IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM SPINES OF RATTAN (*Calamus castaneus*)

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by

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LIST OF SYMBOLS

α	alpha
β	beta
bp	base pair
^{o}C	degree Celsius
cm	centimetre
h	hour
g	gram
kb	kilo base
L	litre
M	molar
min	minutes
mA	micro ampere
μl	microlitre
ml	millilitre
mm	millimetre
n	total number
rpm	rate per minute
v	volt
γ	gamma

LIST OF ABBREVIATIONS

ACT	Actin
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
CaCl2	Calcium chloride
CHS	chitin synthase
CLA	Carnation Leaf-pieces Agar
$CuSO_4$	Cuprum sulphate
CYA	Czapek Yeast Agar
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DS	Disease severity
EI	Enzyme activity index
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GIC	Growth inhibition category
LSU	Large subunit region
HCl	Hydrochloride acid
H_3BO_3	Boric acid
ICTF	International Commission on the Taxonomy of Fungi
ITS	Internal Transcribed Spacer
KH_2PO_4	Potassium hydrogen phosphate
MEA	Malt Extract Agar
MEGA	Molecular Evolution Genetic Analysis
ML	Maximum likelihood
MnSO4	Manganese sulphate
MoO_3	Molybdenum oxide
NA	Nutrient agar
NaCl	Natrium chloride
Na_2HPO_4	Sodium hydrogen phosphate
NCBI	National Centre for Biotechnology Information
NCF	Nomenclatural Committee for Fungi
NaOCl	Sodium hypochlorite
$(NH_4)_2SO_4$	Ammonium sulphate
TBE	Tris-Borate-EDTA buffer

TEF-1α	Translation Elongation Factor-1α
PCA	Plate Count Agar
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGI	Percentage of growth inhibition
rDNA	ribosomal deoxyribonucleic acid
rpb2	RNA polymerase II subunit 2
rRNA	ribosomal Ribonucleic acid
SSU	Small-subunit
UV	Ultraviolet
$ZnSO_4$	Zink sulphide

PENGECAMAN DAN PENCIRIAN KULAT ENDOFITIK DARI DURI ROTAN (*Calamus castaneus*)

ABSTRAK

Kulat endofitik merupakan kulat yang mendiami tisu tumbuhan tanpa memberikan kesan negatif terhadap perumah. Kajian ini dijalankan untuk menentukan sama ada duri *Calamus castaneus* mengandungi kulat endofitik. *Calamus castaneus* (rotan cucor) dipilih kerana palma ini merupakan antara tumbuhan yang lazim ditemui dan penting dalam ekologi hutan Malaysia. Duri rotan adalah struktur pertahanan fisikal terhadap herbivor dan dilaporkan mengandungi patogen manusia. Cir-ciri morfologi digunakan untuk pengecaman kulat pada aras genus atau spesies. Bergantung kepada genus pencilan kulat, beberapa set gen digunakan untuk pengecaman secara molekul dan analisis filogenetik. Berdasarkan kawasan transkripsi dalaman (ITS) dan gliseraldehid 3-fosfat dehydrogenase (GAPDH), lima spesies *Colletotrichum* telah dikenalpasti sebagai *C. horii*, *C. siamense*, *C. fructicola*, *C. endophytica*, *C. boninense* dan *C. cliviae*. Spesies *Diaporthe* telah dikenalpasti secara filogenetik sebagai *D. arengae*, *D. hongkongensis*, *D.* cf. *heveae* 2, *D.* cf. *nobilis*, *D. arecae*, *D. tectonae* dan *Diaporthe* spp. berdasarkan jujukan ITS, faktor pemanjangan translasi-1alpha (TEF-1α) dan β-tubulin. Dua species *Phyllosticta* (*P. capitalensis* dan *P. carochloae*), *Trichoderma* (*T. harzianum* dan *T. koningiopsis*) dan *Neopestalotiopsis* (*N. saprophytica* dan *N. formicarum*) dikenalpasti menggunakan jujukan ITS dan TEF-1α. *Xylaria cubensis* dikenalpasti berdasarkan jujukan β-tubulin dan Aktin (ACT) manakala *Penicillium* (*Pen. indicum* dan *Pen. oxalicum*), *Cyphellophora guyanensis* dan *Arthrinium urticae* dikenalpasti berdasarkan jujukan ITS dan β-tubulin. Jujukan kawasan subunit besar (LSU) dan ITS telah mengenalpasti dua spesies *Helminthosporium* (*H. endiandrea* dan *H. livistonae*) dan *Nemania*

primolutea. *Fusarium lateritium*, *F. decemcellulare*, *F. oxysporum* dan *F. solani* dikenalpasti berdasarkan jujukan TEF-1α dan β-tubulin. *Cladosporium halotolerans* dikenalpasti menggunakan jujukan ITS dan ACT, manakala LSU digunakan untuk mengenalpasti *Pidoplitchkoviella terricola*. Spesies yang dikenalpasti mengunakan jujukan ITS sahaja adalah *Curvularia lunata*, *Bionectria pityrodes*, *Acremonium hennebertii*, *Muyocorpon laterale*, *Acrocalymma fici*, *Acro. medicaginis* dan *Endomelanconiopsis endophytica*. Kebolehan kepatogenan kulat endofitik menunjukkan *C. boninense* dan *Pen. oxalicum* patogenik pada daun *C. castaneus,* bertam (*Eugeissona* sp.) dan kelapa sawit (*Elaeis guineensis*), yang mencadangkan endofit ini berpotensi menjadi patogen tumbuhan. *Trichodrma harzianum* dan *T. koningiopsis* menunjukkan aktiviti antagonistik tertinggi terhadap 10 kulat patogen tumbuhan. Ini menunjukkan kulat endofitik tersebut mempunyai kebolehan untuk merencat pertumbuhan kulat patogen yang diuji. Penghasilan enzim ekstrasel iaitu, lipase, selulase, proteiase, pektinase, amilase dan urease oleh kulat endofit mencadangkan enzim yang dihasilkan boleh berfungsi sebagai pengurai untuk menembusi dan mengkolonisasi tisu tumbuhan dan juga membantu kulat endofit menyebabkan jangkitan dalam ujian kepatogenan. Kesemua enzim ini juga berperanan di dalam mekanisma pertahanan terhadap patogen disamping menghidrolosis bahan makanan dari perumah untuk memperolehi nutrien. Kajian ini mendapati duri *C. castaneus* mengandungi pelbagai kumpulan kulat endofitik, di mana beberapa spesies mungkin menjadi patogen tumbuhan, sebagai kulat antagonis terhadap patogen tumbuhan dan juga berkebolehan menghasilkan pelbagai enzim extrasel. Beberapa kulat endofitik dari duri rotan adalah species yang baru dilaporkan di Malaysia and kulat endofitik ini berkait dengan penyakit tumbuhan, sebagai kulat antagonis dan menghasilkan enzim extrasel.

IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM SPINES OF RATTAN (*Calamus castaneus*)

ABSTRACT

Endophytic fungi are fungi residing within plant tissues without causing any negative effects to the host. The present study was conducted to determine whether spines of *Calamus castaneus* harbour endophytic fungi. *Calamus castaneus* (rotan cucor) was chosen as the palm is among common and ecologically important plant in Malaysian forests. Spines of rattan are regarded as physical defensive structure against herbivores and reported to contained human pathogens. Morphological characteristics were used to tentatively identify the endophytic fungal isolates to genus or species levels. Depending on the genus, different sets of gene were applied for molecular identification and phylogenetic analysis. Based on Internal Transcribed Spacer (ITS) region and Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH), five *Colletotrichum* species were identified as *C. horii*, *C. siamense*, *C. fructicola*, *C. endophytica*, *C. boninense* and *C. cliviae*. *Diaporthe* species were phylogenetically identified as *D. arengae*, *D. hongkongensis*, *D.* cf. *heveae* 2, *D.* cf. *nobilis*, *D. arecae*, *D. tectonae* and *Diaporthe* spp. based on ITS, Translation Elongation Factor-1α (TEF-1α) and β-tubulin sequences. Two species of *Phyllosticta* (*P. capitalensis* and *P. carochloae*), *Trichoderma* (*T. harzianum* and *T. koningiopsis*) and *Neopestalotiopsis* (*N. saprophytica* and *N. formicarum*) were identified using ITS and TEF-1α sequences. *Xylaria cubensis* was identified based on β-tubulin and Actin (ACT) sequences while *Penicillium* (*Pen. indicum* and *Pen. oxalicum*), *Cyphellophora guyanensis* and *Arthrinium urticae*, were identified based on ITS and β-tubulin sequences. Large subunit region (LSU) and ITS sequences were used to identify two

species of *Helminthosporium* (*H. endiandrea* and *H. livistonae*) and *Nemania primolutea*. *Fusarium lateritium, F. decemcellulare, F. oxysporum* and *F. solani* were identified based on TEF-1α and β-tubulin sequences. *Cladosporium halotolerans* was identified using ITS and ACT, while LSU was used for identification of *Pidoplitchkoviella terricola*. Species identified using ITS sequences were *Curvularia lunata*, *Bionectria pityrodes*, *Acremonium hennebertii*, *Muyocorpon laterale*, *Acrocalymma fici*, *Acro. medicaginis* and *Endomelanconiopsis endophytica*. The pathogenic ability of the endophytes showed that *C. boninense* and *Pen. oxalicum* were pathogenic on *C. castaneus*, *bertam* (*Eugeissona* sp.) and oil palm (*Elaeis guineensis*) leaves suggested that the endophytes are potential plant pathogens. *Trichoderma harzianum* and *T. koningiopsis* exhibited the highest antagonistic activity against 10 phytopathogenic fungi indicated the ability to inhibit the growth of tested fungal pathogens. Production of extracellular enzymes including lipase, cellulase, protease, pectinase, amylase and urease by some of the endophytes suggested that the enzymes may function as degraders for penetrating and colonizing plant tissues and helped the endophytes to cause infection in the pathogenicity test. These enzymes may also play a role in defence mechanism against pathogens as well as hydrolyses food substances from the host to obtain nutrients. The study revealed that spines of *C. castaneus* harbour diverse groups of endophytic fungi of which several species may become plant pathogens, as antagonist to fungal plant pathogens as well as able to produce extracellular enzymes. Several endophytic fungi from the spines are new reported species in Malaysia and the endophytes were associated with plant diseases, as antagonist fungi and produced extracellular enzymes.

CHAPTER 1

INTRODUCTION

Rattans are climbing plants covered with spines and is classified in the palm family, known as Palmae or Arecaceae. The climbing behaviour of rattans are adapted by the spines covered on every parts of the plants. However, some species of rattans are not climbers and commonly referred as acaulescent rattans but are also regarded as rattan based on their morphological characteristics that are similar to the climbing rattans (Uhl et al., 1987; Baker et al., 2000).

Rattan plant is covered with spines, which are sharp appendages arose from modification of leaves. The spines can act as plant defences against mammalian herbivores which prevented herbivores from grazing and climbing the plant. The spines might deposited microorganisms, either dangerous or not to animals and humans (Halpern et al., 2007).

One of common rattan plants in Malaysian forest is *Calamus castaneus*. This non-climbing palm is locally known as *rotan cucor* and can be recognised by yellow-based spines covered on the stems and on the middle part of the upper leaves. The spines are arranged in one parallel line while at the bottom of the leaves, the spines are arranged in two parallel line (Dransfield, 1979).

Previously, there are reports of pathogenic bacteria and fungi residing in spines or thorns of spiny plants that are harmful to humans and animals. Halpern et al. (2007) reported 27 species of bacterial isolates were recovered from thorny and spiny plants comprising bacteria from the groups Proteobacteria, Firmicutes and Actinobacteria. These bacteria are either potential pathogens or opportunistic pathogenic bacteria. According to Halpern et al. (2011), not only bacteria are found in thorns, spines or prickles, there are also indications of dermatophytes (fungi that cause superficial infection on skin of human and animal) such as *Fusarium solani* from rose prickles (Kantarcioglu et al., 2010), *Fonsecaea pedrosoi* from *Mimosa pudica* spines (López Martínez & Méndez Tovar, 2007), *Sporothrix schenckii* and *Candida parapsilosis* from rose prickles (Engle et al., 2007). In addition to dermatophytes, other types of filamentous fungi may also be found residing in the spines as well as in other thorny structures. Thus, in the present study, the main aim is to determine whether filamentous fungi occurs in the spine, of rattan palm, *C. castaneus*.

Microbes found in plant parts such as in the roots, stems, leaves as well as spines are known as endophytes. Endophytes are unique as it has the ability to penetrate and colonise internal host tissues without causing any negative effects such as infection to their host (Schulz et al., 1993). Endophytes can be found in every plant parts of any plants with symbiotic lifestyle and various interactions within the host plant (Nair & Padmavathy, 2014). Endophytes may provide many advantages to its host such as promote growth and health of the host plant, protect the host plants from pathogen and able to tolerate abiotic stresses (Hallmann et al., 2007).

Filamentous fungi residing in spines of *C. castaneus* are endophytic fungi and so far, the occurrence of filamentous fungi in the spines of this rattan palm has not been reported. Spines of rattan which serve as defence structure against herbivores or seed predators may contained interesting and novel endophytes as most study of endophytes from defensive structures reported are human pathogens. The information of endophytic fungi in spines will contribute knowledge on the occurrence and biodiversity of endophytic fungi of which several species might be newly reported species in Malaysia. The endophytic fungi from the spines might be associated with plant diseases, as well as human and animal pathogens. The endophytes may also be antagonist fungi which have the potential to be develop as biocontrol agent. The endophytes also may produce extracellular enzymes and other metabolites that have the potential to be used in biotechnological processes.

For identification of endophytic fungi from spines of *C. castaneus*, morphological characterisation is the first step of identification. Morphological identification is based mainly on microscopic characteristics such as shapes and sizes of conidia, septation of the conidia, formation of conidiophores. The main macroscopic characteristics observed are the colour and appearances of the upper and lower fungal colonies. These characters can be used to identify the isolates to genus level, sometimes to species levels. However, some fungal isolates did not produce or produce less number of spores/conidia, or other microscopic characteristics are difficult to observe, molecular identification and phylogenetic analyses are employed (Hsieh et al., 2005).

For molecular identification, Internal transcribed spacer (ITS) region which is the DNA barcode for majority of fungi is commonly used. The ITS is a non-coding region and has high degree of variations that has reasonable ability to distinguish many groups of fungi to species level (Schoch et al., 2012). However, for several fungal genera, multiple genes or markers are required for identification as ITS showed less variation and ITS is not sufficient to identify species in a species complex or existence of cryptic species. Among the genes/markers employed are protein-coding genes, including Translation elongation factor-1α (TEF-1α), β-tubulin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actin (ACT) depending on the fungal genera. These protein-coding genes can be used to distinguish closely related species and cryptic species as well as provide information on phylogenetic relationships (Stielow et al., 2015).

For certain fungal genera such as *Diaporthe*, *Colletotrichum* and fungi that do not produce conidia, phylogenetic analysis is used to resolve species within the genus. Species identity based on phylogenetic analysis is known as phylogenetic species which is in accordance with phylogenetic species concept of fungi. In phylogenetic species concept of fungi, a group is formed whose members shared certain characters descended from a common ancestor. Thus, isolates that grouped in the same branching or clade in a phylogenetic tree are considered as the same species as they shared certain characters (Taylor et al., 2000; Martin et al., 2010).

Phylogenetic refers to study of relationships of species among individuals or group of organisms (Brinkman & Leipe, 2001). In phylogenetic analysis, sequences of common genes are used to estimate the evolutionary relationships of species illustrated by phylogenetic tree. Phylogenetic analysis will lead to identification, naming, and classification of entities, known as taxonomy which is naming a group of organisms based on shared sequence characteristics (Taylor et al., 2000). Phylogenetic tree is also used to distinguish species as well as to resolve species in species complexes (Taylor et al., 2000). Isolates that grouped in the same branching or clade in a phylogenetic tree are considered as the same species as they shared certain characters (Taylor et al., 2000).

Phylogenetic analysis of individual and combined sequences are used to identify fungal isolates and for confirmation of species identity in which the isolates of the same species are grouped together in a same clade, separated from other species in different clades (Martin et al., 2010). Combination of at least two genes/markers are often applied resulted with better species separation especially for cryptic species and with higher branch support values in terminal clades. Phylogenetic analysis is applied for identification of *Diaporthe* spp. and *Colletotrichum* spp. in which five genes/markers are often used for accurate species identification and to resolve cryptic species (Udayanga et al., 2012a; Weir et al., 2012).

Endophytic fungi can become pathogens when environmental conditions are favourable for disease development, often influenced by biotic and abiotic factors (Photita et al., 2004; Schulz & Boyle, 2006; Bacon et al., 2008). Therefore, pathogenicity test were conducted to verify whether the endophytic fungi from spines of *C. castaneus* were able to become pathogens. The pathogenic variability of the endophytic fungal isolates to cause disease symptoms on *C. castaneus* leaves were also determined. In addition, cross-pathogenicity on *bertam* (*Eugeissona* sp.) and oil palm (*Elaeis guineensis*) leaves were also conducted to determine whether the endophytes from spines of *C. castaneus* can caused infection on other plant parts.

Endophytes can be antagonistic fungi that inhibited the growth of other fungi (Mukherjee & Raghu, 1997). Many endophytic fungi have also been reported to be developed as potential biocontrol agents (Mejia et al., 2008; Gao et al., 2010). For example, several endophytic fungi from mangroves plant (*Rhizophora mucronata*) have potential use as antagonist which inhibited the growth of phytopathogenic fungus, *Fusarium solani* (Tuan Hamzah et al., 2018). Thus, there is a posibility that fungal endophytes from spines of *C. castaneus* can act as antagonitic fungi, and inhibited growth of several plant pathogens.

Endophytes have also been reported to have the ability to produce various types of extracellular enzymes (Choi et al., 2005). According to Choi et al. (2005), these enzymes are secreted by the endophytes to utilise different substrates within the host. Among the most common extracellular enzymes are cellulase, pectinase, lipase, protease and amylase were detected and could be used as a preliminary information of the endophyte’s interactions inside the host. Various types of extracellular enzymes may be produced by the endophytes from spines of *C. castaneus* which may represent the endophytes functional roles inside the spines of *C. castaneus*. Cellulase and amylase produced by the endophytes may indicated that the endophytes are saprophytes. Pectinase is produced as an indication of opportunistic or latent pathogens. Lipase is secreted mainly for nutrient acquisition while protease associated with mycoparasitism may suggest that the endophytes are potential biocontrol agent (Benítez et al., 2004; Choi et al., 2005; Feng et al., 2005).

As there is lack of information on the fungal endophytes from spines, the objectives of this study were:

(i) To isolate and identify endophytic fungi from spines of *C. castaneus* using morphological and molecular identification as well as phylogenetic analysis;

(ii) To determine the pathogenicity and virulence of selected endophytic fungi on *C. castaneus*, *bertam* and oil palm leaves;

(iii) To assess the ability of the endophytic fungi as antagonistic fungi to inhibit the growth of selected plant pathogenic fungi; and

(iv) To determine the ability of selected endophytic fungi to produce various types of extracellular enzymes, namely cellulase, pectinase, lipase, protease, amylase and urease.

CHAPTER 2

LITERATURE REVIEW

2.1 Origin, distribution & ecology of rattan

Rattan, a climbing palm originated from the Malay local name, “rotan”, belongs to the Palm family, Palmae or Arecaceae. There are about 600 species of rattan all over the world belonging to 13 genera. Rattan belongs to subfamily Calamoideae, in which the fruits have overlapping reflexed scales and every parts of the palm are covered with spines which adapted by its climbing habit (Dransfield, 1993). The subfamily Calamoideae comprises tree palms, Raffia (*Raphia*), Sago palm (*Metroxylon*) and shrub palms, Salak (*Salacca*) (Uhl et al., 1987). However, some species are not climbers, but shrubby palms included in the rattan genera as the reproductive characters are similar with other climbers species. Rattan can also be in clustering or solitary but there are some species that can be both, such as *Calamus subinermis*. Others are acaulescent, with no discernible stem at all (Dransfield, 1979).

Presence of wide forest habitats makes rattan an endemic plant (Dransfield, 1993). Rattan are mainly found in the Afro-Eurasia tropic and subtropics areas initially grows in primary rain and monsoon forests (Dransfield & Manokaran, 1993). Many species have limited natural ranges, but majority of the world rattans resources are from Indonesia. There are 600 species of rattans estimated worldwide, but only 10% are commercial species. The main product of rattans are furniture, roof, handicraft and food. The tropical rain forest in South-east Asia is the major habitat of rattans. It has been reported that the best quality canes are mainly from Malaysia and Indonesia. In Peninsular Malaysia, 106 species of rattan, belonging to eight genera grow naturally. However, only 30 species are utilized and exploited commercially (Dransfield, 1979).

In Peninsular Malaysia, naturally grown rattan in reserved forests are estimated to be around 32.7 million hectares (Nur Hasmiza & Mohd Nazip, 2010). Rattan is also available in Sarawak, covering an area of 12.5 million hectare with 105 species within eight genera have been identified with *Calamus* species covered around 2,222 hectare of the forest (http://www.forestry.sarawak.gov.my/page-0-0-647-Forest-Plantations.html). As for rattan in Sabah, no recent information is available. However, according to Dransfield (1984), 84 species of rattan were estimated in Sabah. In 1987, a holding company of Sabah foundation has planted several commercial rattan species in plantation over 40,000 hectares in Luasong, west of Sabah (Pinso & Vun, 2000).

Rattan species can be divided into two types; large diameter (>18mm) and small diameter (<18mm). The canes of large diameter consisted of rattan species that usually used for making furniture components, roof, and walking sticks such as *Calamus manan* (rotan manau), *C. ornatus* (rotan mantang), *Daemonorps grandis* (rotan sendang) and *D. melanochaetes* (rotan getah). The small diameter of rattan canes can be used for handicraft items and binding materials such as *C. caesius* (rotan sega), *C. insignis* (rotan batu) and *C. luridus* (rotan kerai). Some of the rattan's fruits are known as dragon's blood as it is being used as dye such as *C. didymophylla*, *C. propinqua* (rotan jernang) and *C. micracantha* (rotan jernang miang) while some are edible such as *Calospatha scortechinii* (rotan demuk), *Calamus paspalanthus* (rotan sirikis), *C. castaneus* (rotan cucor) and *C. lobbianus* (rotan cucor kelabu) (Norani et al., 1985; Abdul Razak & Raja Barizan, 1998).

Native people use the rattan leaves for cigarette papers such as *Daemonorps leptopus* (rotan bacap) and *Calamus longipathus* (rotan kunyung). The rattan leaves from *C. castaneus* (rotan cucor), *Daemonorps calicarpa* (rotan lumpit) and *D.*

kunstleri (rotan bulu landak) can be made into roof and thatch (Dransfield, 1979; Abdul Razak & Raja Barizan, 1998; Nur Hasmiza & Mohd Nazip, 2010).

Diversity of rattan species and their broad geographical area corresponded with their ecological diversity. Rattan can be found in a diverse type of forests and soils throughout their natural habitat. Some species grow in understory forest, while some depend on adequate sunlight penetration for their growth. Hence, rattan can be found in various ecological surroundings such as in marshes, swamps, and periodical inundated forest, some grows in gap vegetation, some can be found on dry narrow hilltops, while others reacts to canopy manipulation consequence from planned logging (Sunderland, 1990; Sunderland, 2001)

Adequate sunlight is required for rattan growth regardless of various ecological conditions. Sunderland (2001) reported rattan seedlings of Southeast Asia species and African species will survive on the forest ground for long periods until it receives sufficient sunlight and began to grow. Rattan seeds will only germinate in sufficient and extensive light conditions. The seedling on the forest floor is regarded as seedling bank and is a general trait of revival of most rattan species and a feature of forest where rattans occur.

2.1.1 *Calamus castaneus*

Calamus is the largest genus of rattan with approximately 370 species recorded worldwide. *Calamus castaneus* belongs to family *Arecaceae*/*Palmae*, subfamily *Calamoideae* and tribe *Calameae* (Uhl et al., 1987). Lineages classification of *Calamus castaneus* is as follows:

Domain: Eukaryota

Kingdom: Viridiplantae

Phylum/Division: Streptophyta

Class: Magnoliophyta

Order: Liliopsida

Family: Arecaceae/Palmae

Subfamily: Calamoideae

Genus: *Calamus*

Species: *castaneus*

In Malaysia, local name for *Calamus castaneus* is “rotan cucor”, and is one of the most common rattan in Peninsular Malaysia (Ruppert et al., 2012). The leaves are used for making roof and thatch while the seeds can be used for medical purposes. *Calamus castaneus* is a non-climbing rattan (**Figure 2.1A**), and the fruit is reddish brown like chestnut colour **(Figure 2.1B1**). Native people in Malaysia uses the fruits as a remedy for cough. It has yellow-based spines (**Figure 2.1B2**) and broad lush green leaves that are grey on the under surface and helps shape the undergrowth vegetation of primary lowland forest. The spines are arranged in one parallel line in the middle part of the leaf, while at the bottom of the leaves, the spines are arranged in two parallel line. It also produces inflorescences up to about 45 cm long (Dransfield, 1979).

Figure 2.1: **(A)** Rattan palm, *Calamus castaneus*; **(B1)** reddish brown fruits; **(B2)** yellow-based spines.

Natural populations *C. castaneus* is relatively preserved as it is less exploited by humans (Kidyoo & McKey, 2012). Thus, this rattan species is easily found in Malaysian forest and can be planted in large-scale plantation. Due to its availability and widely distributed, *C. castaneus* is considered as ecologically important plant in Malaysia. The plant helps to shape the forest vegetation (Putz & Sharitz, 1991) and vital source of food to insects, mammals or birds. Fruits, seeds and fresh shoots of this rattan plant has sweet and acidic taste which attracted primates such as macaques, mammals and birds (Dransfield, 1979; Ruppert et al., 2016), and various insects that consume pollen (Kidyoo & McKey, 2012). The height of *C. castaneus* is about 3 m tall which initiate shades for growth of lower plants (Ruppert et al., 2012).

In natural habitats, spines of *C. castaneus* mainly functioned as leaf litter trapping and aid climbing herbivory as the spines are always pointing upwards. The spines pierced the falling leaves from the canopy, trapped along the stems which leads to ant colonisations (Liu et al., 2019). Rattan spines-ant relationship include interactions of leaf-harvesting for fungal gardens (Wirth et al., 2013); seed-harvesting and seed-dispersal (Berg, 1975; Beattie & Culver, 1981).

Asexual propagation of seeds are common in *C. castaneus* with high germination rate that contribute to many generations and genetic variability affects the plant survival (Ruppert et al., 2012). The plant prefers watercourse area, however, it can also be found in drought soil. The survival and adaptation of this rattan plant might be associated with the presence of the endophytes inside. Many studies have shown the presents of endophytes help the host plants to tolerate stress factors (Potshangbam et al., 2017; Tuan Hamzah, 2018). Thus, any organisms that can be isolated from this plant species would be interesting in discovery of various enzymes and antifungal compounds.

2.2 Aposematisms

Aposematism is referred to as the use of bright colours by an organism to warn potential predators that it is dangerous, poisonous, or unpleasant. The bright colours shown by the organism such as red, yellow, black, brown, or mixture of these colours warns the prey and therefore preventing it from attacking the organism (Lev-yadun, 2009). In plants, aposematic coloration is shown by spiny, thorny, and prickly plants (**Figure 2.2**) that warns animals that the plants are inedible or hard to swallow.

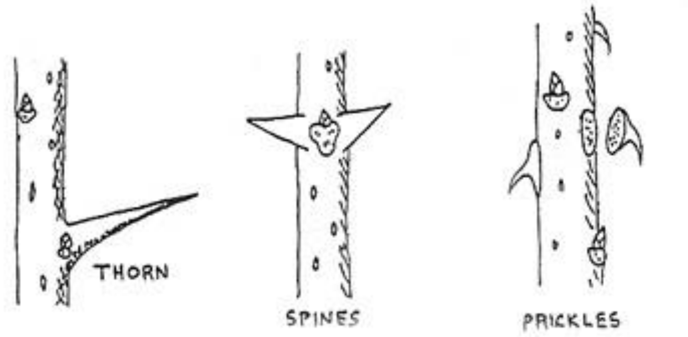

Figure 2.2: Defence mechanism of plant; thorn, spines and prickles (Lev-Yadun, 2016).

These defence mechanisms are modification of the plant appendages whereby spines are modified from leaves, whereas thorns are modification from branches, and prickles resulted from the outgrowth of cortical tissues from the bark (Lev-Yadun & Ne'Eman, 2006; Halpern et al., 2007; Lev-yadun & Gould, 2008). These sharp appendages are commonly colourful, or white with colourful stripes and spots and clearly visible due to the coloration association formed by the tissues, including white markings (Halpern et al., 2007). The sharp structures provide physical protection to the plants by causing injury to body parts of herbivores including mouth and intestinal system (Rebollo et al., 2002). Herbivores will eventually discover and familiar that spines, thorns, and prickle along with the bright colours shown by the structures and thus avoiding the noxious plants (Lev-yadun & Gould, 2008). In addition, plants itself can be aposematic due to its poisonous nature displayed by its coloration such as poisonous mushrooms (Lev-Yadun & Ne'Eman, 2006).

Aposematisms can be seen on spines of *C. castaneus* in which the spines are bright yellow, and sometimes are brown and black in colour. The spines base are also bright yellow. Among rattan plants, *C. castaneus* has the densest spines on stem with more than 300 spines per 20 cm (Liu et al., 2019). Higher density of spines act as deterrence and reduce the efficiency of herbivory. Several studies indicated spines have reduced the biomass losses caused by herbivores and also decreased the cruising of small climbing mammals (Cooper & Ginnett, 1998; Barton & Koricheva, 2010). However, the spines of *C. castaneus* did not deter small climbing mammals as the spines are pointing upwards, which makes it less effective to hinder small climbing mammals (Liu et al., 2019).

There are also plants species without conspicuous defensive structures but equipped with an alternative sharp defensive structure internally such as silica needles and raphides (**Figure 2.3**) (Lev-Yadun, 2009). Deposited silicon enters through plant roots and formed silica needles inside the plant parts (Richmond & Sussman, 2003) while the needles are made of calcium oxalate. Raphides are needle-shape, elongated with two sharps and pointed ends formed in the idioblast cells of certain plants (Fahn, 1990). From scanning and transmission electron microscopes, raphides appeared to be spiny or may have deep line along them (Lev-Yadun, 2009). Silica needle appear singly while raphides occurred as a bundle of needles.

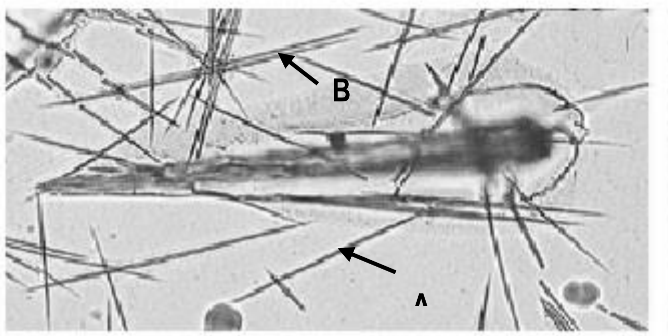

Figure 2.3: Defensive structures. **(A)** Individual needle; **(B)** needle bundle, raphides (Prince, 2012).

2.2.1 Microbes and aposematism

Halpern et al. (2007) conducted a study on spines and thorns from date palm trees and hawthorn in Israel and found that the spines and thorns harbour various aerobic and anaerobic harmful bacteria such as *Bacillus anthracis*, *Pantoea agglomerans*, and *Clostridium perfingens*. The spines that contain the microbes can caused skin injuries, wound at mouth and digestive system when herbivores touch and ingest the plants and at the same time inject the pathogenic microbes. These spines also contained pathogenic fungi, *Sporothrix schenckii* and *Cladophialophora carrionii* that can caused septic inflammation and subcutaneous mycoses on skin by a puncture wound affected by plant thorn injury (Halpern et al., 2007; Martínez & Tovar, 2007).

The silica needle and the spiny structures of raphides served as passage by which plant toxins are secreted into the herbivores tissues that enters from the wounded tissue and at the same time capable to inject pathogenic microorganisms and caused mechanical irritation (Lev-Yadun, 2009). Microorganisms that already exist on the plant surface as well as in the mouth and intestinal system enters through wounds caused by the silica needles and raphides and are able to cause infection. All of the defensive structures which are thorns, spines, prickles, raphides, and silica needles can also shoot in the pathogenic microorganism into the sensitive mouth and later into digestive systems of herbivores (Lev-Yadun, 2009).

As a conclusion, spines, thorns, prickles, silica needles and raphides are able to introduce microbes into herbivores through wound, subsequently pass through the skin that may cause serious infections which is more painful and hazardous than physical wounding (Halpern et al., 2007).

2.3 Endophytic fungi

Endophytic fungi occurred in plant tissues for at least part of their life cycle without effecting their host. It colonizes healthy plant tissues internally with unobtrusive infections and symptomless infected tissues. Therefore, endophytic fungi are defined as fungi that live inside its host's tissues without causing damage or any harm to its host (Schulz et al., 1993) and considered as mutualistic (Carroll, 1988). However, this interaction may occur short-term and interchangeable over time. Hence, endophytic fungi can occur as latent pathogens that reside in the host plant without any symptoms for a part of their life (Petrini, 1991). Endophytic fungi can be found in every plant parts including leaves, twigs, petioles, stems and spines, and has been reported in many plants species (Nair & Padmavathy, 2014).

Endophytic fungi can be categorized as clavicipitaceous endophytes (C-endophytes) and the non-clavicipitaceous endophytes (NC-endophytes) (Rodriguez et al., 2009). The C-endophytes are known as Class 1 endophytes with systemic intercellular infections and consist of endophytes of grasses and naturally found inside plant shoots (Bischoff & White, 2005). The Class 1 endophytes are divided into three types; Type I comprising various types of symptomatic and pathogenic species, Type II with mix interaction, and Type III with asymptomatic endophytes (Clay & Schardl, 2002). These endophytes can be transmitted vertically when mother plants infecting the offspring through seed infection (Saikkonen et al., 2002). The endophytes benefit its host by increases the hosts biomass, improves its hosts stress tolerance (e.g. drought) and protecting its host from animals and herbivory by producing toxic chemicals (Clay, 1988). Nevertheless, these benefits are influence by environmental conditions, plant species and plant genotype (Saikkonen et al., 1998; Faeth & Fagan, 2002). Example of grass endophyte is *Colletotrichum endophytica* which was isolated from two common tropical grasses; *Pennisetum purpureum* (dwarf napier) and *Cymbopogon citratus* (lemon grass) in Thailand (Manamgoda & Udayanga, 2013).

Class 3 endophytes occurred only in the above ground plant tissues of which the endophytes are transmitted horizontally and forms highly localized infections. The Class 3 endophytes are well known for their high diversity within individual host tissues, plants, and populations (Rodriguez et al., 2009). For example, individual leaves may contain one isolate of endophytic fungi per 2 mm^2 of leaf tissue indicated that hundreds of endophytic fungal species may harbour in an individual plant (Arnold et al., 2000).

Diversity of Class 3 endophytes occurred in tropical plants as well as in temperate and boreal plant communities (Higgins et al., 2007; Rodriguez et al., 2009). Although endophytic fungi reside in the host without any symptoms, it can become pathogen when the conditions are suitable for disease development, and this is regarded as latent pathogen. During normal growth conditions, relationships between endophytic fungi and the host are in balance. This neutral relationship depends on biotic factor (host genotype) and abiotic stress factor (soil, temperature, water). When the balance relationship is disturbed, the host's fitness weakened, and later reduced the plant protection and subsequently followed by disease development (Photita et al., 2004; Schulz & Boyle, 2005; Bacon et al., 2008). A study done by Bacon et al. (2008) reported that *Fusarium verticillioides* isolated from symptomless maize exist as endophytes, but could develop disease when unfavourable conditions occurred.

Most of Class 3 endophytes are Ascomycetes (Hyde & Soytong, 2008) and some are Basidiomycetes (Rungjindamai et al., 2008). A study by Raja et al. (2017) showed several genera of Ascomycota including *Alternaria* sp., *Penicillium* sp. and

Thielavia sp. were isolated from leaf of milk thistle (*Silybum marianum*). In another study, many genera of basidiomycetes such as *Bjerkandra* sp., *Ceriporia* sp. and *Phanerochaete* sp. were isolated from rubber tree (*Hevea* spp.) (Martin et al., 2015). The endophytic fungi produce hyphal fragmentation, sexual or asexual spores on dead or aging tissues of which these structures are dispersed by wind, rain, or transported by herbivores or insects (Arnold, 2008; Feldman et al., 2008). High humidity due to dew, rain, and fog, as well as the presence of airborne inoculum accelerate the colonization of Class 3 endophytes (Arnold & Herre, 2003).

In the tropics, there are many studies on endophytic fungi from various types of plants. The studies were done to determine the diversity of a particular group of fungi or to determine the ecological group of a particular group of fungi. Different fungal genera obtained indicated the diversity of fungi that reside in a particular host plant. For example, a study by Bezerra et al. (2015) found 28 isolates of endophytic fungi including *Acremonium curvulum*, *Aspergillus ochraceus*, *Gibberella fujikuroi*, and *Penicillium glabrum* isolated from medicinal plant, *Bauhinia forficate* in Brazil.

Endophytic fungi produced secondary metabolites and bioactive compounds that are utilised by the host for protection against pathogens. These natural compounds have been reported to be beneficial to human as sources of novel secondary metabolites (Debbab et al., 2011), novel drug discoveries, application in agriculture, and as industrial enzymes (Mahfooz et al., 2017) that have the potential to be developed into useful products such as antibiotics, antimicrobial, immunosuppressant, anticancer agents, and biological control agent (Joseph & Priya, 2011).

2.3.1 Benefits of endophytic fungi to the host plants

Endophytic association benefits the host plants and the endophytes itself. Endophytic fungi that live in favourable environment in the plant's internal tissues obtained shelter and protection from the enemies. The host shielded the endophytes from any biotic and abiotic stresses as well as ensure less competition with other microorganisms. It also gets direct nutritional elements within the internal tissues (Rodriguez et al., 2009).

Endophytes obtain metabolites produced by the host without causing apparent effect on the host performance (Hardoim et al., 2015). In return, the endophytes supply its hosts plant with growth promoting substances such as indole acetic acid or gibberellins, that can stimulates the plant growth (Nair & Padmavathy, 2014; Jia et al., 2016). A study by Hamayun et al. (2009) found a new endophytic fungus, *Cladopsorium spaerospernum* isolated from roots of soybean (*Glycine max*) can produce higher amount of bioactive compounds which was gibberellins (GA3, GA4, and GA7) that enhance maximum plant growth of soybean and rice varieties.

Endophytes can also benefit it host plant with phytostimulation by enhancing the nutrient uptakes of the plant host, and also helps in nitrogen fixation (Bo et al., 2015). Commonly nutrients required by plants are obtained from soil, water, atmosphere, and organic matter in chemical forms (Nair & Padmavathy, 2014; Jia et al., 2016). *Phomopsis liquidambari*, an endophytic fungi isolated from inner bark of bishop wood (*Bischofia polycarpa*) was able to increase the contents of organic composites in rice root exudates, alter the composition and oversupply of ammonia-oxidizers, and as nitrogen-fixers whereby it induces the content of nitrogen in soil with lower nitrogen availability (Bo et al., 2015). A study by Malinowski et al. (2000)

reported a leaf fungal endophyte, *Neotyphodium coenophialum* in tall fescue (*Festuca arundinacea*) effect its host's root activity and induce mineral uptake rate, and also helps the host to adapt in phosphorus deficiency.

2.3.2 Antifungal activity of endophytes

Many species of endophytic fungi produced bioactive metabolites, which have inhibitory properties, or contain antimicrobial compounds that shields the host plants from pathogens and herbivores by inhibit the growth of the plant pathogen. The antimicrobial compounds are also capable to inhibit the growth of microbial pathogens of humans and animals (Nair & Padmavathy, 2014). Bin et al. (2014) isolated 61 endophytic fungi including *Colletotrichum* spp., *Phomopsis* spp., *Alternaria* spp., *Phyllosticta* spp., and *Cladosporium* spp. from leaf of mangrove plant (*Aegiceras corniculatum*). Among the species identified, *Colletotrichum gloeosporioides* showed inhibitory activity against two human pathogenic bacteria, *Klebsiella pneumonia* and *Acinetobacter baumanii* (Bin et al., 2014).

The antimicrobial compounds from endophytic fungi has the potential to be used as biological control agent (BCA) against diseases and pests (Nair & Padmavathy, 2014; Jia et al., 2016). Biocontrol agents is preferred as it is environmental friendly, that can reduce negative effects to its surrounding compared to chemical control (Agrios, 2005). Several studies have been conducted to determine the potential of several endophytic fungi as BCA. Chen et al. (2016a) found an endophytic fungi, *Trichoderma gamsii* isolated from healthy ginseng (*Panax notoginseng*) produced volatile organic compounds identified as dimethyl disulphide, dibenzofuran, methanethiol, and ketones, were able to suppress growth of several pathogens, including *Epicoccum nigrum, Scytalidium lignicola, Phoma herbarum,* and *Fusarium flocciferum* that caused root-rot disease on *Panax notoginseng.* Shentu et al. (2014) found *Trichoderma brevicompactum* isolated from garlic, secreted trichodermin (4β-acetoxy-12,13-epoxy-h9-trichothecene), an active metabolite that showed strong antifungal activity against two phytopathogens, *Rhizoctonia solani* and *Botrytis cinerea.*

The mechanisms of antagonism shown by potential BCA by endophytic fungi are similar with other fungi. Among the well-known mechanisms are mycoparasitism, antibiosis, and competition of which the antagonistic microorganisms display these mechanisms to the target pathogens. Previous studies have shown the used of endophytic fungi, *Trichoderma* spp. with mode action of antibiosis in suppressing the growth of pathogenic fungi, *Rhizoctonia solani* and *Botrytis cinerea* (Shentu et al., 2014; Talapatra et al., 2017). Villamizar-Gallardo et al. (2017) suggested that *Botryosphaeria quercum*, an endophytic fungus isolated from cacao pod (*Theobroma cacao*) successfully inhibited *Phytopthora palmivora* and *Moniliophtora roreri* that caused black pod disease and frosty pod diseases respectively by competing for limiting nutrients and space.

Study of antagonistic activity of endophytic fungi from palm has been reported by Song et al. (2016) in which endophytic fungi were isolated from 10 species of palms including *Mascarena lagencuulis* and *Chrysalidocarpus lotescens* in Bangkok, Thailand. In the study, endophytic *F. chlamydopsorum*, *Phialophola* spp. and *Nigrospora* spp. significantly inhibited the growth of *C. coffeanum*, causal pathogen of anthracnose on coffee leaves.

2.3.3 Extracellular enzyme activity

Extracellular enzymes produced by endophytic fungi act as one of the resistance tools against pathogen by inhibiting growth of the pathogen (Terhonen et al., 2016) and hydrolyses food substances to obtain nutrients from the host (Sunitha et al., 2013). In addition, extracellular enzymes help in defence mechanisms by degradation of pathogen cell wall during mycoparasitism (Pozo et al., 2004; Hoell et al., 2005; Kredics et al., 2005). Endophytic fungi produced a particular extracellular enzyme according to their substrate utilization pattern (Carroll & Petrini, 1983). Pectinase may be release if the endophytes are latent pathogens, whereas, cellulase and amylase may be release if they are mutualistic, or saprophytic (Choi et al., 2005).

Commercially important enzymes produce by fungi are more stable, and the production of the enzymes are easier and safer. Production of enzymes from microbes will not affect the environment, as they are biodegradable and usually carry out at mild pH values and at room temperature (Nielsen & Oxenbll, 1998). Many endophytic fungi are able to produce potential sources of enzymes that can be used in many applications such as detergent manufacturing, starch conversion, textile technology, animal feed production, food preparation, leather treatment, and in paper industry (Nielsen & Oxenbll, 1998; Sunitha et al., 2013).

Cellulase is one of the most important enzymes that can be produce by several endophytic fungi for decomposition of cellulose in cell wall of plant, wood, and leaf litter. Cellulase enzyme also assist its host to assimilate cellulose (complex carbohydrate) that readily exist in its host (Lynd et al., 2002). Therefore, it is widely used in making detergents, textile technology, food preparation, and animal feed (Nielsen & Oxenbll, 1998). A study by Choi et al. (2005) discovered several endophytic fungi, namely *Colletotrichum* spp., *Phomopsis* spp., and Sterile mycelia isolated from *Brucea javanica* (woody shrub) were able to degrade cellulose and simpler sugars present in dead leaves and wood as they capable to cause weight loss in wood blocks test. Ribeiro et al. (2018) reported that endophytic *Diaporthe anacardii* isolated from leaves of golden shrimp plant (*Pachystachys lutea*) has the potential for cellulase production.

Pectinases are responsible in degradation of pectin substances, which commonly located between plant lamella and primary cell wall, and further assist in decomposition of plant litter (Gummadi & Panda, 2003). Many endophytic fungi are producer of pectinases and many pectinases have been commercially used in food and beverages industries (Sin et al., 2006). Pectinases are commonly used to speed up the process of fruit juice extraction from fruits such as apple, as it involves hydrolysing plant materials. Pectinase also degraded starch and pectin that made the finished fruit juice clear from murkiness as well as increase the storage stability (Mieszczakowska-Frac et al., 2012). An endophytic fungus, *Talaromyces* sp. from a medicinal plant, *Calophyllum inophyllum* showed optimal activity of pectinase and indicated as one of the potential sources of pectinase in food industries such as in food preparation and confectionaries (Sunitha & Srinivas, 2017).

Lipase is able to hydrolyses ester bonds, triglycerides, and synthesize ester bonds which make it widely used in esterification, alcohol lysis, and transesterification catalysts that usually produced by microorganisms, animals and plants (Illanes, 2008). Endophytic fungi are one of the main sources of lipases as fungi are able to release the enzyme in masses in multiple ways, which is related to the enzyme property and substrate (Pacheco et al., 2015). In addition, lipases produced from fungi are more stable in organic solvents, can performed reaction without cofactors and able to react on various substrates (Hasan et al., 2006). Several endophytic fungi including *Aspergillus chartarum*, *A. ochraceus, Myrmecridium schulzeri, Myrothecium verrucaria* and *Penicillium glabrum* isolated from a medicinal plant (*Bauhinia forficate*) in Brazil was reported as a good producer of lipases (Bezerra et al., 2015).

Protease, glucanase and chitinase are some of the hydrolytic enzymes produce by endophytic fungi that can degrade the cell walls of pathogens (Fouda et al., 2015) and therefore have the potential to be developed as biocontrol agent. Secretion of these hydrolytic enzymes, lyse the pathogen cell wall, enable the penetration of endophytic fungi, and subsequently hydrolyse the pathogen's cell wall (Jia et al., 2016). These enzymes also assisted to overcome plant protection barrier, penetrates and colonizes the host plants and sequentially gain nutrient for their growth (Amirita et al., 2012; Sunitha et al., 2013). Endophytic *Fusarium oxysporum* isolated from healthy flowering banana plants was able to control banana nematodes; *Pratylenchus goodeyi* and *Helicotylenchus multicinthus* that caused crop damage and banana yield drop. Protease secreted by *Fusarium oxysporum* lead to paralysis and mortality of the nematodes by penetrating the nematode's cuticles and damaging the nematodes structures and their eggs (Ng'ang'a et al., 2011).

Amylases produce by certain endophytic fungi degrade starch into simple carbohydrates and subsequently assimilated by the fungi and the host (Fouda et al., 2015). In biotechnology application, amylases hydrolysed starch into sugar syrups which is widely used in food processing. Amylases are also being used in various industrial sectors including pharmaceuticals, textiles and detergent (Zaferanloo et al., 2014). Endophytic *Penicillium chrysogenum* isolated from a medicinal plant (*Asclepias sinaica*) showed high activity of amylases, which involves in polysaccharides and proteins degradation during plant maturity and utilized the starch