MICROSTRUCTURE CHARACTERISTICS OF *Penicillium* Spp. ISOLATED FROM CLINICAL WASTES

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ABSTRACT

*The present study aimed to recognize the microstructure of conidiophores and spores of Penicillium spp. which were isolated from clinical wastes. Penicillium spp. isolates were recovered from the clinical wastes on V8A medium and purified using single spore isolation technique. The culture characteristics were described on five culture media included; Czapek Yeast Extract A*gar *(CYA); Potato Dextrose Agar (PDA); Czapek-Dox* Agar *(CZ); Malt Extract Agar (MEA) and Sabouraud dextrose agar (SDA), while the conidiophores and spores were described using light and Scanning Electronic Microscope (SEM). Penicillium spp. isolated from the clinical waste exhibited some diversity in their culture characteristics. However, the microscopic morphologies were less complicated than that of Aspergillus spp. Among 11 Penicillium species isolated in this study and identified based on culture and microscope morphology. Five species including P. simplicissium, P. waksmanii, P. corylophilum and P. decumbens as well as one species identified as T. wortmannii were described in details using SEM. The study revealed that the microstructure of the fungal spores and conidiophores play an important role in the taxonomy of fungi species based on the phenotypic method.*

Keywords: *Penicillium* spp. SEM, single spore technique, ultrastructure

INTRODUCTION

Penicillium spp. are group of fungi which have subjected for many of developments in their taxonomy during the last century. The identification of fungi by phenotypic method needs to consider several characteristics simultaneously. Fungi have high diversity in their culture characteristics such as colony size (Diameter, mm), texture, surface, zonation and sporulation on different culture media [1]. Besides, the microscope morphologies for the vegetative and reproductive structures such as hyphal colour and structures, as well as spore shape, surface ornamentation and size, are very important for the identification of fungi [2]. These characteristics are more useful to identify *Aspergillus* sp. *Curvularia* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. to species level [3]. However, it has to mention that the phenotypic characteristics of fungi depend on environmental conditions and culture media [4]. Hence, several culture media are recommended to be used in the phenotypic identification. Moreover, the developments in the Scanning Electronic Microscopy (SEM) have improved the recognition of several microstructures of fungal conidiophores and spores [4]. The SEM analysis for fungal spores has high efficiency to show the microstructure in the spore shape and surface ornamentation which enhance the accurate identification of the fungi to the varieties level. Most of *Penicillium* spp. are dominant the environment with high pathogenicity for plant and low against the humans except for immune-compromised patients, where *Penicillium* spp. can cause the death. Among several *Penicillium* spp. the most common infection in patients with human immunodeficiency virus is caused by *P. marneffei, P. chrysogenum* as opportunistic fungi [5,6]. The fungi in the clinical wastes might is generated from the infected specimens or as result of contamination during the storage period of these wastes. The ability of the fungi to contaminate the clinical wastes belongs to the storage conditions and the nature of the clinical wastes in terms of nutrients temperature, pH and moisture which might support their survival [7]. Among the fungi isolated from the clinical wastes, *Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp., *Basipetospora* spp., *Fusarium* spp. and *Scopulariopsis* spp., have isolated from the dental healthcare facilities in Brazil [8]. In Malaysia, few studies were conducted on the fungi from the clinical wastes samples [2, 9]. However, these studies have focused mainly on provide an inventory for the fungi in these wastes. In the present study, the microstructure of *Penicillium* spp. conidiophores and spores was studied by using SEM in order to investigate the similarity between *Penicillium* strains recovered from the clinical wastes.

MATERIALS AND METHODS

Isolation and purification of *Penicillium* spp. from clinical waste samples

The clinical waste samples were obtained from Wellness Centre at Universiti Sains Malaysia (USM), Penang, Malaysia and included gloves, tissue papers, gauze, cotton, needles, pasture pipette, kits, urine strips, blood and serum containers, HCG

kits, strips of glucose test lancets, ACCU-CHEK Safe-T-Pro Plus lancets, yellow tips, microscopic slides, wood sticks and HB cuvettes. *Penicillium* isolates were recovered from the clinical waste samples using direct plate method on Potato Dextrose Agar (PDA) medium and then purified using single spore technique as described in previous work [2].

Identification of fungal isolates

Penicillium isolates were identified according to the culture and microscopic characteristics as described by according to Pitt [10] and NMRC [11]. The flowchart used for the identification of species within *Penicillium* spp. genus is presented in Figure 1. The culture characteristics of *Penicillium* spp. were described on Potato Dextrose Agar (PDA), Czapek Yeast Extract Agar (CYA), Malt Extract Agar (MEA) and Czapek-Dox Agar (CZ). The fungal characteristics on these media which included colony size (Diameter, mm), surface and texture were recorded after 7 days of the incubation period at 28˚C [1]. The morphological characteristics of each fungal isolate determined using the light microscope (100X, Olympus, BX53F-CCD, Serial No. 1A589796, Japan). The microscopic examination of fungal isolates was described after the fungal colonies were sporulated on the different culture media. For this purpose, small mycelia part from the centre and edge of the growing colony was placed onto glass slide contained one drop of distilled water and covered by a cover slip. The characteristics of vegetative and reproductive structures such as hyphal colour and structures, spore shape, as well as spore size were determined. The spore size of 25 spores was determined by using Cell Sens Standard (Version 1.4.1) programme.

Determination of morphological characteristics by Scanning Electron Microscope (SEM)

The detailed morphology of conidiophore and spore shapes of the highest frequency of occurrence fungi were observed using SEM. Pure culture of each fungal isolate was sub-cultured on new PDA medium. The plates were sealed carefully with Para film and incubated at 28˚C for 2 days. The cultured media were transported under aseptic conditions to SEM laboratory at Biological School, USM. Small piece (0.2×0.2 cm) from the edge of grown colony was taken into aluminium Petri dish. The sample was drying using liquid nitrogen, coated with gold powder and then observed using SEM.

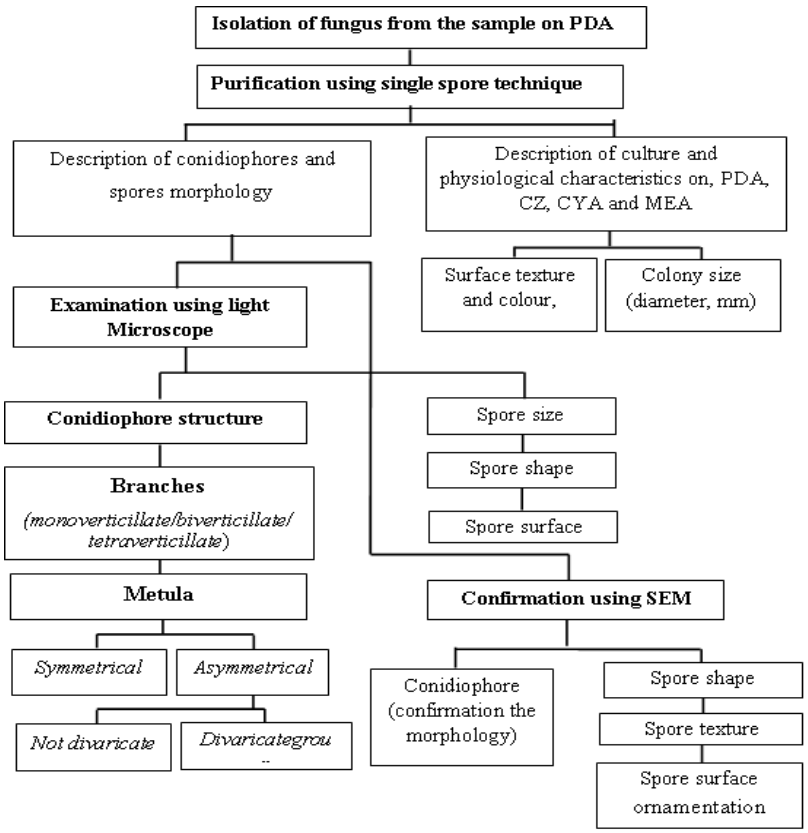

Figure 1. Flowchart of *Penicillium* spp. identification

RESULTS AND DISCUSSION

Penicillium spp. recovered from the clinical waste samples occurred high diversity in their colony morphology on the cultured media. The culture and microscope morphology of 11 *Penicillium* species obtained in the current study are illustrated in

Table 1 and 2. The microstructure of *P. waksmanii, P. simplicissium, P. Decumbens* and *P. corylophilum* as well as *Talaromyces wortmannii* were analysed using SEM because these isolates exhibited similar culture and microscopic morphology among all *Penicillium* spp. investigated in the present work.

Table 1. Culture characteristics of *Penicillium* spp. and *Talaromyces* sp. on different culture media after 7 days at 28˚C

Fungus	Media type	Colony diameter (mm)	Colony character		Zonation (Margin)	Sporulation
			Texture	Surface colour		
P. simplicissimum	CZ	39±5.5	amaranthine smooth	dark green with grey zone	white	high
	CYA	26±3.5	sulcate/amaranthine	dull green	white	high
	MEA	29±1.3	amaranthine smooth	dark green/grey centre	white	high
	PDA	25±1.3	amaranthine centre and radially edge	green with grey centre	colourless	high
P. waksmanii	CZ	22.4±2.4	velvety	dull green to olive	white	moderate
	CYA	27±3	sulcate/wrinkled/annular	green centre	white	moderate
	MEA	27±2.2	sulcate/wrinkled annular	dull green	light grey	high
	PDA	22±3.2	sulcate/wrinkled/annular	olive	white	moderate
P. corylophilum	CZ	21±2.8	velvety	dull green	white	high
	CYA	27±1.3	velvety/wrinkled/sulcate	green with white zone in the centre	white	high
	MEA	20±1.3	velvety/sulcate	olive to dull green	white	high
	PDA	18±2.3	velvety/sulcate	olive to dull green	white	high
P. decumbens	CZ	18±1.3	thin floccose	white to creamy	white	moderate
	CYA	25±2.2	wrinkled sulcate,	grey to greenish	white	high
	MEA	30±1.5	annular/thin floccose	grey to greenish	white	high
	PDA	25±2.1	thin floccose	light green	white	high
P. citrinum	CZ	19±2.2	velvety/sulcate	dark green /grey centre	white	high
	CYA	23±2.5	velvety/sulcate	grey centre	white	high
	MEA	22±2	velvety/wrinkled/sulcate	grey centre	white	high
	PDA	20±2	velvety/sulcate	dull green with grey centre	white	high
P. janthinellum	CZ	38.8±0.3	velvety	dull green with grey centre	white	high
	CYA	32±1.5	velvety/sulcate	grey centre	white	high
	MEA	45±1.3	velvety	grey centre	white	high
	PDA	25.5±3.5	velvety/annular	dark green/ grey centre	white	high
P. digitatum	CZ	41±4.2	velvety/annular	dark green centre	white	high
	CYA	50±3.5	velvety/sulcate	dark green/ grey centre	white	high
	MEA	45±2.3	velvety centre/ sulcate	dark green centre	white	high
	PDA	35±0.5	velvety	dark green centre	white	high
P. aurantiogriseum	CZ	16±3.2	velvety	grey centre	white	high
	CYA	28±4.7	velvety/sulcate	grey centre	white	high
	MEA	21±2.9	velvety/wrinkled	dark green with grey centre	white	high
	PDA	19±1.2	velvety	dark green/ grey centre	white	high
P. verruculosum	CZ	28±1.7	velvety	dark green	white	high
	CYA	39±2.9	velvety/sulcate	beige centre/ exudate	white beige	high
	MEA	29±1.1	velvety, sulcate/wrinkled	dark green/ grey centre/ clear exudate	white	high
	PDA	21±0.6	velvety/sulcate	grey	white	high

P. oxalicum	CZ	51.5±0.5	velvety/annular	grey/ dark green centre	white	high
	CYA	54±0.5	velvety	dull green/olive	white	high
	MEA	45±1.2	velvety	dull green	White/blue	high
	PDA	33±1.1	velvety	dark green/ grey centre	white	high
Penicillium sp. new strain no. 55	CZ	36±2.6	velvety, sulcate	light grey	white	high
	CYA	26±1.3	velvety, sulcate	light grey	white	high
	MEA	21±2.6	velvety, sulcate	light grey	white	high
	PDA	15±1.3	velvety	grey	white	high
T. wortmannii	CZ	8±2.2	crisp and annular	dark green centre	white	low
	CYA	10±2	amaranthine	dark green centre surrounded by white zone	white	moderate
	MEA	22±3.5	annular/amaranthine	dark green centre	white	high
	PDA	12±2.7	velvety	white to creamy	white	low

Czapek-Dox Agar (CZ); Czapek Yeast Extract Agar (CYA); Malt Extract Agar (MEA); Potato Dextrose Agar (PDA)

Table 2. Microscopic morphology of *Penicillium* spp. *Penicillium* spp. and *Talaromyces* sp.

Fungal species	**Conidiophore morphology***	**Conidia diameter (µm)***			**Spore shape and texture surface ****
		mean	**max**	**min**	
P. simplicissimum	Long and rough/diverticillate, a symmetrical/ divaricate, some monoverticillate.	4.2	6	3.3	Ellipsoidal to spherical/ roughened surface and finely wrinkled ornamentation
P. waksmanii	Conidiophore long and smooth, diverticillate terminated with long and smooth metula and phialids	2.9	3.2	2.4	Spherical/finely roughened surface, distinctly wrinkled ornamentation
P. corylophilum	Conidiophore long, smooth and tetra-verticillate with sub-terminal metula	2.7	3.2	2.2	Spherical to sub-spheroidal, smooth surface and finely wrinkled ornamentation
P. decumbens	Conidiophore long, thin and smooth, with monoverticillate branches, phialids long and cylinder	2.4	2.9	1.7	Ellipsoidal, smooth surface and finely wrinkled ornamentation
P. citrinum	Conidiophore short, smooth to finely roughened, monoverticillate	2.3	2.8	1.8	Spherical, small, smooth to finely roughened surface
P. janthinellum	Conidiophore short, thin and smooth, monoverticillate. Phialids cylindrical	3.3	4.6	2.6	Spherical to ellipsoidal, smooth surface
P. digitatum	Conidiophore long, thin and smooth surface, biverticillate, phialids cylindrical in shape	3.4	4.1	2.5	Ellipsoidal to cylindrical with smooth surface
P. aurantiogriseum	Conidiophore long, smooth to finely roughened, biverticillate, metula arranged in a symmetrical shape, divergent with acute angle, phialids slender	2.8	3.2	2.4	Spherical with smooth surface
P. verruculosum	Conidiophore thin and smooth and monoverticillate	3.3	4.1	2.2	Ellipsoidal, with smooth surface
P. oxalicum	Conidiophore thin and smooth and monoverticillate	5.1	6.1	4.3	Ellipsoidal, large, smooth surface
Penicillium sp. new strain no. 155	Conidiophore long, smooth and monoverticillate	3.4	4.2	2.8	Spherical to ellipsoidal, smooth to roughened surface
T. wortmannii	Conidiophore long, smooth and branched (biverticillate), metula arranged as symmetrical shape, divergent at acute angles	3	3.6	2.3	Ellipsoidal/ pyriform to fusiform/ smooth to spinulose

*As shown using light microscope with 100X of magnification with Cell Sens Standard (CSS) programme (Version 1.4.1);**as shown using Scanning Electron Microscope (SEM)

P. simplicissium colonies have as light green with white zone on PDA, a dark green colour with newer, whitish tint mycelial at the peripheral of the colony on CYA, while were dull green with white zone CZ and MEA, (Fig. 2). The fungus has long and rough, diverticillate, a symmetrical/ divaricate conidiophore with a terminal and sub-terminal tetrad of divergent metula, some of conidiophore were monoverticillate similarly as described by Pitt (1991). Metula was long and rough (Fig. 3 A). The

spore size ranged from 3.3 to 6 µm with an average of 4.2 µm (Table 2), while the spore shape was spherical to ellipsoidal / roughened surface and finely wrinkled ornamentation (Fig.3 B).

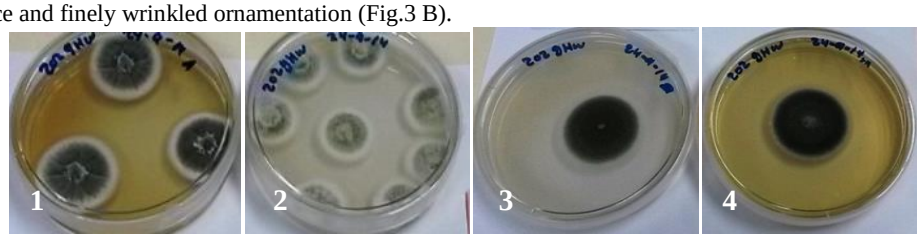

Figure 2. *P. simplicissmum* on culture media after 7 days at 28˚C; **1)** CYA; **2)** PDA; **3)** CZ; **4)** MEA

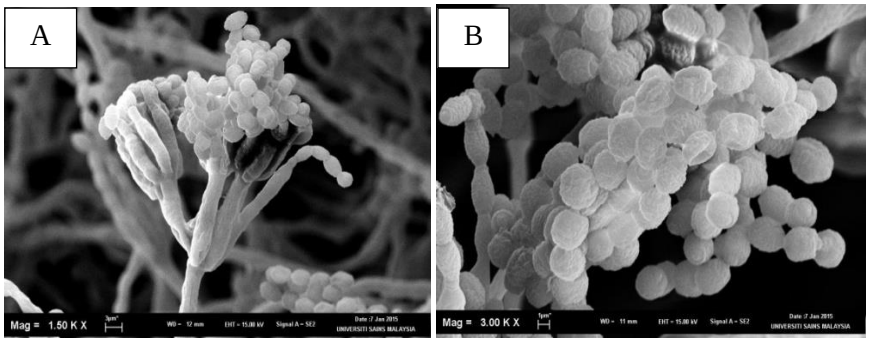

Figure 3. *P. simplicissmum* as occurred by SEM*;* **A)** conidiophores (1500X); B) spores (3000X).

P. waksmanii colonies exhibited an olive colour with white edge on CZ. The colony on MEA appeared as a dull green with light grey zone, while was dark olive colour with yellowish to greenish edge on PDA, and greenish grey with white edge on CYA (Fig. 4). It has a long and smooth conidiophores which was monoverticillate terminated with long and smooth metula and phialids similarly as described by Pitt (1991) (Fig. 5A). The spore size ranged from 2.4 to 3.3 µm and in an average of 2.9 µm. The spore shape was spherical, distinctly wrinkled ornamentation and finely roughened surface (Fig. 5 A).

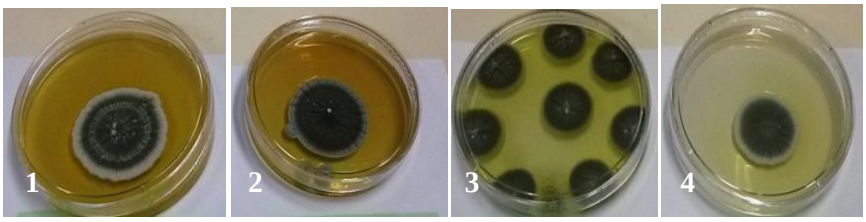

Figure 4. *P. waksmanii* on culture media after 7 days at 28˚C; **1)** CYA; **2)** MEA; **3)** PDA; **4)** CZ

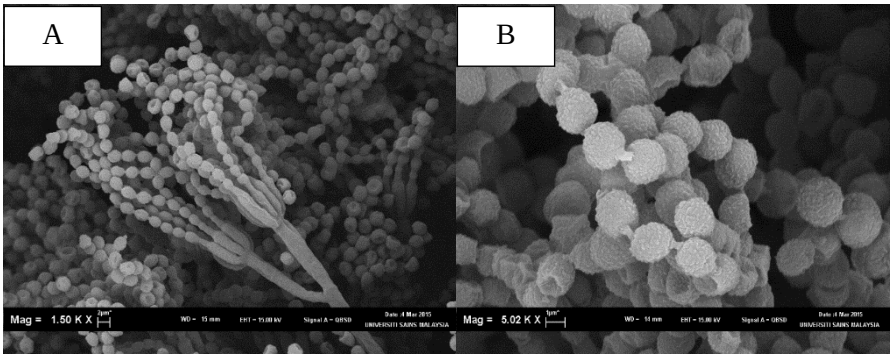

Figure 5. *P. waksmanii* as occurred by SEM*;* **A)** conidiophores (1500X); B) spores (5020X)

The culture characteristics of *P. corylophilum* on CYA was dark dull green with narrow white edge, while were similar on CZ, MEA and PDA (Fig. 6). It has a long, smooth and monoverticillate conidiophore with sub-terminal metula as described by Pitt (1991) (Fig. 7 A). The spore size ranged from 2.2 to 3.2µm with average 2.7 µm with spherical to sub-spheroidal shape and smooth surface as well as finely wrinkled ornamentation (Fig. 7 B).

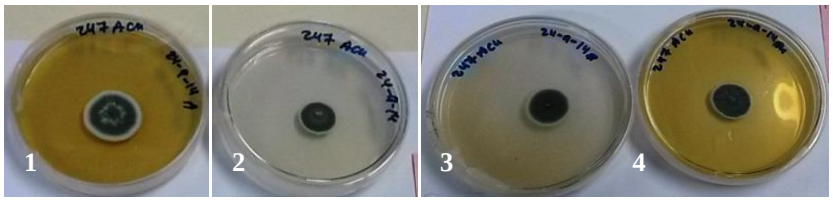

Figure 6. *P. corylophilum* on culture media after 7 days at 28˚C; **1)** CYA; **2)** PDA; **3)** CZ; **4)** MEA

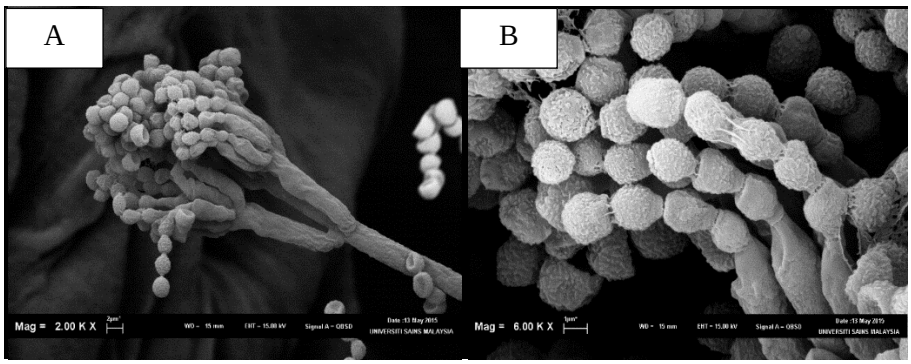

Figure 7. *P. corylophilum* as occurred by SEM; A) Conidiophore (2000X); B) spores (6000X).

P. decumbens has a small colony with grey to greenish colony on CYA, while has light green colour on PDA and white to cream on CZ as well as dull green on MEA (Fig. 8). The conidiophore was long, smooth and thin, monoverticillate branches with long and cylinder phialids (Fig. 9 A). It has ellipsoidal, smooth surface and finely wrinkled ornamentation spores, ranged from 1.9 to 2.7µm with 2.4 µm in average (Fig. 9 B).

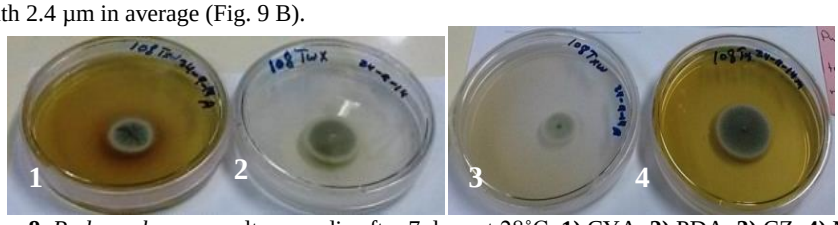

Figure 8. *P. decumbens* on culture media after 7 days at 28˚C; **1)** CYA; **2)** PDA; **3)** CZ; **4)** MEA

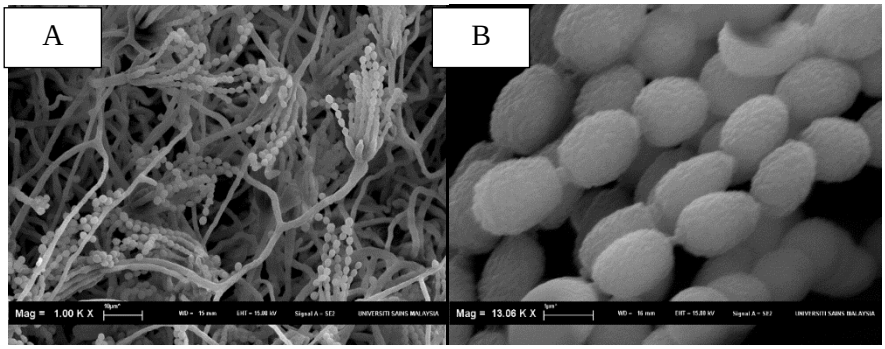

Figure 9. *P. decumbens* as occurred by SEM; A) Conidiophore (1000X); B) spores (13060X).

T. wortmannii has small colonies, with green centre on CYA, while was white to creamy colour on PDA, it has weak growth with green centre on CZ and dull green colony on MEA (Fig. 10). The conidiophore was smooth and branched (biverticillate), the metula arranged in a symmetrical shape, divergent at acute angles (Fig. 11 A). The spore size was between 2.3 and 3.6 µm with 3 µm in average and ellipsoidal/ pyriform to fusiform shape/ smooth surface (Fig. 11 B).

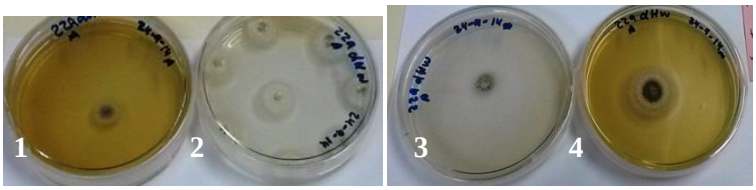

Figure 10. *T. wortmannii* on culture media after 7 days at 28˚C, **1)** CYA; **2)** PDA; **3)** CZ; **4)** MEA

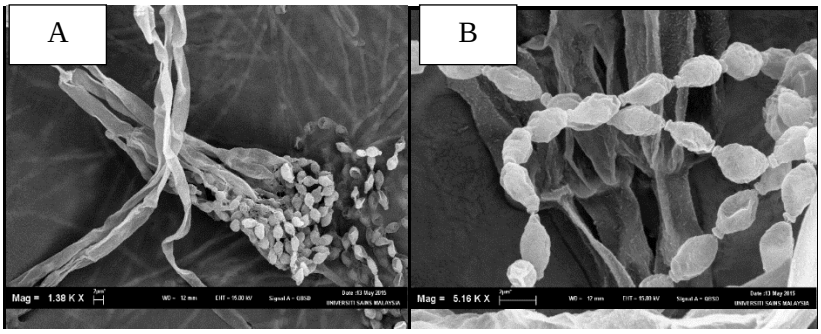

Figure 11. *T. Wortmannii* as occurred by SEM, A) Conidiophore (1380X); B) spores (5160X).

The microscopic morphology and cultural characteristics of fungal species represent the important keys for taxonomy by phenotypic method [12]. This technique is an effective and essential tools for identification of fungi. It has been used by 89% of laboratories to identify the fungi [12]. Nonetheless, the phenotypic technique based on the observation by light microscope is often creating misconception. Therefore, SEM is a useful tool to get the correct classification of the fungi based on the microstructure of conidiophores and spores [13, 14].

CONCLUSION

It can be concluded that the *Penicillium* spp. in the clinical wastes exhibited high diversity in their species, with a similarity in the culture characteristics and conidiophores as well as spores structure. However, the SEM analysis was more useful for detect the fine structure of the fungal isolates belonged to *Penicillium* spp. and thus facility the identification process.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Ministry of Science Technology and Innovation (MOSTI) for the research project financial support under FRGS Grant No. 203/PTEKIND/6711438) and APEX Grant 1002/PJJIUH/910324).

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