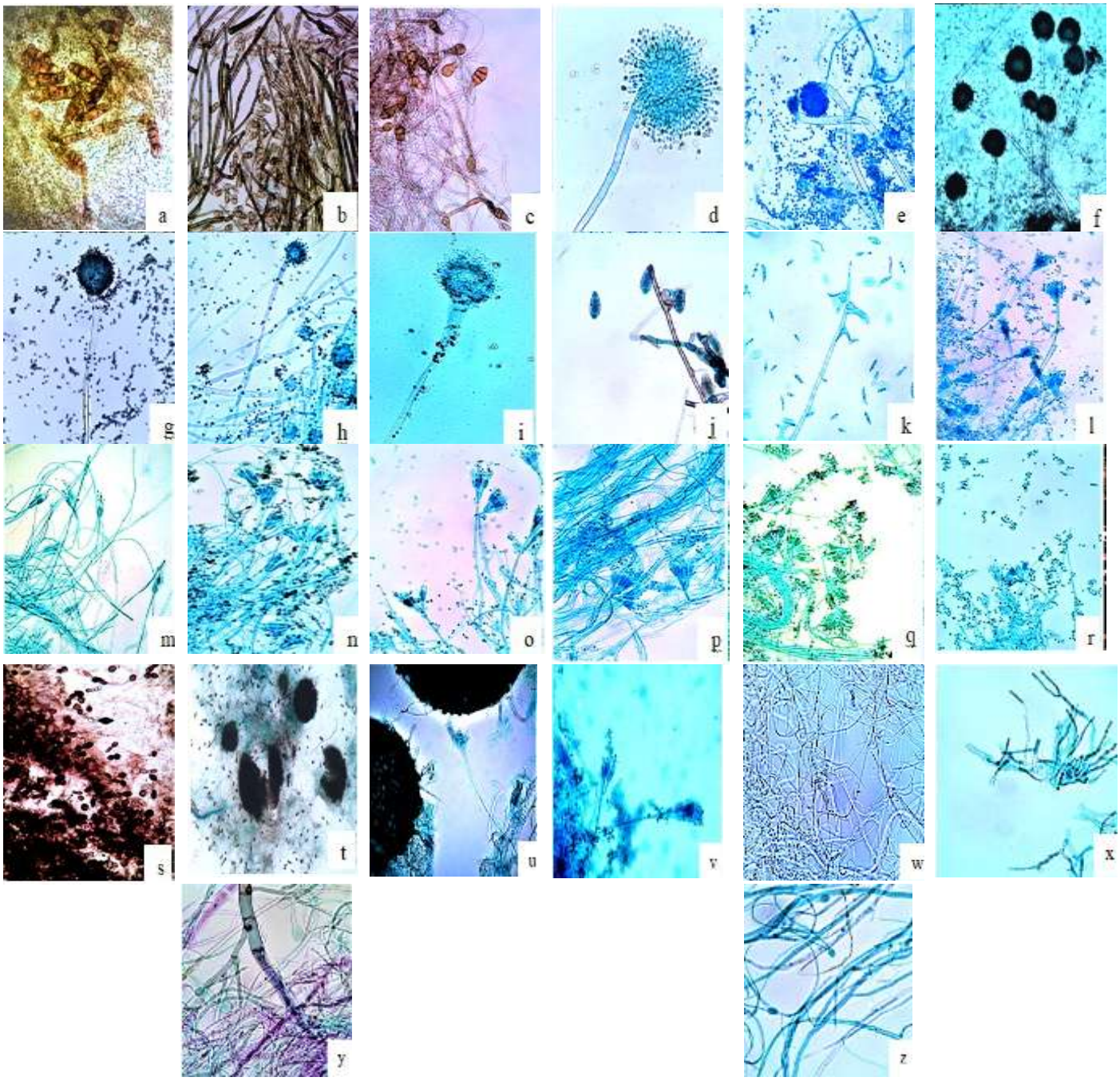

Fig. 1. Photographs of conidia of isolated fungi (a-v): **a.** *Alternaria alternata*; **b.** *A. palandui*; **c.** *A. tenuissima*; **d.** *Aspergillus flavus* type 1; **e.** *A. flavus* type 2; **f.** *A. niger* type 1; **g.** *A. niger* type 2; **h.** *Aspergillus* sp. 1; **i.** *Aspergillus* sp. 2; **j.** *Curvularia lunata*; **k.** *Fusarium sporotrichioides*; **l.** *Penicillium commune*; **m.** *P. digitatum*; **n.** *P. italicum*; **o.** *Penicillium* sp. 1; **p.** *Penicillium* sp. 2; **q.** *Penicillium* sp. 3; **r.** *Penicillium* sp. 4; **s.** *Harknessia* sp.; **t.** *Sphaeropsis* sp.; **u.** *Eupenicillium* sp. 1; **v.** *Eupenicillium* sp. 2; and conidiophores under microscope (w-z). **w.** *Diaporthe hongkongensis;* **x.** *D. perseae*; **y.** *Lasiodiplodia pseudotheobromae*; and **z.** *L. theobromae*. (Bar=50 μm).

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