MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Colletotrichum* SPECIES ISOLATED FROM ANTHRACNOSE OF RED-FLESHED DRAGON FRUIT (*Hylocereus polyrhizus*)

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by

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LIST OF UNITS, SYMBOLS AND ABBREVIATIONS

µm	Micrometer
ACT	Actin
AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAL	Calmodulin
CHS-1	Chitin synthase 1
CVX	Cactus Virus X
dNTP	Deoxyribonucleic triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCPSR	Genealogical Concordance Phylogenetic Species Recognition
GS	Glutamine synthetase
h	Hour
ha	Hectare
HIS-3	Histone3
IDF	Insoluble Dietary Fibre
ITS	Internal Transcribed Spacer
kb	Kilobase
MAT	Mating type
ML	Maximum Likelihood
mM	Millimolar

mtDNA	Mitochondrial deoxyribonucleic acid
NaOCl	Sodium hypochlorite
NJ	Neighbour Joining
NNI	Nearest Neighbour Interchange
OA	Oatmeal Agar
PCA	Potato Carrot Agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
psi	Pressure per square inch
rDNA	Ribosomal deoxyribonucleic acid
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
s	Second
SDF	Soluble Dietary Fibre
SNA	Synthetic nutrient-poor agar
TBE	Tris-Boric acid-EDTA
UV	Ultraviolet light
V	Volt

PENCIRIAN SECARA MORFOLOGI DAN MOLEKUL SPESIES *Colletotrichum* DIPENCIL DARIPADA ANTRAKNOS PADA BUAH NAGA ISI MERAH (*Hylocereus polyrhizus*)

ABSTRAK

Buah naga isi merah (*Hylocereus polyrhizus*) dianggap sebagai tanaman buahan kesihatan dan boleh membawa pulangan ekonomi yang tinggi kepada Malaysia. Keluasan kawasan penanaman tanaman ini semakin meningkat setiap tahun dan ditanam dengan meluas di Malaysia. Walaubagaimanapun, tanaman *H. polyrhizus* mudah dijangkiti oleh pelbagai penyakit disebabkan oleh kulat, bakteria dan virus dan menjadi masalah besar kepada penanaman *H. polyrhizus* di Malaysia. Salah satu penyakit penting disebabkan oleh kulat yang menjangkiti tanaman *H. polyrhizus* adalah antraknos, disebabkan oleh spesies *Colletotrichum*. Oleh itu, objektif utama kajian ini adalah untuk memencil, mengenal pasti dan mencirikan spesies *Colletotrichum* yang berasosiasi dengan *H. polyrhizus* berdasarkan pada ciri-ciri morfologi, penjujukan DNA bagi kawasan Penjarak Transkripsi Dalaman (ITS+5.8S) dan gen ß-tubulin, ujian kepatogenan dan menentukan variasi genetik pencilan *Colletotrichum* menggunakan analisis filogenetik jujukan ITS+5.8S dan ß-tubulin. Sejumlah 73 pencilan *Colletotrichum* diperoleh daripada batang *H. polyrhizus* yang berpenyakit daripada enam negeri di Semenanjung Malaysia (Johor, Kedah, Negeri Sembilan, Pahang, Pulau Pinang and Selangor). Berdasarkan ciri-ciri morfologi, 63 pencilan dikenal pasti sebagai *C. gloeosporioides* dan 10 pencilan dikenal pasti sebagai *C. truncatum*. Pencarian kesamaan menggunakan 'Basic Local Alignment Search Tool' (BLAST) jujukan ITS+5.8S dan ß-tubulin mengesahkan identiti *C. gloeosporioides* (63 pencilan) dan *C. truncatum* (10 pencilan) seperti yang telah dikenalpasti menggunakan ciri-ciri morfologi. Berdasarkan analisis filogenetik, pencilan-pencilan *C. gloeosporioides* dan *C. truncatum* dibahagikan kepada dua

kelompok yang berasingan. Walaupun pencilan-pencilan *C. gloeosporioides* daripada antraknos batang *H. polyrhizus* berkumpul dalam kelompok yang sama tetapi berbeza dari segi genetik dengan strain epitip *C. gloeosporioides*. Keputusan ini menunjukkan pencilan-pencilan *C. gloeosporioides* daripada antraknos batang *H. polyrhizus* berkemungkinan daripada spesies yang lain yang tergolong di dalam kompleks spesies *C. gloeosporioides*. Pencilan-pencilan *C. truncatum* daripada antraknos batang *H. polyrhizus* berkelompok dengan strain epitip *C. truncatum* di dalam satu kelompok monofiletik dan dari segi genetiknya sama dengan strain epitip tersebut, oleh itu mengesahkan identiti pencilan-pencilan tersebut sebagai *C. truncatum*. Berdasarkan ujian kepatogenan, 57 pencilan *C. gloeosporioides* dan 10 pencilan *C. truncatum* adalah patogenik terhadap batang *H. polyrhizus* dengan rawatan luka dan kedua-dua spesies *Colletotrichum* berjaya dipencilkan semula daripada lesi yang terhasil. Postulat Koch ditepati dan pencilan-pencilan tersebut disahkan sebagai penyebab antraknos batang *H. polyrhizus*. Melalui ujian jangkitan bersilang terhadap buah *H. polyrhizus*, kesemua wakil pencilan daripada *C. gloeosporioides* (15 pencilan) dan *C. truncatum* (5 pencilan) adalah patogenik terhadap buah *H. polyrhizus* dengan rawatan luka. Pemencilan semula *C. gloeosporioides* dan *C. truncatum* daripada lesi antraknos pada buah berjaya dilakukan. Keputusan ujian kepatogenan dan jangkitan bersilang menunjukkan kedua-dua spesies *Colletotrichum* mampu menjangkiti batang dan buah *H. polyrhizus*. Kesimpulannya, dua spesies *Colletotrichum* penyebab antraknos batang *H. polyrhizus* diperoleh, iaitu kompleks spesies *C. gloeosporioides* dan *C. truncatum* dan maklumat yang diperoleh boleh digunakan untuk merangka strategi pengawalan penyakit.

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Colletotrichum* SPECIES ISOLATED FROM ANTHRACNOSE OF RED-FLESHED DRAGON FRUIT (*Hylocereus polyrhizus*)

ABSTRACT

Red-fleshed dragon fruit (*Hylocereus polyrhizus*) is regarded as a health fruit crop and can bring high economic returns to Malaysia. The cultivation areas of this crop is increasing yearly and is now widely cultivated in Malaysia. However, *H. polyrhizus* is vulnerable towards multiple diseases caused by fungi, bacteria and virus, which is the major problem for *H. polyrhizus* cultivation in Malaysia. One of the important diseases caused by fungi infecting *H. polyrhizus* is anthracnose caused by *Colletotrichum* species. Thus, the main objectives of this study were to isolate, identify and characterize the *Colletotrichum* species associated with anthracnose of *H. polyrhizus*. The analyses were based on morphological characteristics, DNA sequencing of Internal Transcribed Spacer regions (ITS+5.8S) and β-tubulin gene, the genetic variation among the *Colletotrichum* isolates using phylogenetic analysis of ITS+5.8S and β-tubulin sequences and pathogenicity test. A total of 73 isolates of *Colletotrichum* were recovered from diseased *H. polyrhizus* stems from six states (Johor, Kedah, Negeri Sembilan, Pahang, Pulau Pinang and Selangor) in Peninsular Malaysia. Based on morphological characteristics, 63 isolates were identified as *C. gloeosporioides* and 10 isolates were identified as *C. truncatum*. Basic Local Alignment Search Tool (BLAST) similarity search of ITS+5.8S and ß-tubulin sequences confirmed the identity of morphologically identified *C. gloeosporioides* (63 isolates) and *C. truncatum* (10 isolates). Based on phylogenetic analysis, *C. gloeosporioides* and *C. truncatum* isolates were divided into two separate groups. Although *C. gloeosporioides* isolates from stem anthracnose of *H. polyrhizus* were

grouped in the same main clade but they were genetically not the same with *C. gloeosporioides* epitype strain. The results indicated that *C. gloeosporioides* isolates from stem anthracnose of *H. polyrhizus* might belong to other species in the *C. gloeosporioides* species complex. *Colletotrichum truncatum* isolates from stem anthracnose of *H. polyrhizus* clustered with *C. truncatum* epitype strain in a monophyletic clade and genetically the same as the epitype strain, hence confirming the isolates identity as *C. truncatum*. Based on pathogenicity test, 57 isolates of *C. gloeosporioides* and 10 isolates of *C. truncatum* were pathogenic to *H. polyrhizus* stem using wounded treatment and both *Colletotrichum* species were successfully reisolated from the anthracnose lesions. Koch's Postulates were fulfilled and the isolates were confirmed as the causal pathogens of stem anthracnose of *H. polyrhizus*. From cross-infection test on *H. polyrhizus* fruit, all representative isolates of *C. gloeosporioides* (15 isolates) and *C. truncatum* (five isolates) were pathogenic to *H. polyrhizus* fruits using wounded treatment. Reisolation of *C. gloeosporioides* and *C. truncatum* from anthracnose lesion on the fruits were successfully carried out. Results of pathogenicity and cross-infection tests showed that both *Colletotrichum* species can infect *H. polyrhizus* stem and fruit. As a conclusion, two *Colletotrichum* species causing stem anthracnose of *H. polyrhizus* were recovered, namely *C. gloeosporioides* species complex and *C. truncatum* and the information obtained can be used to formulate disease control strategies.

CHAPTER 1

INTRODUCTION

Dragon fruit or pitaya (*Hylocereus* spp.) is a type of climbing cactus from the family Cactaceae, originated from Latin America. There are three species of dragon fruits, namely red-fleshed dragon fruit (*H. polyrhizus*), white-fleshed dragon fruit (*H. undatus*) and yellow skin dragon fruit (*S. megalanthus*) but only *H. polyrhizus* and *H. undatus* can grow well in Malaysia (Hamidah and Zainuddin, 2007). Dragon fruit's planting areas in Malaysia has increased since 2002 with 47.3 ha and increased to 927.4 ha in 2006 (Cheah and Zulkarnain, 2008). In 2011, areas planted with dragon fruits have reached nearly 1200 ha which indicates the risen popularity of dragon fruit in Malaysia's fruit industry (Zainuddin, 2011) and *H. polyrhizus* is the most widely planted species in Malaysia (Cheah and Zulkarnain, 2008; Masyahit *et al.*, 2009a).

With an increase in dragon fruit plantation, disease occurrence has also increased. One of the most serious fungal diseases of dragon fruit is anthracnose caused by *Colletotrichum* species (Masratul Hawa *et al*., 2008a; Masyahit *et al.*, 2009a). The disease can be identified by reddish-brown to dark brown lesions with chlorotic haloes on the stems and fruits of dragon fruit (Masyahit *et al*., 2009a), sometimes with presence of concentric rings of orange or darker conidial masses and acervuli on mature lesions (Palmateer *et al*., 2007). Anthracnose disease is mainly caused by *Colletotrichum* species and these pathogens have a wide host range. Some of the most common pathogenic species causing anthracnose are *C. acutatum, C. dematium, C. gloeosporioides, C. graminicola* and *C. truncatum*, which can cause huge economic loses to various crops in the tropics, subtropics and temperate regions

(Wharton & Diéguez-Uribeondo, 2004; Agrios, 2005; Crouch *et al*., 2009). The occurrence of anthracnose disease caused by *Colletotrichum* species in Malaysia have also been reported on banana (*Musa* spp.) (Latiffah *et al*., 2009; Intan Sakinah *et al*., 2013) and papaya (*Carica papaya*) (Rahman *et al*., 2007).

The identification and characterization of *Colletotrichum* species have been based mainly on morphological characteristics such as colony morphology, the shapes and sizes of conidia, the presence or absence of setae and also shapes and sizes of appressoria (Sutton, 1992; Cai *et al*., 2009). These characteristics have been used by several early *Colletotrichum* taxonomists such as Mordue (1967; 1971a-c), Sutton (1992), Bailey and Jeger (1992) and Freeman *et al.* (1998) to differentiate *Colletotrichum* species from different crops. However, it has been reported that the morphological characteristics are influenced by environmental and incubation conditions such as temperature, light conditions and cultural media (Cannon *et al*., 2000; Cai *et al*., 2009). Due to these limitations, molecular characteristics such as DNA sequencing may be more reliable in determining differences between *Colletotrichum* species.

For molecular identification and characterization of *Colletotrichum* species, the internal transcribed spacer (ITS+5.8S) regions and ß-tubulin gene are commonly used. The ITS+5.8S regions are located in a conserved part of the nuclear rDNA comprising internal transcribed spacers (ITS 1 and 2) flanking the transcribed unit, 5.8S (Mills *et al*., 1992) while ß-tubulin is a protein coding gene and contains variable introns and exons spans (Glass and Donaldson, 1995). The ITS+5.8S regions and ß-tubulin gene were chosen because both are widely used for identification of *Colletotrichum* species, as well as to evaluate intra- and interspecific

relationships among and within species in the genus *Colletotrichum* (Lee *et al*., 2007; Whitelaw-Weckert *et al*., 2007; Anderson *et al*., 2012).

Studies on *Colletotrichum* species causing anthracnose on *H. polyrhizus* in Malaysia are not well documented. Even though there are a few studies done on the disease infecting *H. polyrhizus*, species identification was based solely on morphological and cultural characteristics (Masratul Hawa *et al.*, 2008a; Masyahit *et al.*, 2009a). Molecular identification and phylogenetic analysis are very important to identify the species correctly as well as to determine the genetic diversity of the *Colletotrichum* species infecting the crop. Furthermore, phylogenetic analysis is very much needed to characterize species within *Colletotrichum* species complex in which species within the species complex shows similar morphology and can only be differentiated by DNA sequences (Cannon *et al*., 2008).

Pathogenicity test to fulfill the Koch's Postulates is essential to determine whether the species isolated from the disease symptoms are the causal pathogen or non-pathogen (Agrios, 2005). Furthermore, many species in the genus *Colletotrichum* have a wide host range in which a single species can infect more than one host, and one host can be infected by multiple species (Freeman *et al*., 1998). Pathogenicity test is also useful to determine the degree of virulence of the *Colletotrichum* isolates as different isolates can show different level of aggressiveness in which the severity of infection may vary among the isolates (Abang *et al*., 2002; Than *et al*., 2008a).

Thus, this study was undertaken to characterize the *Colletotrichum* isolates from infected red-fleshed dragon fruit using morphological and molecular

characteristics to determine the anthracnose causing *Colletotrichum* species, and to conduct pathogenicity test.

Therefore**,** the specific objectives were:

1) To isolate and identify the *Colletotrichum* isolates from anthracnose lesions on *H. polyrhizus* stems based on morphological characters and DNA sequences of ITS+5.8S regions and ß-tubulin gene.

2) To evaluate the genetic diversity of *Colletotrichum* isolates by using phylogenetic analysis based on ITS+5.8S and ß-tubulin sequences.

3) To conduct pathogenicity tests on healthy *H. polyrhizus* plants to determine the causal pathogen of anthracnose.

CHAPTER 2

LITERATURE REVIEW

2.1 Dragon fruit

Dragon fruit is a tropical fruit belongs to a group of epiphytic climbing cacti of the family Cactaceae. Dragon fruit is also known as pitaya of pitahaya in Latin (Le Bellec *et al*., 2006), night blooming cereus and strawberry pear in English (Mizrahi *et al*., 1997; Crane and Balerdi, 2005), thanh long in Vietnamese language (N'Guyen, 1996), päniniokapunahou or päpipi pua in Hawaiian language (Zee *et al.*, 2004) and kaeo mangkon Thai language (Clark *et al*., 2005). In Bahasa Malaysia, dragon fruit is known as mata naga (Cheah and Zulkarnain, 2008). The term 'dragon fruit' probably derived from the character of the fruit's skin which is covered with 'scales' or bracts that give it a 'dragon-like' appearance (Hoa *et al.* 2006).

In the early 2000, dragon fruit is considered as a promising fruit crop because it posseses good qualities and characteristics such as attractive colors and shapes, great potential health benefits and high nutritional values (Crane and Balerdi, 2005; Le Bellec *et al*., 2006). This exotic crop is planted all over the world, especially in the tropical and subtropical regions. This crop has been commercially cultivated in Australia (Jacobs, 1999), Brazil (de Andrade *et al*., 2007), Colombia (Le Bellec *et al.*, 2006), Costa Rica (Esquivel, 2004), Egypt (Mohamed-Yasseen, 2002), Germany (Herbach *et al*., 2006), Indonesia (Damar Jaya, 2010), Israel (Raveh *et al*., 1993; Nerd and Mizrahi, 1998), Japan (Shimomura and Fujihara, 1980), Mauritius (Govinden, 2007), Mexico (De Dios, 2005), Nicaragua (Barbeau, 1990), Taiwan

(Yen, 2007), Thailand (Clark *et al*., 2005), the USA (Merten, 2003; Crane and Balerdi, 2005), Vietnam (N'Guyen, 1996; Thao *et al*., 2006) and Malaysia (Cheah and Zulkarnain, 2008). Vietnam is the largest commercial producer of dragon fruit (Nerd and Mizrahi, 2002; Crane and Balerdi, 2005).

Dragon fruits are grouped into four genera, namely *Cereus*, *Hylocereus*, *Selenicereus* and *Stenocereus* (Britton and Rose, 1963; Mizrahi et al., 1997). However, only a few species are commonly found in the market, which are yellow skin dragon fruit (*S. megalanthus*), white-fleshed dragon fruit (*H. undatus*) and red-fleshed dragon fruit (*H. polyrhizus*) (Le Bellec *et al.*, 2006). Species from the genus *Hylocereus* are widely cultivated and more popular compared to other species from other genera probably due to their ornamental value, attractive fruits and health benefits.

In the genus *Hylocereus*, there are 16 species that are appreciated for their beautiful large flowers that blooms at night and most of the species can produce fruits, but only a few species are cultivated for their fruits (Innes and Glass, 1992; Le Bellec *et al.*, 2006). *Hylocereus* species are diploid (2n = 22) (Lichtenzveig *et al.*, 2000; De Dios, 2004). The stems of *Hylocereus* are normally elongated, 3-angled or 3-winged and the branches emitting aerial roots, the areoles bearing a tuft of felt and several short spines (Britton and Rose, 1963). There are five species of *Hylocereus* that are of economic importance, described by Britton and Rose (1963) as follows:

- *Hylocereus polyrhizus* (Web.) Britton & Rose

  Hylocereus polyrhizus has green stems (Figure 2.1a) and the flowers that can be measured up to 30 cm long (Figure 2.1b) with margins and reddish outer perianth segments, especially at the tips and rather short and yellowish stigma

lobes. It produces scarlet colour fruit with 10–12 cm long and 130–350 g in weight, oblong shaped and covered with scales that vary in size (Figure 2.1c). The fruit is known to have red flesh with many small edible black seeds with pleasant flesh texture and good taste.

Figure 2.1: Description of *H. polyrhizus*. (a) Long, vigorous stems. (b) White flower with yellowish stigma. (c) Red coloured fruit with red flesh. (Source: Flickr, 2012)

- *Hylocereus costaricensis* (Web.) Britton & Rose

Hylocereus costaricensis has vigorous, stout vines and waxy-white stems (Figure 2.2a), while the flowers are nearly similar with *H. polyrhizus* (Figure 2.2b). The fruit is scarlet red, 10-15 cm long and weighs about 250-600 g, ovoid in shape and covered with scales of various sizes. The flesh is red-

purple with many small edible black seeds with pleasant texture and good taste (Figure 2.2c).

Figure 2.2: Description of *H. costaricensis*. (a) Long, vigorous stems. (b) White flower with yellowish stigma. (c) Scarlet coloured fruit with red-purple flesh.
(Source: Vegparadise, 2012)

- *Hylocereus purpusii* (Weing.) Britton & Rose

Hylocereus purpusii has vigorous green vines (Figure 2.3a) and bears very large flowers that are 25 cm long (Figure 2.3b), with more or less reddish outer perianth segments, golden middle perianth segments and white inner perianth segments. The fruit is scarlet and oblong covered with large scales, 10-15 cm long and weigh 150-400 g (Figure 2.3c). The flesh is red with many small black seeds and has subtle pleasant texture (Figure 2.3, c)

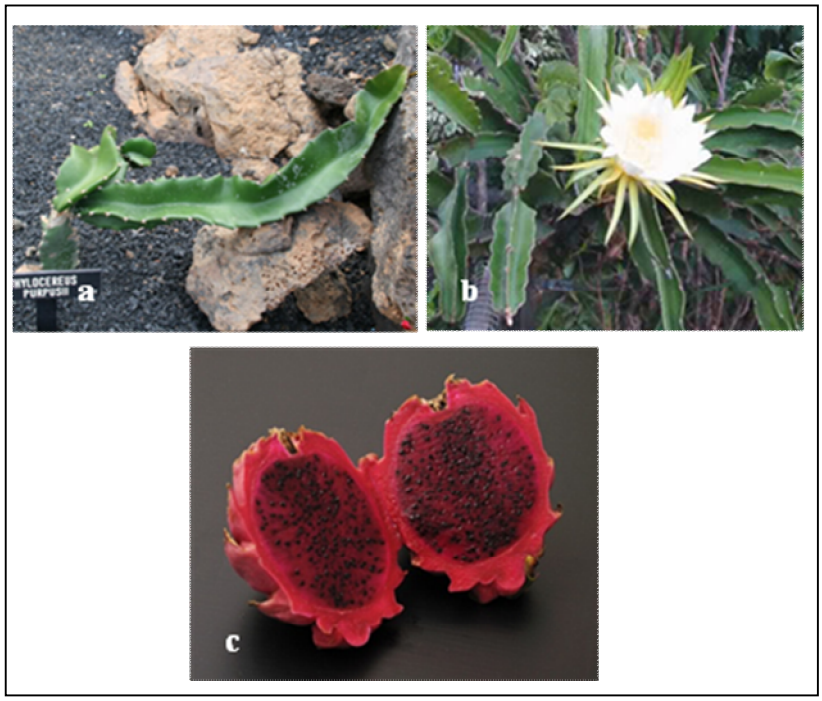

Figure 2.3: Description of *H. purpusii*. (a) Green stems. (b) Flower with golden middle perianth. (c) Red coloured fruit with red flesh. (Source: Photobucket, 2012)

- *Hylocereus undatus* (Haw.) Britton & Rose

Hylocereus undatus has long and green stems, slightly prickly at the margins (Figure 2.4a). It bears flowers that can be measured up to 29 cm long, with green or yellow-green outer perianth segments and pure white inner perianth segments (Figure 2.4b). The fruits are rosy-red, oblong and covered with large and long scales with red and green tips, 15 – 22 cm in length and weight 300 – 800 g, the flesh is white with many small black seeds (Figure 2.4c). The flesh has pleasant texture and good taste.

Figure 2.4: Description of *H. undatus*. (a) Long, green stems. (b) Flower with pure white inner perianth (c) Red coloured fruit with white flesh. (Source: Cactiguide, 2012)

- *Hylocereus trigonus* (Haw.) Saff.

Hylocereus trigonus has green with margins, slender and prickly stems (Figure 2.5a) with areoles located on the top of the rib's undulation. The spines are greenish at first, and then turned dark brown. The flower has greenish outer perianth and white inner perianth (Figure 2.5b). The fruit is red, ovoid or oblong with nearly smooth skin, which is 7-9 cm in diameter and weighs about 120-150 g. The flesh is white with many small black seeds and has pleasant texture and subtle flavour (Figure 2.5c).

Figure 2.5: Description of *H. trigonus*. (a) Slender, prickly stems. (b) Flower with green outer perianth and white inner perianth (c) Red coloured fruit with white flesh. (Source: Deserttropicals, 2012)

2.1.1 Origin, distribution and ecology

Species from the genus *Hylocereus* mostly originate from Mexico and Columbia (Latin America), while other species originate from the West Indies (Britton and Rose, 1963) and till today, they are distributed worldwide, including the tropical and subtropical regions, and among all the species, *H. undatus* is the most cosmopolitan species (Le Bellec *et al*., 2006). The fruits of *Hylocereus* sp. served as a food source and considered as the main traditional fruits in their native region (Mizrahi *et al.*, 1997; De Dios, 2004, Luders and McMahon, 2006).

*Hylocereu*s sp. was first introduced in Asia about 100 years ago when the French brought it into Vietnam and grown as an ornamental plant for the King (Luders and McMahon, 2006). At the end of 1990s, *H. undatus* was brought into Malaysia from Vietnam by Golden Hope Company located in Perak (Halimi and Satar, 2007). In the early 1999, dragon fruits were commercially cultivated in Malaysia, specifically in Sitiawan, Perak; Kuala Pilah, Negeri Sembilan and Kluang, Johor. Until today, dragon fruit is cultivated in various types of land including low and high lands, mined land, rice-planted land and also housing yard (Halimi and Satar, 2007). Two dragon fruit species commonly cultivated in Malaysia are *H. polyrhizus* and *H. undatus* (Hamidah and Zainuddin, 2008).

Hylocereus polyrhizus is the only species being cultivated on a large scale in Malaysia. This is probably because *H. polyrhizus* does not rely on day length to induce flowering compared to *H. undatus* which is a long-day plant that requires longer day length to induce flowering (Luders and McMahon, 2006). Thus, *H. polyrhizus* can produce fruits throughout most of the year. *Hylocereus polyrhizus* fruit is also the most popular compared to other species although the price is higher in the market (Hamidah and Zainuddin, 2008). The pulp is also juicier and sweeter, which is preferred by consumers.

Generally, fruiting cacti are able to grow in soils with high minerals, lime and decaying organic matters (Luders and McMahon, 2006). Different species of dragon fruit are found in a wide range of different ecological conditions due to its ability to adapt in various conditions (Le Bellec *et al*., 2006). It prefers a warm, moist climate with rich, organic soil (Zee *et al*., 2004). In their native country, Mexico, the plant can grow in very rainy regions with 340 – 3500 mm/year rainfall and at up to 2750 m altitude above sea level (De Dios, 2004). Most *Hylocereus* spp. can survive in

different temperatures, for example, up to 38-40ºC (Barbeau, 1990). However, some species cannot tolerate temperature below 12ºC as it can cause necrosis of the stems (Erwin, 1996).

Hylocereus spp. favours growing in half-shaded condition, provided shading naturally by trees, but some species such as *H. undatus*, *H. costaricensis* and *H. purpusii* can adapt in the sun (Le Bellec et al., 2006). As for *H. polyrhizus*, it favours growing in 30% shaded areas (Raveh *et al*., 1998). In addition, *Hylocereus* spp. can grow in different types of well-drained soil (Barbeau, 1990; N'Guyen, 1996). Nevertheless, extreme conditions such as very hot sun, insufficient of water and sub-freezing temperatures may damage the crop (Mizrahi and Nerd, 1999; Le Bellec *et al.*, 2006).

2.1.2 Nutritional values, health benefits and uses

Information regarding physico-chemical characteristics, health benefits and nutritional values of dragon fruit are very well documented and generated a great deal of interest to researchers worldwide. Red pulp of dragon fruit, especially the pulp of *H. polyrhizus*, has generated a lot of interest as a source of natural red colour for the food colouring (Harivaindaran *et al.*, 2008) and lipsticks in cosmetic industry (Mahani and Halimi, 2007). Betacyanins is the pigment which contributes to the red colour of *H. polyrhizus* fruit pulp and is highly sought after not only as a source of natural colouring, but also for the antioxidant properties (Stintzing *et al.*, 2003; Strack *et al.*, 2003). The red betacyanin pigments in *H. polyrhizus* comprises betanin, phyllocactin and hylocerenin (Wybraniec *et al.*, 2001; Wybraniec and Mizrahi, 2002; Harivaindaran *et al*., 2008; Nassim-Naderi *et al*., 2012).

Previous studies have shown that betacyanins in *H. polyrhizus* could maintain their appearance over pH range from 4 to 7 and this could be an ideal colouring pigment for the foods with low acidity such as dairy products (Jamilah *et al*., 2011). Both the pulp and peel of the fruit can be used to extract the betacyanin and based on previous findings, *H. polyrhizus* crop is considered a valuable source of water-soluble and natural dye as well as additives in food industries (Rebecca *et al*., 2008; Harivaindaran *et al*., 2008). In addition, the betacyanins forming enzymes found in the peel has the potential to be developed as moisturizer in cosmetic products (Stintzing *et al*., 2002).

Hylocereus polyrhizus fruit contains high vitamin C and water soluble fibre (Ruzainah *et al*., 2009). The peel has a good ratio of Insoluble Dietary Fibre (IDF) to Soluble Dietary Fibre (SDF) (3.8:1.0), thus indicates that it is a dietary fibre with a very good physiological effect even better than wheat bran and oat bran (Jamilah *et al.*, 2011). In other studies, both pulp and peel of *H. polyrhizus* fruit were found to be rich in polyphenols and antioxidants, in which the peel showed higher antioxidant activities (Wu *et al.*, 2006; Wee and Wee, 2011). *Hylocereus polyrhizus* can also provide an alternative source of commercial pectin, which is widely used in the food industry as thickener, emulsifier, texturizer and stabilizer. According to a study by Norazelina *et al.* (2012), low methoxyl type pectin with yield of 20% was successfully extracted, which makes it feasible for commercial use.

Medicinal qualities of *H. polyrhizus* have showed promising results in alleviating common stomach disorder and persons suffering from hypercholesterolemia, diabetes and anaemia have been suggested to consume the fruit (Raveh *et al*., 1998; Wu *et al*., 2006). The peel of *H. polyrhizus* was proven of having the ability to inhibit the growth of melanoma cells, a type of skin cancer (Wu

et al., 2006). Wichienchot *et al.* (2010) reported that oligosaccharides from dragon fruit, including *H. polyrhizhus*, might be suitable to be included in various food products, prebiotic products, products design for overweight individuals and diebetic prevention products as food supplements. This is because the oligosaccharides showed functional properties such as prebiotic effects, reduced caloric intake and insulinaemia, compared to digestible carbohydrate (Wichienchot *et al.*, 2010). Furthermore, consumption of red-fleshed dragon fruit has the potential to reduce dyslipidemia and play a role in prevention of cardiovascular disease (Mohd Adzim Khalili *et al.*, 2009). A study by Tenore *et al.* (2012) showed that polyphenols extracted from pulp and peel of *H. polyrhizus* demonstrated antimicrobial properties, thus were suggested to be used for the formulation of neutraceutical products. Apart from the pulp and peel, oil extracted from the seeds has been proven to possess mild laxative property (Ariffin *et al.*, 2009).

The pulp of a dragon fruit comprised 70 %-80 % of the ripened fruit, is the most popular and commonly used as edible part of the dragon fruit (Gunasena *et al.*, 2007). Red-fleshed dragon fruit is commonly consumed fresh or chilled, and it can be processed into various industrial and food products, namely juices, sherbets, cordial, yoghurt, ice cream, jelly, candy, pastry, dried fruit and jam. In Malaysia, wine making using dragon fruit is an increasingly popular industry. The flower buds are used to make soups or mixed in salads and tea (Gunasena *et al.*, 2007). *Hylocereus polyrhizus* fruits contain protein, fat, fiber, carotene, calcium, phosphorus, iron and vitamins that are beneficial for health (Morton, 1987; Mahani and Halimi, 2007; Ariffin *et al.*, 2009).

2.1.3 Pests and diseases

Dragon fruit is relatively tolerant of pest's infestation, but a few infestations have been observed and some may cause considerable damage to the crop. Dragon fruit crops are vulnerable towards diseases that can cause high economic damages. The diseases that are commonly infecting dragon fruit including bacterial and fungal diseases, and also a viral disease.

There are several species of insects that can cause damages to dragon fruit crop. Green fruit beetle (*Cotinus mutabilis*) and leaf-footed bug (*Leptoglossus zonatus*) have been reported to cause deformation on dragon fruit stem by perforating the stem and sucking the sap, respectively (Barbeau, 1990). A beetle species from the genus *Xylopetrus* also inflict damage to dragon fruit plant, although not severe (Zainuddin, 2005). Species of ants belonging to the genera *Atta* (Barbeau, 1990) and *Solenopsis* (N'Guyen, 1996; Le Bellec, 2003) cause major damage to the plants, including the flowers and fruits. The activity of bees (*Apis mellifera*) can hinder manual pollination of the crop by harvesting all pollens in only a few hours (Le Bellec, 2004). Rats and birds can also cause serious damage to flowers (Le Bellec, 2003) and fruits (N'Guyen, 1996). Other pests include the different species of aphids, scale insects (Le Bellec *et al*., 2006), snails and slugs.

Bacterial diseases are the most serious diseases affecting dragon fruit and may cause serious loss and damage to the crops. Previous studies reported that *Xanthomonas campestris* causes severe stem rot on dragon fruit (Barbeau, 1990; N'Guyen, 1996; Zee *et al*., 2004; Crane and Balerdi; 2005; Le Bellec *et al*., 2006; Hamidah and Zainuddin, 2007). The symptoms include soft watery, rotting area on the stems (Hoa, 2008). *Erwinia caratovora* has also been reported to cause serious

stem rot disease on dragon fruit (Barbeau, 1990; N'Guyen, 1996; Le Bellec *et al*., 2006; Cheah and Zulkarnain, 2008). The symptoms are similar to the one caused by *X. campestris* which started with water-soaked lesions on the stems and gradually becoming soft rot. *Enterobacter cloacae* have been reported by Masyahit *et al*. (2009b) as the main pathogenic agents of yellowish to brownish soft and watery symptoms on infected dragon fruit stems and fruits.

One of the most severe fungal diseases of dragon fruit is anthracnose. The causal pathogen of dragon fruit stem and fruit anthracnose is *Colletotrichum gloeosporioides* (Crane and Balerdi, 2005; Palmateer *et al*., 2007; Masratul Hawa *et al*., 2008a; Masyahit *et al*., 2009a). Typical symptoms appeared as brownish to yellowish lesion with chlorotic haloes on the stems and fruits (Masyahit *et al*., 2009a). Another fungal species causing stem disease is *Cuvularia lunata* in which the symptoms of small, circular spots developed into faint pink-to-beige necrotic lesions on the dragon fruit stems (Masratul Hawa *et al*., 2009).

Species from the genus *Fusarium* have also been reported to cause diseases to dragon fruit. *Fusarium oxysporum* has been reported to cause basal rot of dragon fruit with symptoms started with soft rot near the soil line and developed upward to infect all the rootstocks (Crane and Balerdi, 2005; Kostov and Ye, 2006; Choi *et al*., 2007). The scion was not rotted, however it will die due the rootstock infection (Choi *et al*., 2007). Masratul Hawa *et al*. (2008b) reported a disease on *H. polyrhizus* caused by *F. proliferatum* in which the symptom appears as brownish to reddish lesions with water-soaked margins on the stems. *Fusarium lateritium* was reported to cause postharvest fruit rot of dragon fruit (Le *et al*., 2000; Paull, 2007).

Fruit rot caused by *Bipolaris cactivora* was also reported on imported dragon fruit from Vietnam. The symptoms appeared as water-soaked area with olive to black powdery spots that coalesced to develop soft rot (He *et al.*, 2012). Similar disease and causal pathogen was also observed in Taiwan (Wang and Lin, 2005), Japan (Taba *et al.*, 2007), South Florida, USA (Tarnowski *et al.*, 2010) and Zhanjiang district of Guandong province, China (Liu *et al.*, 2011). *Bipolaris cactivora* also caused fruit blotch of dragon fruit in Israel (Israel *et al.*, 2011).

Dothiorella sp. is also pathogenic to dragon fruit in which the fungus caused circular brown spots on the stems (Hamidah and Zainudin, 2008). If the disease is severe, the spots coalesced and greatly reduced the stem's surface area for photosynthesis (Zee *et al.*, 2004). Other species of pathogenic fungi that caused diseases on dragon fruit including *Aspergillus niger*, and *A. flavus* (Le *et al.*, 2000; Kostov and Ye, 2006), *Botryosphaeria dothidea* (Valencia-Botin *et al.*, 2003), *Gloeosporium agaves* and *Macssonina agaves* (Le Bellec *et al.*, 2006), *Phomopsis* spp., and *Oidium* spp. (Hamidah and Zainuddin, 2008).

A viral disease has also been reported to attack dragon fruit stem. Cactus Virus X (CVX) has been reported to cause mottling disease of dragon fruit in Taiwan (Liou *et al.*, 2001). It is a systemic mild mottling of the stems and the symptoms including chlorotic and yellowing of stems that leads to stem mottling and necrosis (Liou *et al.*, 2001; 2004; Liao *et al.*, 2003). In Japan, dragon fruit stems infected with CVX showed mosaic symptom (Natsuaki and Shinkai, 2001).

2.1.4 Anthracnose disease

Anthracnose disease is one of the important diseases infecting dragon fruit. Masratul Hawa *et al*. (2008a) and Masyahit *et al*. (2009a) reported the occurrence of anthracnose disease on *H. polyrhizus* caused by *C. gloeosporioides* in Malaysia. In other countries, anthracnose disease caused by *C. gloeosporioides* have been reported on white-fleshed species (*H. undatus*) in Okinawa Perfecture, Japan (Taba *et al*., 2006) and Florida, USA (Palmateer *et al*., 2007). The yellow skin dragon fruit in Brazil was also reported to be infected by similar disease (Takahashi *et al*., 2008). Anthracnose disease on dragon fruit is characterized by reddish to brown or black lesions with chlorotic haloes on stems and fruits. Formation of conidia masses can sometime be seen on the lesions in concentric form (Masyahit *et al*., 2009a).

Anthracnose is a disease that infects the fruits, foliages and stems (Agrios, 2005). The typical symptoms of anthracnose are black and round to irregular necrotic lesions on the stems, leaves and fruits with orange conidial masses and acervuli in the middle of the lesions (Afanador-Kafuri *et al*., 2003; Palmateer *et al*., 2007). Two distinct type of anthracnose disease can occur; disease occurring on developing crop and fruit in the field (preharvest), and those damaging mature fruit during storage (postharvest) (Freeman *et al*., 1998). The common causal agents of anthracnose disease are the fungi belonging to the genus *Colletotrichum* and these pathogens have a wide host range, with incidence of multiple pathogens on a single host as well as a single species of pathogen on multiple hosts (Freeman *et al*., 1998).

Besides *Colletotrichum* and its teleomorph *Glomerella*, there are other genera of plant pathogenic fungi that caused anthracnose related diseases, namely *Sphaceloma*, *Gnomonia*, *Diplocarpon*, and *Elsinoe* (Agrios, 2005). Plant pathogenic

fungi from genera *Microdochium*, *Pezicula* and *Pseudopeziza* may also cause anthracnose on various hosts (Kenneth Horst, 2008).

2.2 *Colletotrichum* systematics

The genus *Colletotrichum* (teleomorph: *Glomerella*) is classified into Superkingdom Eukaryota, Kingdom Fungi, Subkingdom Dikarya, Phylum Ascomycota, Subphylum Pezizomycotina, Class Sordariomycetes, Subclass Sordariomycetidae and Order Glomerellaceae (NCBI, retrieved on).

The description of the genus *Vermicularia* by Tode in 1790 contributed to the early information gathered on *Colletotrichum* (Sutton, 1992). Later, the generic name *Colletotrichum* was introduced by Corda in 1837. The sexual stage or teleomorph of *Colletotrichum* is *Glomerella* which was introduced by von Schrenk and Spaulding in 1903 that includes the type species, *G. cingulata* (Sutton, 1992). The generic synonyms, including *Caulochora*, *Chiloella*, *Haplotherium*, *Hypostegium* and *Neozimmermannia* were listed by von Arx and Müller (1954). According to Sutton (1992), about 20 species from the genus *Glomerella* were reported to be associated with *Colletotrichum* anamorphs.

Mycologists and taxonomists have redescribed the genus under several different names and included species or genus thought to be closely related to *Colletotrichum*, but actually they were different (Sutton, 1980; 1992). For example, the genus *Discladium* was placed in *Colletotrichum* by Wilson (1914), but Vassiljevsky and Karaklin (1950) excluded *Discladium* from *Colletotrichum* based on the differences in the preliminary stages of conidiomatal development of both

genera. Previously, there were about 900 ‘species’ assigned to *Colletotrichum* (Sutton, 1992) including its synonyms. Von Arx (1957) reduced the ‘species’ of *Colletotrichum* from approximately 750 to only 11 based on morphological characters. To date, there have been more than 40 accepted *Colletotrichum* species and several new species have been identified, based on morphological and molecular characters (Sutton, 1980; 1992; Cai *et al.*, 2009; Hyde *et al*., 2009; Damm *et al.*, 2012a,b; Weir *et al*., 2012; Yang *et al*., 2012).

Several features including size and shape of conidia and appressoria, presence or absence of setae, acervuli, and also cultural characters such as colony color, pigmentation, colony texture and growth rate have been used for *Colletotrichum* species identification (Simmonds, 1965; TeBeest *et al*., 1997; Photita *et al*., 2005; Than *et al*., 2008a,b; Thaung, 2008; Masyahit *et al*., 2009; Awa *et al*., 2012). However, morphological characteristics alone are not sufficient to identify and differentiate *Colletotrichum* species due to variation in morphology resulted from environmental influences. *Colletotrichum* species have few distinguishable morphological characters, such as shapes of conidia and appressoria which were commonly used to differentiate species but in some cases, these characters were not variable enough to differentiate between closely related species (TeeBeest *et al*., 1997; Freeman *et al*., 2000; Sutton, 2000; Thaung, 2008; Hyde *et al*., 2009b).

Using host range or host specificity to differentiate *Colletotrichum* species may not be reliable because some of the *Colletotrichum* species such as *C. acutatum* infects various host plants. However, some *Colletotrichum* species are host-specific such as *C. kahawae* and *C. musae* (Prihastuti *et al*., 2009; Su *et al.*, 2011). In the past years, Koch’s postulates was carried out to determine the pathogenicity of various *Colletotrichum* species. For example, the causal pathogen of anthracnose and fruit rot

disease of guava fruits in Nigeria was determined to be *C. gloeosporioides* (Amusa et al., 2005). Nair *et al.* (2010) showed that anthracnose of Australian native pasteur legume (*Cullen australasicum*) was caused by *C. trifolii* and the study believed that the pathogen infection might be specific to that particular species of pasteur legume.

Accurate identification of *Colletotrichum* is difficult to achieve when several taxonomists, grouped the taxa into species complexes. For example, isolates of *C. boninense* were often mistakenly identified as *C. gloeosporioides* due to similar conidial sizes (Moriwaki *et al.*, 2002, 2003; Johnston *et al.*, 2005). There were several *Colletotrichum* species complexes reported, including *C. acutatum* (Damm *et al.*, 2012b), *C. boninense* (Damm *et al.*, 2012a) and *C. gloeosporioides* (Weir *et al.*, 2012) species complexes.

Thirty-one *Colletotrichum* species in the *C. acutatum* species complex that were previously differentiated by morphological and cultural characteristics, were re-identified using multigene phylogeny analysis of six genes namely complete internal transcribed spacer regions (ITS+5.8S), actin (ACT), ß-tubulin, chitin synthase (CHS-1), histone 3 (HIS-3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Damm *et al.*, 2012b). The phylogenetic analysis using the six genes managed to determine 21 species in the *C. acutatum* species complex that were previously unrecognised. Among the 31 species are *C. chrysanthemi, C. cosmic, C. fiorinae, C. tamarilloi, C. walleri, C. lupine* and *C. phormii* (Damm *et al.*, 2012b).

Another study showed that 86 strains of *C. boninense* previously identified using conidial and setae morphology belongs into 18 separate species, including 12 species previously undescribed (Damm *et al.*, 2012a). In the study, multigene phylogeny analysis using seven genes namely ITS+5.8S, ACT, ß-tubulin, CHS-1,

HIS-3, calmodulin (CAL) and GAPDH was carried out to determine species in the *C. boninense* species complex. Among the 18 species determined are *C. boninense, C. hippeastri*, *C, karstii, C. beeveri, C. brasiliense, C. constrictum, C. dacrycarpi, C. oncidii, C. parsonsiae* and *C. torulosum* (Damm *et al*., 2012a).

Colletotrichum species started to be epitypified in 2007 and since then, 42 species have been epitypified and some with living cultures (Hyde *et al*., 2009a). Freshly isolated cultures can be compared with living culture of the epitype strains and their sequence data, making epitypification of important *Colletotrichum* species, such as *C. gloeosporioides*, an essential aspect for correct species identification (Cannon *et al*., 2008; Phoulivong *et al*., 2010). Thus, combination of epitypification with other methods of identification, such as morphological and molecular characterization, can solve many problems regarding taxonomy and eventually change the understanding of species relationship among species in the genus *Colletotrichum* (Phillips *et al*., 2007; Shenoy *et al*., 2007b; Than *et al*., 2008a; Cannon *et al*., 2008; Hyde and Zhang, 2008; Phoulivong *et al*., 2010; Su *et al*., 2011).

2.2.1 Morphological characterization of *Colletotrichum*

Traditional taxonomic system for identification of *Colletotrichum* species was based on morphological characteristics that include characters on natural substrates, such as size and shape of acervuli, conidia, conidiophores and setae; size and shape of conidia, conidiophore and setae in culture and size and shape of appressoria (Mordue, 1967; Sutton and Waterson, 1970; Mordue, 1971a-c; Dyko and Mordue, 1979; Cai *et al*., 2009). The conidia of *Colletotrichum* are hyaline, smooth-

walled and aseptate (von Arx, 1957; Mordue, 1967; Sutton and Waterson, 1970; Mordue, 1971a-c; Dyko and Mordue, 1979). The shapes of conidia can be differentiate into two groups, cylindrical and curved conidia (Damm *et al*., 2009; Phoulivong *et al.*, 2010), and the size and shape varies according to species. For example, *C. gloeosporioides* produced cylindrical conidia with rounded ends (Mordue, 1971a) while *C. acutatum* also produced cylindrical conidia but with tapered ends (Dyko and Mordue, 1979). *Colletotrichum capsici* and *C. dematium* both have falcate conidia with gradually tapered to each ends (Mordue, 1971b; Sutton, 1980). Appressoria forms from mycelia or conidia and the shapes varies from irregular, crenate or lobed, bullet-shaped to clavate or obovate (Mordue, 1967; Sutton and Waterson, 1970; Mordue, 1971a-c; Dyko and Mordue, 1979).

Cultural characteristics on agar media, such as colony color, pigmentation, colony texture and growth rate, have also been applied for identification of some *Colletotrichum* species (von Arx, 1957; Mordue, 1971a-c; Dyko and Mordue, 1979). For example, Baxter *et al*. (1983) recorded that although the conidial morphology of *C. lindemuthianum* and *C. gloeosporioides* are similar, but the cultural characters are distinctly different. *Colletotrichum lindemuthianum* produces dark pigmentation on potato-carrot agar (PCA) and grows slower than *C. gloeosporioides* (Baxter *et al*., 1983; Sutton, 1980). Other characteristics used for identification is the existence of teleomorph. It is not really reliable as most *Colletotrichum* species exist on the host without the teleomorph (Freeman *et al.*, 1998).