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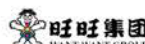
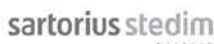
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Microfungi On Green Leaves And Leaf Litters From Santubong National Park, Sarawak

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ABSTRACT

Microfungi are highly abundant and diverse group of organisms with a worldwide distribution, but very little is known about the microfungi in Sarawak, thus less exploited for their beneficial uses. Microfungi on leaf litters from Santubong National Park in Sarawak were studied to assess their diversity and identify potentially beneficial rare species for human use and healthy functioning of the ecosystem. Leaf litters were collected from five (5) different locations in Santubong National Park. The leaves were processed for fungal isolation using the leaf washing method and leaf segment plating method. The leaf segments were plated on a relatively weak media (Water agar) to achieve maximum diversity. After seven (7) weeks of incubation at room temperature, twelve (12) taxa of microfungi with eight (8) Deuteromycotina, three (3) Ascomycotina and 1 (one) sterile mycelia were isolated and identified. The identified microfungi includes *Myriogenospora atramentosa*, *Periconia byssoides*, *Cylindrocladium* spp, *Penicillium* sp, *Trichoderma viride*, *Trichoderma* sp, *Chaetomium globosum*, *Fusarium* sp, *Oidiodendron* sp, and *Codinae* sp. There are many other microfungi that are still waiting to be discovered and exploited in Sarawak.

Keywords: Ascomycotina, Deuteromycotina, leaf litter fungi, Microfungi, Santubong National Park

1. INTRODUCTION

Many studies have been done on the identification and diversity of macrofungal species in Malaysia (Eyssartier *et al.*, 2009; Zainuddin *et al.*, 2010; Bolhassan *et al.*, 2012; Tan *et al.*, 2013) and Sarawak in particular (Sepiah *et al.*, 2009a, 2009b; Yamashita *et al.*, 2009; Pang *et al.*, 2010; Peay *et al.*, 2010; Dentinger *et al.*, 2012), that of the microfungi have been very limited in number both in peninsular Malaysia and Sarawak.

Sarawak is one of the only two (2) states of Malaysia on the Borneo Island which have been rated as a megabiodiversity centre in the world. There is a high level of biological diversity in this region which has attracted researchers both within and outside Sarawak including international researchers. Many rare species with some endemic to Sarawak has been found in this region and thus their conservation initiated. Sarawak is mostly covered by the mixed dipterocarp forest with the plant family dipterocarpaceae dominating the forest plant species.

Despite a lot of studies that have been carried out in Sarawak, microfungi; a group of organism with an important role in the functioning of the ecosystem (Mueller and Bill, 2004), have been systematically out of the research spectrum. This has also led to the unfortunate exclusion of the microfungi from conservation programs in this region.

Microfungi are involved in the breakdown of nutrients on the forest floor which are then available for uptake by the plants. They are important contributors to the diversity of organisms in the forest (Snaddon *et al.*, 2011). Microfungi have been used in medicine, agriculture, bioremediation, industrial processes, food industry, pharmaceuticals (Kumar *et al.*, 2013) and other facet of human life. Six (6) out of twenty (20) of the most commonly used drugs are of fungal origin (Gloer, 1997 In Sculz *et al.*, 2012).

Leaf litters have been greatly studied for the presence and diversity of the microfungal species they harbour (Kumar *et al.*, 2013) recording some species that are rare to encounter and many others that are cosmopolitan. Many of these microfungi species can be directly harnessed for their beneficial activities like the use of *Trichoderma* spp. as a biological control agent, *Periconia* spp for production of piperine, a potential cancer preventive drug (Verma *et al.*, 2011) and other useful activities. There have been individual and collaborative researches on microfungi associated with leaves in the forest, but data on tropical forest microfungi is still insufficient.

This study therefore, as a pioneer study, inventoried the microfungi associated with leaf litters in Santubong National park in Western Sarawak, revealing the diversity of microfungal species as well as their rareness and commonness. This will be an indicator of the enormous microfungal diversity in Sarawak.

2. MATERIALS AND METHODS

Sampling site

The sampling site is located in the Santubong National Park, Western Sarawak, on the Borneo Island; the second largest tropical Island in the world (Ting *et al.*, 2012). The park is located 30km North of Kuching, the capital city of Sarawak. The park was gazetted as a National park on 28th February, 2007 covering an area of 1,410 ha and its vegetation is dominated by lowland mixed dipterocarp forest (UNIMAS Wallace Expedition, 2013) with the plant family dipterocarpaceae dominating the plant species. This site is characterised by a tropical climate with an average annual temperature of 27 °C and average annual rainfall of 4095 mm (Climate-data, 2013).

Sample collection

As our interest was solely on microfungi generally and not host specific, mixed leaf litters were collected (Bills and Polishook, 1994; Polishook *et al.*, 1996; Kshattriya *et al.*, 1992 1994 In Shanthi and Vittal, 2010; Kominoski *et al.*, 2007; Jabiol and Chauvet, 2012). 25 senescent leaves were randomly collected from each of the five collection points. Some green leaves were also collected. The samples were placed into sterile plastic bags and the date of sampling and location in the forest were noted. The samples were then returned to the laboratory for processing.

Isolation and culture of microfungi

The leaves were cut into 1 cm² and kept in a 500 ml conical flask. For each location, about 50 pieces of leaf samples were prepared. These samples were then washed with sterile distilled water for five (5) times to make sure that any extraneous materials are removed from the leaves. The leaf segment were then blotted out on a Whatmann's No 1 filter paper to remove excess water before plating on the media in 9 cm petri dishes containing about 20mls of corn meal agar (CMA) media.

The leaf segments were plated on the media, 5 segments per plate evenly distributed, with either the adaxial or abaxial surface touching the media. Five replicates were prepared for the samples of each location and the plates were incubated on bench top at 25±2°C. Fungi on the

samples were observed every day after the first 3 days . This was done until after 7 weeks when the media started to dehydrate and no more colony was seen emerging.

All fungal colony was observed under the Motic stereo microscope model MZ 168. Slides were prepared and viewed under an Olympus compound microscope model CX-31 using acid fuchsin as the stain.

The frequency of occurrence of each fungal taxa observed was calculated and recorded. The frequency of occurrence was determined as the total number of leaf segments from which a fungi was present divided by the total number of segments used for isolation (Laran *et al.*, 2002 In Bezerra *et al.*, 2013). Based on this, the microfungi were categorised into five (5) groups viz; most common (81-100%); common (61-80%); frequent (41-60%); occasional (21-40%) and rare (1-20%) (Kumar *et al.*, 2013).

Identification of the microfungi

Identification to genus/species level was done using the following keys: Ellis (1971) and (1976), Domsch *et al.*, (1980), Carmichael(1980), Hanlin (1997), Watanabe (2002) and other relevant resources. Given the complexity unavoidably associated with the identification of microfungi, most of the identifications were done to the genus level only and some others remained unidentified.

3. RESULTS AND DISCUSSION

In total, 14 species belonging to nine (9) genera were identified mainly from lesioned green leaves and senescent leaves (Table 1).

Table 1 showing the different microfungi identified.

S/N	Species	Type of specimen	Subdivision of Fungi	I.F*
1	<i>Chaetomium</i> sp	Senescent leaf	Ascomycotina	O
2	<i>Codinae fertilis</i>	Lesioned green leaf	Deuteromycotina	O
3	<i>Colletotrichum truncatum</i>	Lesioned green leaf	Deuteromycotina	R
4	<i>Cylindrocladium malesianum</i>	Lesioned green leaf	Deuteromycotina	R
5	<i>Cylindrocladium</i> sp	Lesioned green leaf	Deuteromycotina	R
6	<i>Cylindroladium scoparum</i>	Lesioned green leaf	Deuteromycotina	C
7	<i>Menisporosis</i> sp	Lesioned green leaf	Deuteromycotina	O
8	<i>Myriogenospora atramentosa</i>	Lesioned green leaf	Ascomycotina	R
9	<i>Oidiodendron echinulatum</i>	Senescent leaf	Deuteromycotina	O
10	<i>Oidiodendron</i> sp	Senescent leaf	Deuteromycotina	R
11	<i>Penicillium</i> sp 1	Senescent leaf	Deuteromycotina	R
12	<i>Penicillium</i> sp 2	Senescent leaf	Deuteromycotina	R
13	<i>Periconia byssoides</i>	Lesioned green leaf	Deuteromycotina	R
14	<i>Sterilia mycelia</i>	Lesioned green leaf	Deuteromycotina	R

I . F= Isolation Frequency.

MC= Most Common, C=Common, F=Frequent, O=Occasional, R=Rare.

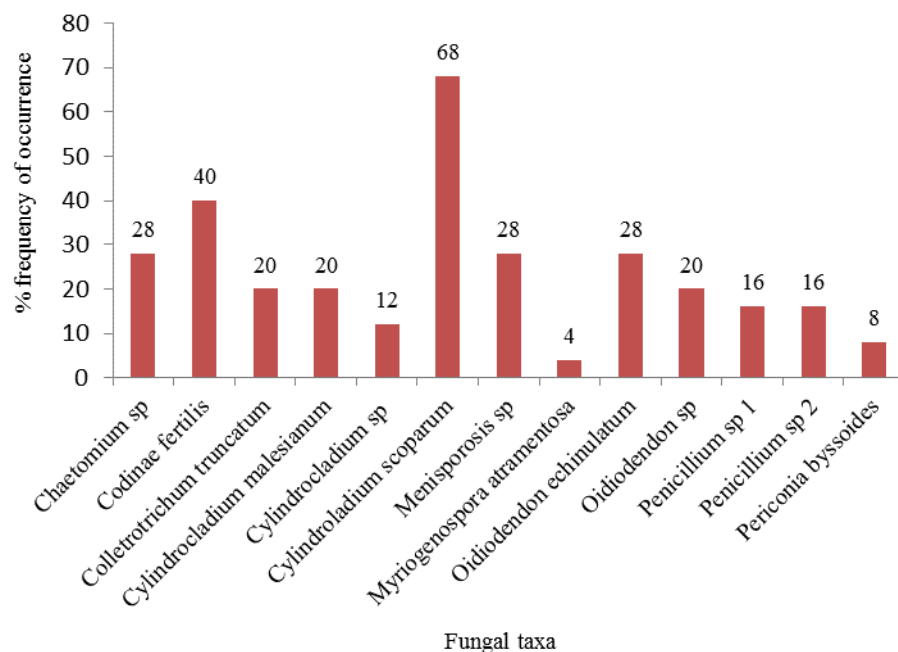


Figure 1: Percentage (%) frequency of occurrence of each species/genus identified.

Majority (86%) of the identified fungal species belonged to the subdivision Deuteromycotina and others (14%) belonged to the Ascomycotina (Table 1). The Deuteromycotina were represented by 12 species while the Ascomycotina was only represented by *Chaetomium* sp and *Myriogenospora atramentosa*. The high frequency of isolation of the Deuteromycotina can be attributed to the fact that majority of the Deuteromycotina are terrestrial fungi (Schoenlein-Crusius *et al.*, 1998). The Deuteromycotina are either saprophytes or parasites, thus confirming their isolation from lesioned green leaves and senescent leaves. This result is corroborated by the report of Schoenlein-Crusius and Milanez, (1989) and Schoenlein-Crusius *et al.*, (1998). Schoenlein-Crusius *et al.* (1998) found that out of 81 species of microfungi on leaves of *Alchornea triplinervia*, 50 species belonged to the Deuteromycotina. Similar result was also obtained by Schoenlein-Crusius *et al.*, 1990 on leaves of *Quercus robur*. Upadhyaya (2013) and Rodrigues (1994) also recorded a high occurrence of Deuteromycotina microfungi on the leaves *Taxus baccata* and the Amazonian palm *Euterpe oleracea* respectively.

The most frequently encountered genus in this study was *Cyindrocladium*, followed by *Penicillium* and *Oidiodendron* simultaneously. The genus *Cyindrocladium* was the most diverse with 3 species. *Cyindrocladium scoparium* had the highest frequency of occurrence of 68% followed by *Codinae fertilis*, then *Chaetomium* sp, *Menisporosis* sp, and *Oidiodendron* sp all with 28% occurrence, and *Myriogenospora atramentosa* being the least frequent species with an isolation frequency of 4% (Figure 1). Nine (9) species/genera were categorised as rare. This includes *Periconia byssoides*, *Menisporosis* sp, *Colletotrichum truncatum*, *Cyindrocladium malesianum*, *Cyindrocladium* sp, *Oidiodendron echinulatum*, *Oidiodendron* sp, *Penicillium* sp 1, *Penicillium* sp 2, and the *Sterilia mycelia*. A similar results have also been reported by other researchers (Kumar *et al.*, 2013; Shanthi and Vittal, 2010). *Cyindrocladium scoparium* was the only species common in this study and four (4) species were only found occasionally. This result is also supported by Iracema-Helena *et al.*, 1998's report that *Cyindrocladium scoparium* was commonly encountered.

4. CONCLUSION

This present study revealed that, though the sample size was comparatively low, a diverse group of microfungi are to be found on leaves in Santubong National Park with increased sampling efforts and samples.

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Lactobacillus acidophilus* modulates the virulence of *Clostridium difficile*: Inhibition of Autoinducer-2 of *C. difficile* ribotype O27*Bohyun Yun¹ Dong Jun Park², and Sejong Oh^{1*}**¹Division of Animal Science, Chonnam National University, South Korea²Korea Food Research Institute, South Korea**ABSTRACT**

Clostridium difficile is a spore-forming, gram-positive anaerobic bacillus that produces two exotoxins (toxin A and toxin B). Many pathogens synthesize an extracellular signal named LuxS or autoinducer-2 (AI-2), such as *E. coli* O157:H7, *C. perfringens*, and *C. difficile*. AI-2 has been implicated in control of virulence in pathogenic bacteria. Several studies have reported that probiotics are capable of interfering with QS in pathogens by decreasing AI-2 production. Particularly, *L. acidophilus* GP1B exhibited the highest inhibitory activity among all the *Lactobacillus* strains tested. The results obtained in this study suggest that probiotic preparations have potential for both prevention and treatment of *C. difficile*-associated diarrhea.

1. INTRODUCTION

Clostridium difficile-associated disease (CDAD) is increasing in incidence and severity. Moreover, its treatment is becoming more difficult. According to numerous recent studies, *C. difficile* has been isolated from raw foods intended for human consumption in the United States (Songer et al. 2009), Canada (Weese et al. 2009), and Europe (Bakri et al. 2009). Thus making *C. difficile* a potentially foodborne pathogen. (Gould and Limbago 2010). Many pathogens have the *luxS* gene, such as *E. coli* O157:H7, *C. perfringens*, and *C. difficile*. The autoinducer-2 (AI-2) has been implicated in the virulence factors in *C. perfringens*. Probiotics are defined as live microorganisms. When consumed in adequate amounts, there is a comparison in health benefits for the host (FAO/WHO, 2002). It is mostly studied in gastrointestinal health as well (Lenoir-Wijnkoop et al. 2007). Potential health effects in human intervention trials include relief of inflammatory bowel disease (Limdi et al. 2006), antibiotic-associated gastrointestinal symptoms (Guglielmetti et al. 2011) and anti-inflammatory effect (Maslowski et al. 2009). The purpose of this study is to evaluate the inhibitory activity of AI-2 in *C. difficile* culture by Lactic acid bacteria.

2. MATERIALS AND METHODS**Bacterial strains and culture conditions**

Brain Heart Infusion broth (Difco) supplemented with 0.5% yeast extract (Difco) and 0.1% L-cysteine (Sigma) (BHIS) was inoculated with *C. difficile* 027 and incubated overnight under anaerobic conditions at 37 °C. *L. acidophilus* GP1B were grown at 37 °C in MRS broth.

Screening of *Lactobacillus* isolates

Lactic acid bacteria stains were isolated from Korean infant feces, yogurt, cheese, and kimchi (traditional Korean fermented vegetable). Identification of the isolated test subjects was achieved by the 16S rDNA sequence analysis. For storage, stock cultures were maintained at -80 °C in 10% skim milk broth containing 0.2% yeast extract.

AI-2 activity assays

The bacterial supernatants were assayed using the techniques developed by (Surette and Bassler 1998). *C. difficile* 027 was grown overnight at 37 °C in BHIS, diluted 1:100 in the same fresh medium containing cell supernatants of Lactic acid bacteria (LAB). Then it was then grown at 37 °C. The supernatant passed through a 0.2 µm pore size cellulose nitrate membrane filter (Sartorius, Goettingen, Germany). The reporter strain, *V. harveyi* BB170, was grown overnight in autoinducer bioassay (AB) medium, and then diluted 1:5000 into fresh AB medium. Then, the cell-free supernatant from the *C. difficile* 027 was added to the diluted AB medium at a concentration of 10% (v/v). The luminescence values were measured with a Synergy HT multimode plate reader (BioTek, Winooski, VT). Each experiment was conducted in triplicate.

3. RESULTS AND DISCUSSION**Screening of lactic acid bacteria with high AI-2 inhibitory activity**

Lactic acid bacteria (LAB) was detected for *C. difficile* 027 AI-2 inhibition activity. A broad inhibition activity spectrum was observed across the 47 strains of lactic acid bacteria. Different LABs showed various degrees of AI-2 inhibition, with ranged from 0 to 89-fold regular inhibition. Among the 47 strains, six LAB strains (*L. acidophilus* GP1B, *L. helveticus* 1213, *L. acidophilus* CH5, *Lactococcus* *latis* 01, *L. plantarum* CSBS, and *L. rhamnosus* LGG) were selected as the high inhibition group. *L. acidophilus* GP1B exhibited the highest inhibitory activity among the six LAB strains tested (Fig. 1).

L. acidophilus GP1B was isolated from swine intestine, and subsequent studies have shown probiotic characteristics such as cholesterol lowering activity and bacteriocin production. In order to confirm the species of *L. acidophilus* GP1B, the sequence of the PCR product was compared with known 16S rRNA gene sequences in GenBank by multiple-sequence alignment. Sequencing of the 16S rRNA genes showed that they possessed 99% nucleotide sequence similarity to *L. acidophilus* (Fig. 2).

Inhibitory effect of *L. acidophilus* GP1B on AI-2 production in *C. difficile* 027

Antipathogenic activity of the cell-extract (CE) and cell-free supernatant (CFS) of *L. acidophilus* GP1B against *C. difficile* 027 was evaluated as the inhibitory effect of CE and CFS of *L. acidophilus* GP1B on AI-2 production in *C. difficile* 027.

10 to 40 µg/ml CE of *L. acidophilus* GP1B exhibited high inhibitory activities for all concentrations of CE and CFS of *L. acidophilus* GP1B (Fig. 3). Using AI-2 assay, the inhibitory effects of *L. acidophilus* GP1B on AI-2 production of *C. difficile* 027 was determined in the present study. When *C. difficile* 027 was grown in BHI broth supplemented with 10 µg/ml CE of *L. acidophilus* GP1B, bacterial growth was not inhibited. In addition, the extracellular AI-2 concentration was significantly reduced, to 35% at 6 h. The results indicate that CE of *L. acidophilus* GP1B inhibited AI-2 production in *C. difficile* 027.

Probiotics are live microorganisms that provide beneficial effects to the health of the host (Kun et al. 1999); they confer protection by inhibiting the growth of food-borne pathogens (Chateau et al. 1993). Also, single- and mixture-probiotics are effective to inhibit pathogens activity (Chapman et al. 2012). It has been reported that probiotics inhibit QS and virulence factor of *E. coli* O157:H7. (Kim et al. 2008; Kim et al. 2012).