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Phenotypic identification of *Penicillium* spp. isolated from clinical wastes based on microstructure characteristics

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ABSTRACT

Aims: The present study aimed to recognize the microstructure of conidiophores and spores of *Penicillium* spp. which were isolated from clinical wastes.
Methodology and results: The isolates of *Penicillium* spp. were obtained from the solid clinical wastes on V8A medium and purified by single spore method. The culture characteristics were described in five culture media included; Czapek Yeast Extract Agar *(*CYA); Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar (SDA) and Czapek-Dox Agar (CZ) while the conidiophores and spores were described using light and Scanning Electronic Microscope (SEM). *Penicillium* spp. observed some differences in their culture characteristics. 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 detail using SEM.
Conclusion, significance and impact of study: 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, ultrastructure, single spore technique

INTRODUCTION

Penicillium spp. is a group of fungi which have subjected to many of developments in their taxonomy during the last century. The identification of fungi by phenotypic method needs to consider several characteristics simultaneously. Fungi occur different culture characteristics in terms of texture, colony size (diameter, mm), surface, zonation and sporulation on different culture media (Promputtha *et al*., 2005). Besides, the microscope morphologies for the mycelium structures and surface ornamentation, shape and size of the spores, are very important for the identification of fungi (Noman *et al*., 2016). These characteristics are more useful to identify *Aspergillus* sp. *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. to species level (Emine *et al*., 2010). However, it has to mention that the phenotypic characteristics of fungi depend on environmental conditions and culture media (Guarro *et al*., 1999). 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 (Guarro *et al*., 1999). 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. The fungi in the clinical wastes might are generated from the infected specimens or as result of contamination during the storage period of these wastes, since, the conditions of storage in terms of nutrients, temperature, pH and moisture support their survival (U.S. EPA1990).

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 (Vieira *et al*., 2010). *Penicillium* spp. has a low pathogenicity for humans and have isolated from different sources in the environment. Nonetheless, these fungi are virulent pathogens and can cause death among the immune-compromised patients (Oshikata *et al*. 2013) such as *P. marneffei*, *P. chrysogenum* (Barcus *et al*. 2005; Vanittanakom *et al*., 2006). A very few studies were conducted on the fungi from the clinical wastes in Malaysia (Abdul-Rahman *et al*., 2008; Noman *et al*.,

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2016). However, these studies have focused mainly on providing an inventory of the fungi in these wastes. In the present study, the microstructure of *Penicillium* spp. conidiophores and spores were studied by using SEM in order to improve the phenotypic identification of *Penicillium* strains recovered from the clinical wastes.

MATERIALS AND METHODS

Recovering *Penicillium* spp. from solid clinical waste

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, kits, strips of glucose test lancets, Safe-T-Pro Plus lancets, yellow tips, slides, wood sticks and HB cuvettes. *Penicillium* isolates were isolated based on the direct plate method on Potato Dextrose Agar (PDA) medium and purified by the method of single spore as described in previous work (Noman *et al*., 2016).

Identification of fungal isolates

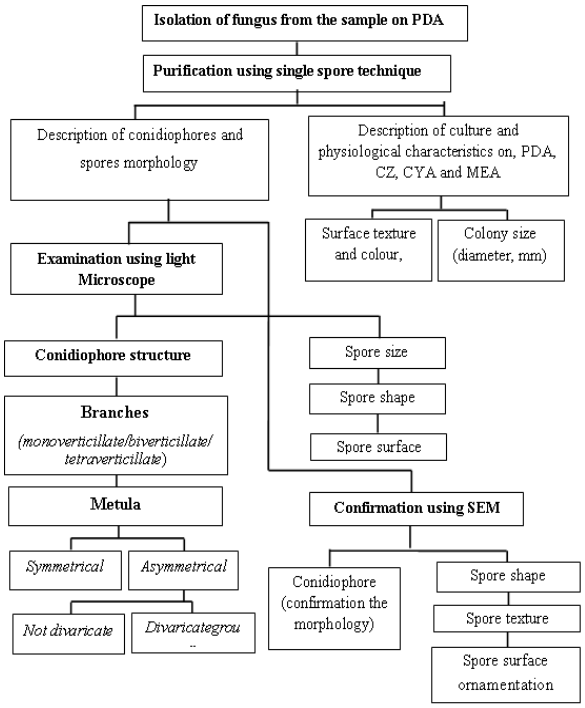

Figure 1: Flowchart of *Penicillium* spp. identification.

Penicillium isolates were identified according to the culture and microscopic characteristics as described by Pitt (2010) and NMRC (2015) according to the flow chart depicted in Figure 1. The culture characteristics of *Penicillium* spp. were described on Czapek Yeast Extract Agar (CYA), Potato Dextrose Agar (PDA), 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 obtained after the incubation period of 7 days at 28 °C (Promputtha *et al*., 2005). The morphological characteristics of each fungal isolate were recoded based on the observation under light microscope as described in previous work (Noman *et al*., 2016).

Microstructure of fungal isolates

The microstructure of spore shape and conidiophore were recorded based on the observation using Scanning Electron Microscope (SEM). A small piece (0.2×0.2 cm) was taken from each colony, dried using liquid nitrogen, coated with gold powder and then observed using SEM.

RESULTS AND DISCUSSION

Penicillium spp. obtained from the clinical solid waste observed to be 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 Tables 1 and 2. The microstructure of *P. waksmanii*, *P. simplicissium*, *P. Decumbens*, and *P. corylophilum*, as well as *Talaromyces wortmannii*, were observed by using SEM because these isolates exhibited similar culture and microscopic morphology among all *Penicillium* spp. investigated in the present work.

Penicillium simplicissium colonies have as light green with white zone on PDA, while exhibited a green to whitish mycelial on CYA. On MEA and CZ the colony was dull green colour with white zone, (Figure 2). The fungus morphology was recognized with long and rough, diverticillate, a symmetrical/ divaricate conidiophore as mentioned by Pitt (1991). The fungus has rough and long metula (Figure 3A). Besides, it has spores spherical to ellipsoidal / roughened surface and finely wrinkled ornamentation with size between 3.3 and 6 µm (Table 2) (Figure 3B).

Penicillium waksmanii colonies on CZ has white edge and olive colour. The colony on MEA appeared as a dull green with light grey zone, on PDA it has yellowish edge and dark olive colony. Moreover, on CYA it appears as greenish grey with white edge (Figure 4). It has long and smooth conidiophores which as recognized by Pitt (1991) (Figure 5A). The spores were spherical with 2.9 µm of size as well as distinctly wrinkled ornamentation and finely roughened surface (Figure 5A).

The culture characteristics of *P. corylophilum* on CYA was dark dull green with narrow white edge, while were similar on CZ, MEA and PDA (Figure 6). It has a smooth and long conidiophore as recognized by Pitt (1991) (Figure 7A). The spores were spherical shape with size of 2.7 µm, has a smooth surface and finely wrinkled ornamentation (Figure 7B).

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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)

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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)

Penicillium decumbens grown with a small colony, grey to greenish colour on CYA, on PDA it was light green, while on CZ was white to cream colour and on MEA it was dull green colour (Figure 8). The fungus has smooth, long and thin conidiophore, monoverticillate branches with long and cylinder phialids (Figure 9A). It has ellipsoidal, smooth surface and finely wrinkled ornamentation spores with 2.4 µm of the size (Figure 9B).

Talaromyces wortmannii colonies were on the culture medium, it exhibited a green colour on CYA, and creamy colour on PDA, it has weak growth with green centre on CZ and dull green colony on MEA (Figure 10). The fungus has branched smooth conidiophore *(*biverticillate), with a symmetrical arrangement of the metula (Figure 11A). The spores were ellipsoidal to pyriform and fusiform shape with 3 µm of the size and smooth surface (Figure 11B).

The microscopic and culture morphology of the fungal isolates represent the important keys for taxonomy by phenotypic method (Diba *et al*., 2007). This technique is an effective and essential tool for identification of fungi. It has been used by 89% of laboratories to identify the fungi (Diba *et al*., 2007). Nonetheless, the phenotypic technique based on the observation by the light microscope is often creating a misconception. Therefore, SEM is a useful tool to get the correct classification of the fungi based on the microstructure of conidiophores and spores (Clarke and Griffiths, 1970; Eduard *et al*., 1988). The utilisation of SEM in the phenotypic methods might be equivalent to the molecular technique, for instance, according to the morphological identification of *C. lunata,* Ellis (1971) and Sivanesan (1987) recognized two strains *C. lunata* var. *aeria* (has smooth conidia and stromata in culture) and *C. lunata* var. *lunata* (has smooth to roughly conidia but no stromata in culture). In comparison, the molecular techniques recognize these strains which have different 16S rRNA sequencing (Nakada *et al*., 1994; Cunha *et al*., 2013). It was noted that the PDA and V8A are among the media which supported the fungal growth.

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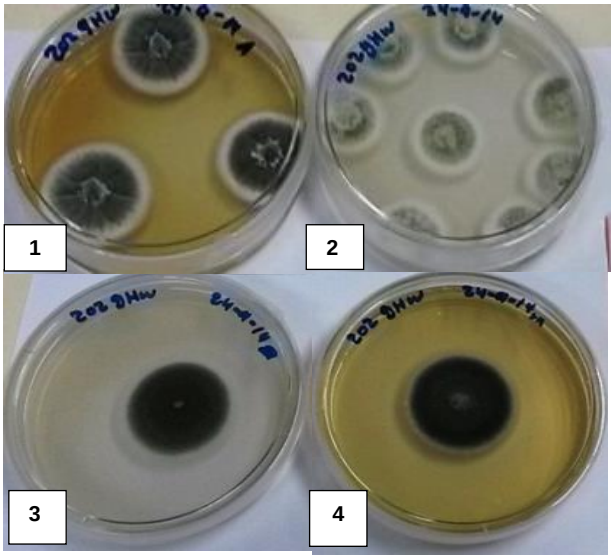

Figure 2: *P. simplicissmum* on culture media after 7 days at 28 °C; 1) CYA; 2) PDA; 3) CZ; 4) MEA.

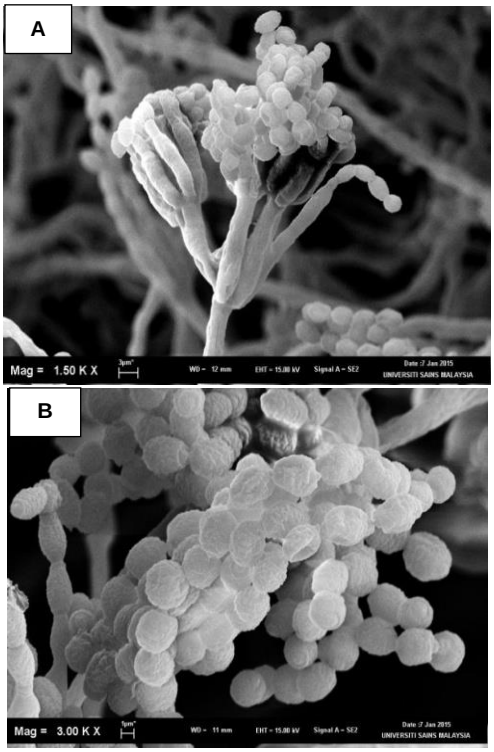

Figure 3: *P. simplicissmum* as observed by SEM*;* A) conidiophores (1500×); B) spores (3000×).

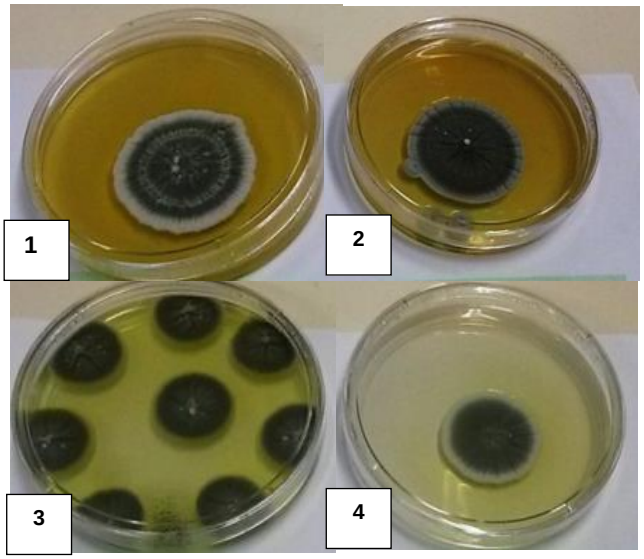

Figure 4: *P. waksmanii* on culture media after 7 days at 28 °C; 1) CYA; 2) MEA; 3) PDA; 4) CZ.

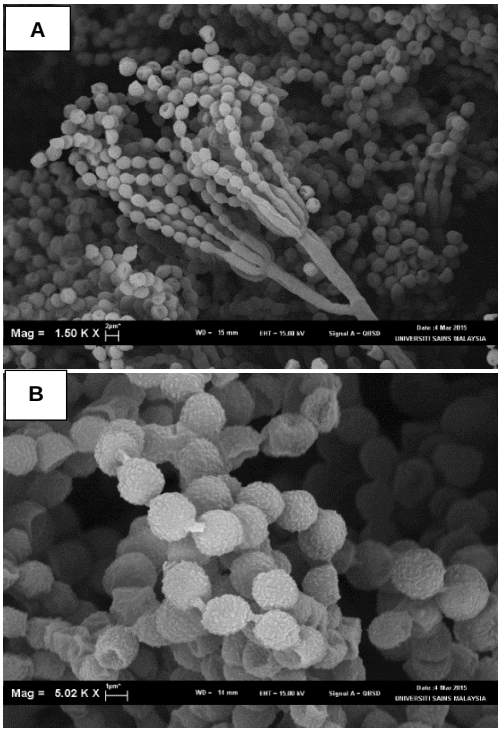

Figure 5: *P. waksmanii* as observed by SEM*;* A) conidiophores (1500×); B) spores (5020×).

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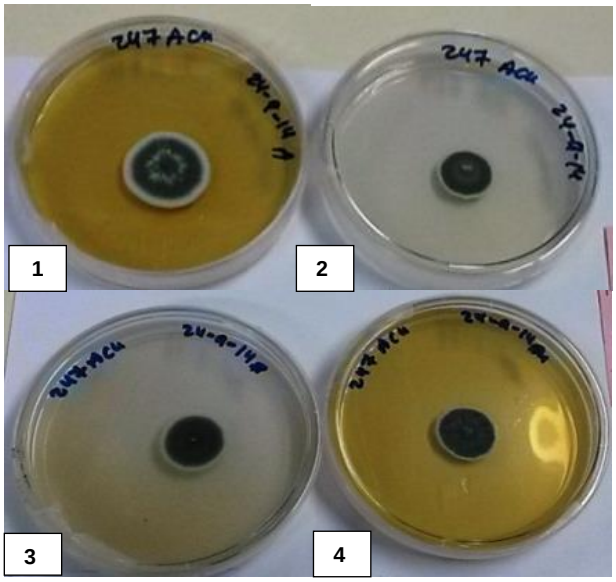

Figure 6: *P. corylophilum* on culture media after 7 days at 28 °C; 1) CYA; 2) PDA; 3) CZ; 4) MEA.

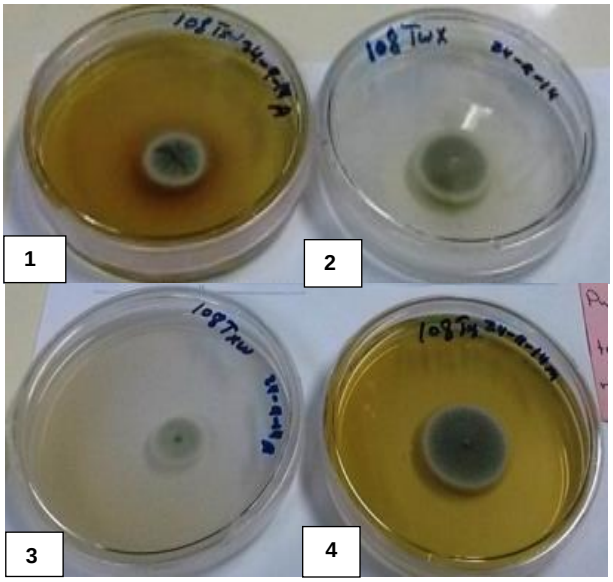

Figure 8: *P. decumbens* on culture media after 7 days at 28 °C; 1) CYA; 2) PDA; 3) CZ; 4) MEA.

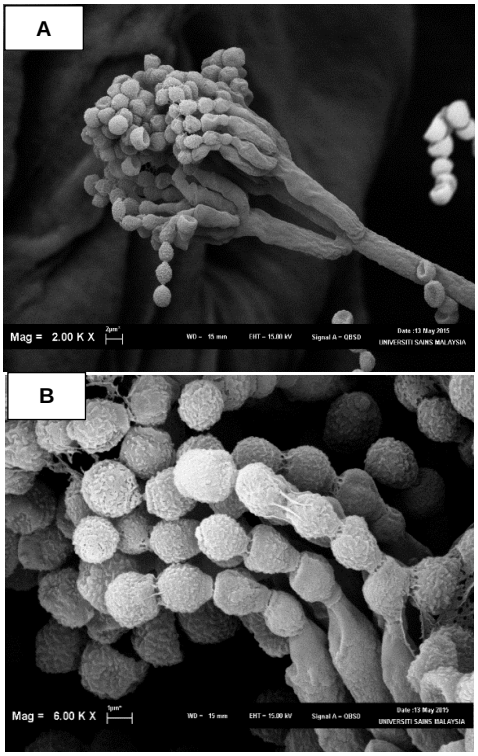

Figure 7: *P. corylophilum* as observed by SEM*;* A) Conidiophore (2000×); B) spores (6000×).

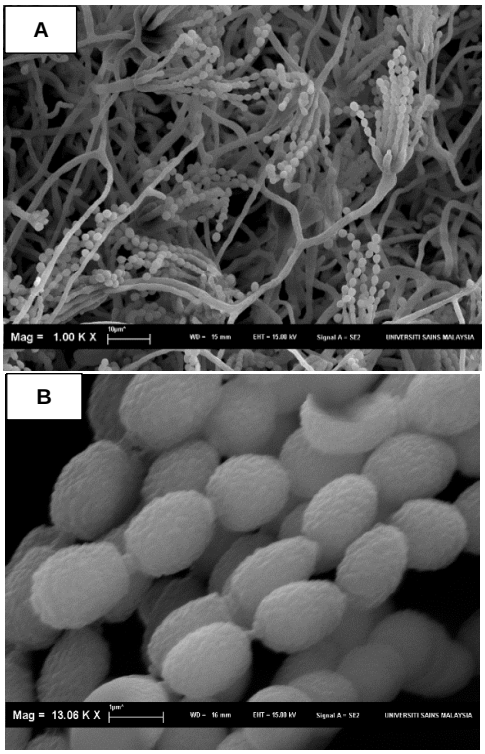

Figure 9: *P. decumbens* as observed by SEM; A) Conidiophore (1000×)*;* B) spores (13060×).

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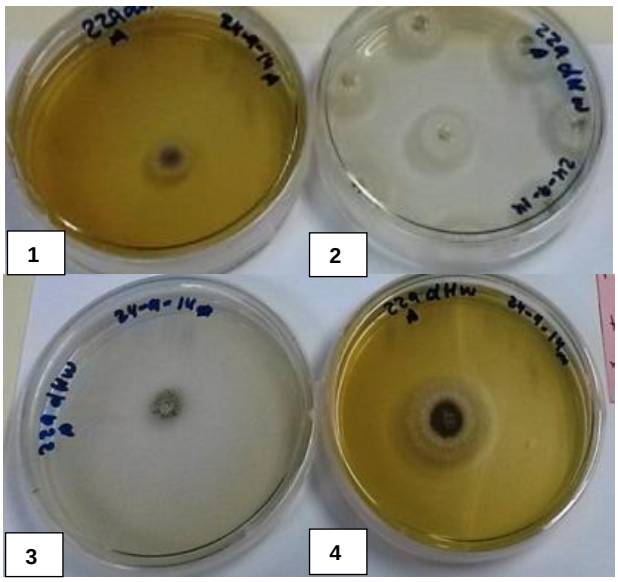

Figure 10: *T. wortmannii* on culture media after 7 days at 28 °C, 1) CYA; 2) PDA; 3) CZ; 4) MEA.

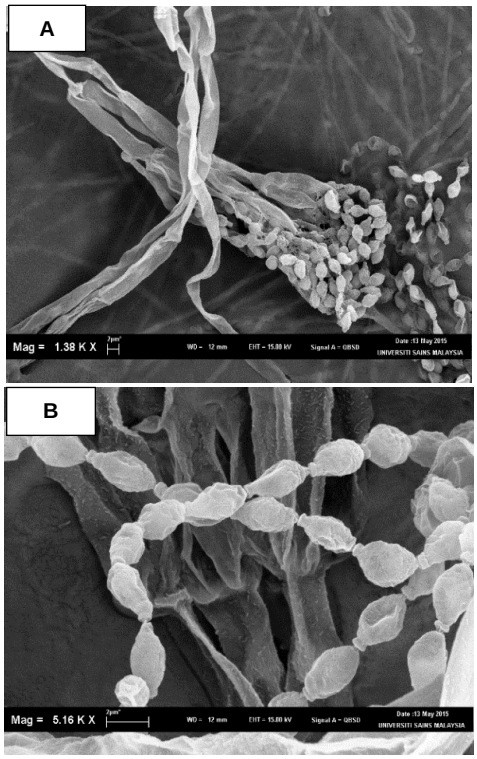

Figure 11: *T. wortmannii* as observed by SEM, A) Conidiophore (1380×); B) spores (5160×).

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 detecting the fine structure of the fungal isolates belonged to *Penicillium* spp. and thus facility the identification process.

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