DIVERSITY OF *Fusarium semitectum* (BERKELEY AND RAVENEL) ASSOCIATED WITH RED-FLESHED DRAGON FRUIT (*Hylocereus polyrhizus* [WEBER] BRITTON AND ROSE) IN MALAYSIA

MASRATUL HAWA BINTI MOHD

UNIVERSITI SAINS MALAYSIA

2010

DIVERSITY OF *Fusarium semitectum* (BERKELEY AND RAVENEL) ASSOCIATED WITH RED-FLESHED DRAGON FRUIT (*Hylocereus polyrhizus* [WEBER] BRITTON AND ROSE) IN MALAYSIA

by

MASRATUL HAWA BINTI MOHD

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

January 2010

ACKNOWLEDGEMENTS

In the name of Allah, Most Gracious, Most Merciful

All praise and glory to Almighty Allah (S.W.T) who gave me courage and patience to perform this work. Peace and blessing of Allah be upon last Prophet Muhammad (Peace Be upon Him).

It is my pleasure to express my sincere and deepest gratitude to my supervisor, Professor Baharuddin Bin Salleh for his patience, motivation, enthusiasm, and immense knowledge. Above all and the most needed, he provided me unflinching encouragement and support in various ways. I am indebted to him more than he knows.

I gratefully acknowledge Dr. Latiffah Binti Zakaria as my co-supervisor for her invaluable advice, suggestion, supervision, and crucial contribution, which made her a backbone of this research. Her involvement with her originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. A special thank also extended to Dr. Maziah Binti Zakaria and Dr. Hideyuki Nagao for their guidance, encouragement and opinion.

My deep appreciation and profound gratitude goes to my laboratory colleagues Azmi, Nur Ain Izzati, Siti Nordahliawate, Nor Azliza, Nik Mohd Izham, Leong Sau Kueen, Hew Pui Yee, Heng Mei Hsuan, NurulHuda, Wardah, Nor Fazila, Nurul Farizah, Siti Nursyila, Hazrati, Hafizi, Norlia, Farhana, Darnetty,

Bintra and Sundis for their kind and valuable assistance in Plant Pathology Laboratory.

It is a pleasure to pay tribute also to Mr. Kamarudin, Mr. Johari, Mr. Muthu, Miss Jamilah and staffs in the Department of Plant Pathology and School of Biological Sciences for their help and technical assistance. I gratefully thank Ministry of Science, Technology and Innovations (MOSTI) for the National Science Fellowship (NSF) as a financial support within two years of my study.

I wish to record my very special sincere, appreciation and thank to my family especially Papa (Mohd Bin Baharom) and Mama (Hanizah Binti Hamzah) who give me the warm encouragement and have been with me all the times to spur my spirits. Their prayers and moral support will be always in my heart.

Last but not least, my most sincere thanks extended to someone special in my life Ahmad Afif Bin Azmi who gave me endless inspiration, patience, emotional and encouragement to continue my study. The best part of the life is sharing it with the one you love. Thanks to all of you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
®	Registered
°C	Degree of Celsius
µl	Microliter
µm	Micrometer
µM	Micromolar
2*n*	Diploid
AFLP	Amplified fragment length polymorphism
B_1	Thiamine
B_2	Riboflavin
B_3	Nicotinamide
B_5	Ca pantothenate
B_6	Pyridoxine
B_c	Ascorbic acid
BM	Basal medium
bp	Base pair
$CHCI_3$	Chloroform
C_8H_9CIO	Chloroxylenol
$C_6H_8O_7$	Citric acid
C_2H_5OH	Ethanol
$C_3H_5(OH)_3$	Glycerol
CI	Choline
CLA	Carnation leaf-piece agar
cm	Centimeter
cm^2	Centimeter square
CM	Complete medium
CMAC	Corn meal agar with chlorate
CNL12	IGS primer
CNS1	IGS primer

crn	Chlorate resistant nitrate utilizing
CRSs	Chlorate resistant sectors
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
CVX	*Cactus Virus X*
ddH_2O	Double-distilled water
DFP	Deoxyfusapyrone
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNAs	Double stranded ribonucleic acids
EF-1α	α-Elongation factor
EtBr	Ethidium bromide
$Fe(NH_4)_2(SO_4)_2.6H_2O$	Ferrous ammonium sulfate hexahydrate
FP	Fusapyrone
f. sp.	Formae speciales
g	Gram
h	Hour
ha	Hectare
H_3BO_3	Boric acid
HC	Heterokaryon compatibility
het	Heterokaryon incompatibility
H_2O	Water
HSC	Heterokaryon self-compatible
hsi	Heterokaryon self-incompatible
HSI	Heterokaryon self-incompatible
HX	Hypoxanthine
ICBN	International Code for Botanical Nomenclature
IGS	Intergenic spacer
in	Inch
ITS	Internal transcribed spacer
kb	Kilobase

KCl	Potassium chloride
$KClO_3$	Potassium chlorate
kg	Kilogram
KH_2PO_4	Potassium hydrogen phosphate
L	Liter
mA	Miliampere
MAT	Mating type
mg	Miligram
$MnSO_4$	Manganese (II) sulfate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min	Minute
ml	Mililiter
mm	Milimeter
mm^3	Milimeter cube
MM	Minimal medium
MMC	Minimal medium with potassium chlorate
N	Nitrogen
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOCl	Sodium hypochlorite
ng	Nanogram
NH_4	Ammonium
nit	Nitrate nonutilizing
NTSYS	Numerical Taxonomy and Multivariate Analysis System
p	p value (<0.05)
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDAC	Potato dextrose agar with chlorate
PPA	Peptone pentachloronitrobenzene agar
RAM	Random amplified microsatellite

RAPD	Random amplified polymorphic DNA
RBC	Rose bengal medium with chlorate
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
s	Second
SA	Soil agar
SFP	Single feature polymorphism
SIS	Single image stereograms
SMC	Simple matching coefficient
SNP	Single nucleotide polymorphism
spp.	Species
STR	Short tandem repeat
TBE	Tris-Boric acid-EDTA
UPGMA	Unweighted pair group method with arithmetical mean
U	Unit
UV	Ultraviolet light
V	Volt
var.	Variety
VC	Vegetative compatibility
VCG	Vegetative compatibility group
vic	Vegetative incompatibility
VIC	Vegetative incompatibility
W	Watt
WA	Water agar
WAC	Water agar with chlorate

KEPELBAGAIAN *Fusarium semitectum* (BERKELEY DAN RAVENEL) YANG BERASOSIASI DENGAN BUAH NAGA ISI MERAH (*Hylocereus polyrhizus* [WEBER] BRITTON DAN ROSE) DI MALAYSIA

ABSTRAK

Buah naga isi merah *(Hylocereus polyrhizus)* merupakan tanaman yang baru diperkenalkan tetapi berpotensi tinggi dalam industri buah-buahan di Malaysia. Walau bagaimanapun, tanaman ini telah dijangkiti dengan parah oleh pelbagai jenis kulat termasuk kulat dari spesies *Fusarium*. Satu daripada kulat yang paling banyak dipencilkan daripada bahagian tumbuhan yang diserang penyakit ialah *F. semitectum*. Oleh yang demikian, objektif utama kajian ini adalah untuk memencil, mengenal pasti dan mencirikan *F. semitectum* yang berasosiasi dengan *H. polyrhizus* berdasarkan pada ciri-ciri morfologi, kepatogenan, kumpulan keserasian vegetatif (VCG) dan polimorfisme panjang fragmen pembatasan (RFLP) peruang intergen (IGS) pada DNA ribosom (rDNA). Sejumlah 134 pencilan diperoleh daripada *H. polyrhizus* yang berpenyakit daripada sembilan negeri di Malaysia (Johor, Kelantan, Melaka, Negeri Sembilan, Pulau Pinang, Perak, Sabah, Sarawak dan Selangor) dan 79 pencilan (59%) telah dikenalpasti sebagai *F. semitectum* berdasarkan ciri-ciri morfologi. Lain-lain spesies *Fusarium* (55 pencilan; 41%) yang diperoleh juga telah dikenal pasti tetapi tidak dimasukkan dalam kajian ini. Berdasarkan pencirian mikroskopik dan makroskopik, kesemua 79 pencilan *F. semitectum* dikelaskan kepada dua kumpulan, iaitu Kumpulan Morfologi I dan II. Pencilan daripada Kumpulan Morfologi I menghasilkan makrokonidia yang lebih panjang

(3-septa: 31.03 ± 2.57 µm; 5-septa: 40.17 ± 1.85 µm), konidia 1-7 septa (5-septa adalah yang paling banyak), tanpa klamidospora, dengan sporodokia, miselium gebu-berkapas, koloni berwarna jingga, pigmen jingga ke oren dan pertumbuhan yang cepat, manakala pencilan daripada Kumpulan Morfologi II menghasilkan makrokonidia yang lebih pendek (3-septa: 24.98 ± 1.87 µm; 5-septa: 35.24 ± 2.07 µm), konidia 1-5 septa (3-septa adalah yang paling banyak), dengan klamidospora (56%) atau tanpa klamidospora (44%), tanpa sporodokia, miselium gebu-berkapas dan gebu-berserbuk, koloni berwarna perang ke coklat, pigmen coklat ke coklat terang dan pertumbuhan yang perlahan. Dengan menggunakan empat cara inokulasi yang berbeza iaitu semburan dan sapuan ampaian konidia (teknik tanpa luka); suntikan ampaian konidia dan penembusan dengan pencungkil gigi yang ditumbuhi kulat (teknik luka), kesemua 30 pencilan yang diuji adalah tidak patogenik kepada *H. polyrhizus*. Berdasarkan pencirian secara genetik (VCG), sejumlah 69 VCGs diperoleh daripada 79 pencilan *F. semitectum* dengan 0.87, yang dianggap sebagai kepelbagaian yang sangat tinggi. Melalui analisis RFLP, kesemua 79 pencilan *F. semitectum* dapat dibahagikan kepada Kumpulan RFLP I dan II yang selaras dengan pencirian secara morfologi, iaitu Kumpulan Morfologi I dan II. Variasi intraspesifik dan polimorfisme yang tinggi antara kesemua pencilan *F. semitectum* dibuktikan dengan 49 haplotip IGS yang telah dikenal pasti. Kajian ini merupakan laporan yang pertama tentang kejadian dan kepelbagaian *F. semitectum* yang berasosiasi dengan *H. polyrhizus*.

DIVERSITY OF *Fusarium semitectum* (BERKELEY AND RAVENEL) ASSOCIATED WITH RED-FLESHED DRAGON FRUIT (*Hylocereus polyrhizus* [WEBER] BRITTON AND ROSE) IN MALAYSIA

ABSTRACT

Red-fleshed dragon fruit *(Hylocereus polyrhizus)* is a newly introduced but highly potential crop in Malaysian fruit industry. However, this crop has been seriously infected by several fungi including *Fusarium* species. One of the most prominent and frequently fungus isolated from diseased parts of the plants was *F. semitectum*. Therefore, the main objective of this study was to isolate, identify and characterize *F. semitectum* associated with *H. polyrhizus* based on morphological, pathogenicity, vegetative compatibility group (VCG) and restriction fragment length polymorphism (RFLP) of intergenic spacer (IGS) region of the ribosomal DNA (rDNA). A total of 134 isolates were recovered from diseased *H. polyrhizus* from nine states (Johor, Kelantan, Melaka, Negeri Sembilan, Penang, Perak, Sabah, Sarawak and Selangor) in Malaysia and 79 isolates (59%) were identified as *F. semitectum* based on morphological characteristics. The other 55 isolates (41%) of *Fusarium* species obtained were not included in the present study. Based on microscopic and macroscopic characteristics, all the 79 isolates of *F. semitectum* were classified into two groups i.e. Morphotypes I and II. Isolates of Morphotype I produced longer macroconidia (3-septate: 31.03 ± 2.57 µm; 5-septate: 40.17 ± 1.85 µm), 1-7 septate (5-septate was the most common) absence of chlamydospores, presence of sporodochia, abundant-floccose mycelium, peach colony

appearance, peach to orange pigmentations and fast growing, while isolates of Morphotype II produced shorter macroconidia (3-septate: 24.98 ± 1.87 µm; 5-septate: 35.24 ± 2.07 µm), 1-5 septate (3-septate was the most common), with (56%) or without chlamydospores (44%), without sporodochia, abundant-floccose and abundant-powdery mycelium, beige to brown colonies, brown to dark brown pigmentations and slow growing. By using four different inoculation methods i.e. spraying and swabbing with conidial suspensions (unwounded techniques); injecting conidial suspensions and pricking with colonized tooth picks (wounded techniques), all 30 isolates of *F. semitectum* tested were not pathogenic to *H. polyrhizus*. Based on genetic characteristic (VCG), a total of 69 VCGs were assigned among the 79 isolates of *F. semitectum* with genetic diversity 0.87 which was considered very highly diverse. By RFLP analysis, all the 79 isolates of *F. semitectum* were divided into RFLP Groups I and II, in accordance with the Morphotypes I and II, respectively. High level of intraspecific variations and polymorphisms were observed among all isolates of *F. semitectum* with 49 IGS haplotypes were recognized. This is the first report on the occurrence and diversity of *F. semitectum* associated with *H. polyrhizus*.

CHAPTER 1

GENERAL INTRODUCTION

Dragon fruit, especially red-fleshed *(Hylocereus polyrhizus),* is a newly introduced fruit crop in Malaysian fruit industry. This fruit has recently drawn much attention worldwide, not only because of its attractive red colour and economic value as food products, but also for its antioxidative activity (Wybraniec and Mizrahi, 2002). The suitability of tropical climate, rainfall requirements, light intensity and soil types (Luders and McMahon, 2006) may attribute to the successful cultivation of this exotic fruit in Malaysia. *H. polyrhizus,* rich in micronutrients, has recently generated a great deal of consumer interest and being popularized as a healthy fruit (Wu *et al.*, 2005).

In Malaysia, dragon fruit has been initially introduced on large scale at the end of 1990s by Golden Hope Company at Sungai Wang Estate, Perak. Until 2006, Malaysia has around 927.4 ha (363.2 ha production areas) growing areas with total production about 2534.2 tons (valued around US$ 3.5 million) (Cheah and Zulkarnain, 2008). Dragon fruit is now being cultivated almost in all states of Malaysia where Johor has the largest areas with 326.7 ha (Cheah and Zulkarnain, 2008) (Table 1.1).

Table 1.1: Acreage of dragon fruit plantations for each state in Malaysia

State	Acreage (Ha)
Johor	326.7
Negeri Sembilan	139.6
Pahang	93.4
Perak	82.2
Melaka	47.2
Pulau Pinang	38.5
Kedah	35.9
Selangor	31.7
Terengganu	27.7
Perlis	-
Sabah	102.5
Sarawak	-
Wilayah Persekutuan Labuan	2.0
Total	**927.4**

(Source: Cheah and Zulkarnain, 2008)

Generally, dragon fruit is classified into three varieties which are *H. polyrhizus* (red-fleshed with scarlet skin), *H. undatus* (white-fleshed with scarlet skin) and *Selenicereus megalanthus* (white-fleshed with yellow skin) (Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007). However, only *H. polyrhizus* and *H. undatus* are commercially viable and cultivated in Malaysia. The three varieties of dragon fruit have similarities and differences among each other (Table 1.2). At present, dragon fruit has a great potential in Malaysian fruit industry and can be a profitable crop to venture. A kilogram of the red-fleshed fruit is sold at a price between RM10.00 to RM15.00 for grade A. A hectare of well- managed dragon fruit plantation can yield about 70,000 kg. If the wholesale price is RM4.00 per kg fruit, then the net profit from dragon fruit cultivation is estimated at RM 280,000 per hectare per year (Mahani and Halimi, 2007). The variability in size, taste and colour of this fruit indicates the strong need for co-ordination in commercialization. This crop has a relatively quick return for

Table 1.2: Similarities and differences between varieties of *H. polyrhizus*, *H. undatus* and *S. megalanthus* (Mahani and Halimi, 2007)

Variety and Character	*Hylocereus polyrhizus*	*Hylocereus undatus*	*Selenicereus megalanthus*
Stem	 • Stem with green or bluish colour • Stem with triangular cross-section • More spines	 • Stem with green colour • Stem with triangular cross-section; margin with a whitish layer • Less spines	 • Stem with green colour • Stem with triangular cross-section • More spines • Small and thin stem
Flower	 • The margin of flower with reddish perianth segments	 • The margin of flower with greenish perianth segments	 • The margin of flower with reddish perianth segments
Fruit	 • Scarlet skin, red-fleshed, black seeds • Red, wide, short, closely arrangement of scales • Oblong, 350-600 g • 13.7% of brix (sweetness)	 • Scarlet skin, white-fleshed, black seeds • Green, taper, long, distance arrangement of scales • Oblong, 500-600 g • 11.9% of brix (sweetness)	 • Yellow skin, white-fleshed, black seeds • No scales, horny spines • Oblong, 100-250 g • 18% of brix (sweetness)

tropical fruits, since it can begin bearing in its second year and reaching full production in 5 years (Hamidah and Zainuddin, 2007). Low inputs of water and fertilizer and appropriate management of diseases and pests could render the high quality of fruit and the profit could be further increased. Another special feature of dragon fruit is long life cycle i.e. almost 100 years with appropriate protection and management (Crane and Balerdi, 2005).

Recently, dragon fruit in Malaysia was reported to be seriously infected with several complex diseases (Lau *et al.*, 2003; Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007; Cheah and Zulkarnain, 2008; Hew *et al.*, 2008; Masratul Hawa *et al.*, 2008a, b; Masyahit *et al.*, 2009). Like other countries, dragon fruit in Malaysia is threatened by the most serious disease caused by bacteria (Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007; Cheah and Zulkarnain, 2008). The recent reports are anthracnose disease caused by *Colletotrichum gloeosporioides* (Masratul Hawa *et al.*, 2008a; Masyahit *et al.*, 2009) and another new disease caused by *Fusarium proliferatum* (Masratul Hawa *et al.*, 2008b). Since this is a newly domesticated crop, there are not many well-documented diseases known to affect dragon fruit worldwide. Most researches on dragon fruit in Malaysia are concentrating on its physico-chemical characteristics (Novita *et al.*, 2006, 2008; Chuah *et al.*, 2008; Realiza *et al.*, 2008), health benefits (Ching and Yusof, 2005) and nutritional values (Harivaindaran *et al.*, 2008; Rebecca *et al.*, 2008; Ariffin *et al.*, 2009). However, the scientific documentation particularly on diseases is still lacking (Martini *et al.*, 2004).

The genus *Fusarium* is an important group of fungi due to its diversity, cosmopolitan and responsible for numerous plant diseases (Nelson *et al.*, 1981; Liddell, 1991; Nelson *et al.*, 1994; Summerell *et al.*, 2003; Salleh, 2007). *Fusarium* species are considered as imperfect fungi due to the lacking of sexual phase (Barnett, 1960). They are known as facultative parasites that live as parasites or saprophytes depending on their hosts (Huang and Sun, 1997). Most species of *Fusarium* are pathogenic to plants. At least one *Fusarium*-associated disease is found on many plants (Leslie and Summerell, 2006). In Malaysia, *Fusarium* species have been associated and caused diseases on several economically important crops (Table 1.3).

Table 1.3: Diseases of economically important crops in Malaysia caused by *Fusarium* species

Host (Diseases)	*Fusarium* species
Tobacco (slanting death)	*F. oxysporum, F. solani*
Rice (bakanae)	*F. fujikuroi*
Asparagus (crown and root rot)	*F. oxysporum, F. proliferatum*
Pepper yellows (slow decline)	*F. solani*
Watermelon (vascular wilts)	*F. oxysporum* f. sp. *niveum*
Banana (vascular wilts)	*F. oxysporum* f. sp. *cubense*
Banana (crown rot)	*Fusarium* spp.
Roselle (vascular wilts)	*F. oxysporum*
Sugarcane (pokkah boeng)	*F. sacchari*
Maize (stalk, ear and kernel rot)	*Fusarium* spp.
Long bean (vascular wilts)	*F. oxysorum*
Pineapple (fruitlet core rot)	*Fusarium* spp.
Coffee (canker)	*F. xylarioides*
Orchids (die-back)	*F. proliferatum*

(Source: Salleh, 2007)

The identification and systems of classification of *Fusarium* species are very complex. Although, more than 80 species have been identified, there are still problems to identify *Fusarium* into species morphologically because of the different classification systems used by researchers throughout the globe (Leslie and Summerell, 2006). However, morphological characteristics are still considered as reliable and the most important criteria to identify *Fusarium* into species (Leslie *et al.*, 2001; Summerell *et al.*, 2003).

Genetic characteristic using vegetative compatibility group (VCG) is one of the useful methods for determination of genetic diversity and variability among *Fusarium* species (Leslie *et al.*, 1992; Leslie, 1993). Vegetative compatibility (VC) or heterokaryon compatibility (HC) means that two hyphae can anastomose and fuse to form a stable heterokaryon (Puhalla and Spieth, 1985; Klittich and Leslie, 1988). The isolates that can form a stable heterokaryon are considered to be vegetatively compatible and included into the same vegetative compatibility group (VCG) while those that cannot form such heterokaryons are vegetatively incompatible and included in different VCGs. VC systems basically act to restrict the transfer of nuclear and cytoplasmic elements during growth (Leslie, 1993).

Molecular tools are widely used by many taxonomists and phytopathologists. The results obtained by molecular tools, sometimes can be used to support the results of other methods for identification. In molecular systematic, restriction enzymes have been most commonly used to provide defined fragments of DNA, and differences in fragment size and number have

given rise to a range of techniques defined as restriction fragment length polymorphism (RFLP) analysis (Waller *et al.*, 2001). Combination of PCR and RFLP is suitable method for taxonomic studies in *Fusarium* that can show polymorphisms within the isolates and useful in discriminating between extremely closely related species or subspecies (Smith *et al.*, 1995).

Generally, the current studies were carried out to isolate, identify and characterize the most frequent fungal isolates i.e. *F. semitectum* isolated from diseased *H. polyrhizus* in Malaysia by several approaches. The specific objectives are highlighted and explained below:

1. To isolate, identify and characterize *F. semitectum* associated with diseased *H. polyrhizus* in Malaysia based on morphological characteristics.
    - Isolates of *F. semitectum* were the highest number recovered from diseased *H. polyrhizus* from nine states (Penang, Perak, Selangor, Melaka, Negeri Sembilan, Johor, Kelantan, Sabah and Sarawak) in Malaysia. All isolates were identified and characterized by using microscopic (production of the macroconidia, microconidia, conidiophores, chlamydospores and sporodochia) and macroscopic characteristics (colony appearances, pigmentations and growth rates).

2. To determine the pathogenicity of *F. semitectum* towards *H. polyrhizus* based on Koch's postulates.
    - Healthy dragon fruit seedlings were used for inoculation tests. Four different methods of inoculation were tested i.e. spraying and swabbing with conidial suspensions (unwounded techniques); injecting conidial suspensions and pricking with colonized tooth picks (wounded techniques).

3. To investigate the genetic diversity of *F. semitectum* and to determine if techniques for studying vegetative compatibility developed for other *Fusarium* species could be adapted to *F. semitectum.*
    - Nitrate nonutilizing *(nit)* mutants were used as a forced marker to reveal the genetic diversity and variability of *F. semitectum* isolates. Different *nit* mutants from each isolate of *F. semitectum* were paired and grouped into same or different vegetative compatibility groups (VCGs) based on the formation of heterokaryon. Since, there are no reports on the classification of *F. semitectum* isolates into VCGs, the current study was undertaken to ascertain whether this technique could be applied to *F. semitectum*.

4. To characterize isolates of *F. semitectum* by PCR-RFLP analysis in order to assess intraspecific variation within the isolates.
    - CNL12 and CNS1 primers were used to amplify intergenic spacer (IGS) region of the rDNA of *F. semitectum* isolates. Eight different restriction enzymes (*Alu*I, *Bsu*15I, *Bsu*RI, *Eco*88I, *Hin*6I, *Msp*I, *Pst*I and *Taq*I) were selected and used for digestion of PCR products. Cluster analysis was performed to group isolates of *F. semitectum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Dragon Fruit

The dragon fruit is a group of tropical epiphytic cacti and are also known as pitaya or pitahaya (Latin America) (Le Bellec *et al.*, 2006), strawberry pear and night-blooming cereus (English) (Mizrahi *et al.*, 1997), päniniokapunahou or päpipi pua (Hawaii) (Zee *et al.*, 2004; Paull, 2007), paw wong fa kor (China) (Feng-Ru and Chung-Ruey, 1997), kaeo mangkon and luk mangkon (Thailand) (Clark *et al.*, 2005), nanettikafruit or thanh long (Vietnam) (N' Guyen, 1996), and mata naga (Malaysia) (Cheah and Zulkarnain, 2008; Masyahit *et al.*, 2009). Practically unknown fifteen years ago, dragon fruit today occupies almost all exotic fruit markets (Mizrahi *et al.*, 1997; Imbert, 2001). Dragon fruit is considered to be a new, promising fruit species and cultivated on different scales in different parts of the world. This success can be explained in part by the fruit qualities and characteristics (attractive colours and shape), nutritional values, health benefits and also by the commercial policies of some producing and exporting countries such as Vietnam, Colombia and Israel.

2.1.1 Origin, distribution and ecology

Although dragon fruit originated from North, Central and South America (Britton and Rose, 1963; Barbeau, 1990), today, this crop is cultivated all over the world, including the tropical and subtropical regions. Currently, this exotic crop has been commercially cultivated in Argentina (Wright *et al.*, 2007), Australia (Jacobs, 1999), Brazil (de Andrade *et al.*, 2007), China (Feng-Ru and Chung-Ruey, 1997), Colombia (Le Bellec *et al.*, 2006), Costa Rica (Haber, 1983; Esquivel, 2004), Egypt (Mohamed-Yasseen, 2002), Germany (Stintzing *et al.*, 2001; MoBhammer *et al.*, 2005; Herbach *et al.*, 2006), Hawaii (Zee *et al.*, 2004; Paull, 2007), Israel (Raveh *et al.*, 1993; Nerd and Mizrahi, 1997, 1998), Japan (Shimomura and Fujihara, 1980), Mauritius (Govinden, 2007), Mexico (Reyes-Ramos, 1995; Ortiz, 1999; De Dios, 2005; Valiente-Banuet *et al.*, 2006), Nicaragua (Barbeau, 1990), Poland (Wybraniec *et al.*, 2001), Taiwan (Liou *et al.*, 2001; Wu *et al.*, 2005; Yen, 2007), Thailand (Clark *et al.*, 2005), the USA (Nobel and De la Barrera, 2002; Merten, 2003; Crane and Balerdi, 2005), Vietnam (N' Guyen, 1996; Hoa *et al.*, 2006; Nguyen, 2006), and Malaysia (Mahani and Halimi, 2007; Cheah and Zulkarnain, 2008; Masyahit *et al.*, 2009). Vietnam is the biggest commercial producer of dragon fruit in Asia since it was introduced by the French 100 years ago (McMahon, 2003).

The dragon fruit crop prefers a dry tropical or subtropical climates with an average temperature of 21-29ºC, but can withstand temperatures of 38-40ºC, and as low as 0ºC for short periods. Rainfall requirements are 600-1300 mm with alternating wet and dry seasons. This crop likes a lot of sunshine, but can

be damaged by high levels of light intensity. Therefore, it requires some shading. There is a positive response in growth to organic matter and the sand content of the soil (Luders and McMahon, 2006).

2.1.2 Botanical classification

Dragon fruit belongs to several genera, particularly *Hylocereus* of the botanical family Cactaceae. The crop is characterized by climbing plants with aerial roots that bear a glabrous berry with large scales (Fournet, 2002). *Hylocereus* species are diploid (2*n* = 22) (De Dios, 2004; Lichtenzveig *et al.*, 2000). The dicotyledonous members of family Cactaceae (Caryophyllales) comprise 120-200 genera consisting of 1500-2000 species found especially in the semi-desert and hot tropical regions of Latin America (Spichiger *et al.*, 2000). Members of Cactaceae are mainly appreciated for their ornamental qualities, but also include nearly 250 cultivated species of fruit-bearing and industrial crops (Fouqué, 1969). However, only a few species are of economic value.

The use of generic and vernacular names of dragon fruit renders a great deal of difficulties to their botanical classification. However, all dragon fruit are grouped into four main genera i.e. *Stenocereus* Britton and Rose, *Cereus* Mill., *Selenicereus* (A. Berger) Riccob and *Hylocereus* Britton and Rose (Mizrahi *et al.*, 1997; Britton and Rose, 1963). There are many contradictions concerning the botanical classification of *Hylocereus* (Mizrahi *et al.*, 1997; Daubresse, 1999)

that are probably explained by the similar morphological characteristics and / or environmental conditions. In the present study, the classification according to Britton and Rose (1963) that incorporated some recent genetic analyses (Tel-Zur *et al.*, 2004) is adopted (Figure 2.1). Britton and Rose (1963) proposed 16 species in the genus *Hylocereus* (Figure 2.2). In our study, we only focused on the most widely cultivated and promising species in Malaysia i.e. red-fleshed dragon fruit *(H. polyrhizus)*.

A. Stem bluish or whitish or grayish; areoles spiniferous; margin foliaceous, scales red

- Margin of joints horny; spines short, conic; oblong fruit, red-fleshed……...*H. purpusii*
- Margin of joints not horny; spines few, conic
    - Branches slender, oblong fruit, red-fleshed……..….***H. polyrhizus***
    - Branches stout, ovoid fruit, purple-red-fleshed……*H. costaricensis*

AA. Stems bright green; areoles spiniferous; margin foliaceous, scales green

- Margin of joints horny; ribs of stem crenate;
  oblong fruit, white-fleshed……..……*H. undatus*
- Margin of joints not horny; oblong-obovoid fruit
  becoming nearly smooth, white-fleshed…….……..*H. trigonus*

Figure 2.1: Key to species of the genus *Hylocereus* according to Britton and Rose (1963)

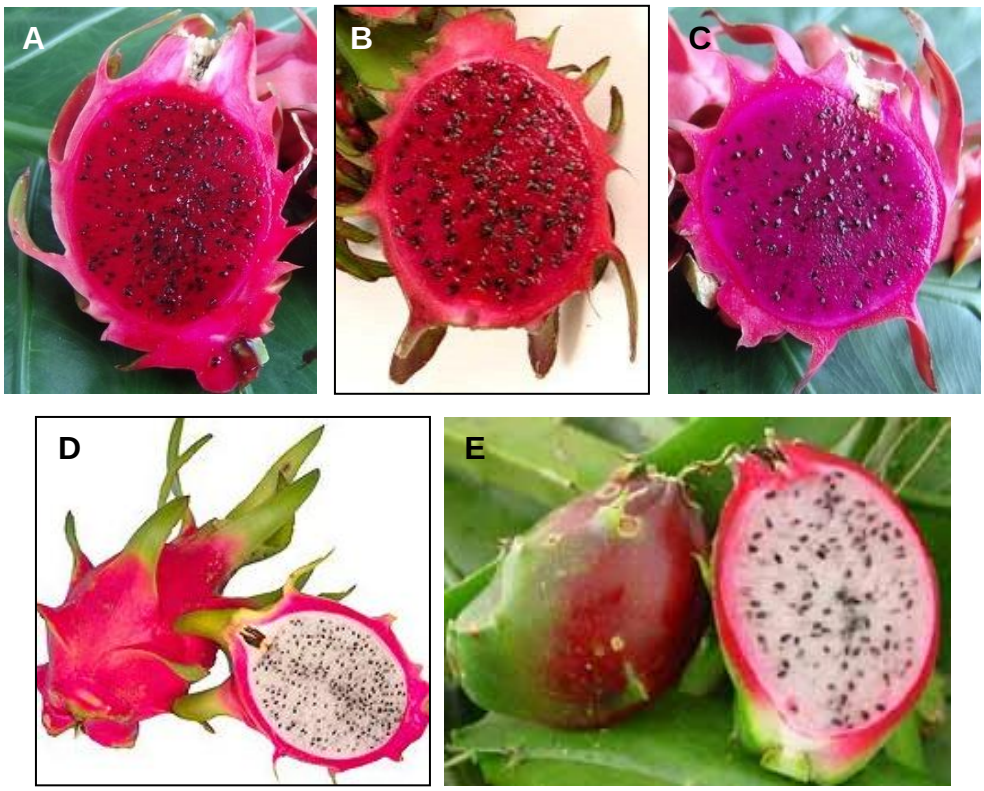

Figure 2.2: Five species of *Hylocereus*. (A) *H. purpusii*; (B) *H. polyrhizus*; (C) *H. costaricensis*; (D) *H. undatus*; (E) *H. trigonus* (Britton and Rose, 1963)

2.1.3 *Hylocereus polyrhizus* (Weber) Britton and Rose

Hylocereus polyrhizus (Weber) Britton and Rose has very long (25–30 cm) flowers with margins; outer reddish perianth segments, especially at the tips (Figure 2.3A) and rather short and yellowish stigma lobes (Figure 2.3B). Its flower is hermaphrodite with both staminate (male, pollen-producing) and carpellate (female, ovule-producing) parts are in the same flower (Le Bellec *et al.*, 2006). Its scarlet fruit (length: 10–12 cm; weight: 350–600 g) is oblong and covered with scales that vary in size (Le Bellec *et al.*, 2006). The fruit is

recognized by red-fleshed with many small black seeds embedded in the pulp (Figure 2.2B). It produces pleasant flesh texture and good taste with less sweatness. *H. venezuelensis* is closely related to *H. polyrhizus*, the only difference being whole (*H. polyrhizus*) or bifid stigma lobes (*H. venezuelensis*) (Britton and Rose, 1963). *H. polyrhizus* is a fast growing, perennial, terrestrial and vine-like cactus. It has triangular (3-sided, sometimes 4- or 5-sided), fleshy, jointed and many-branched stems (Figure 2.3C). Each stem segment has three flat, wavy wings (ribs) with corneous margins and may have 1-3 small spines. The stem sections of *H. polyrhizus* produced aerial roots that adhere to the surface upon which they grow or climb (Crane and Balerdi, 2005) (Figure 2.3D).

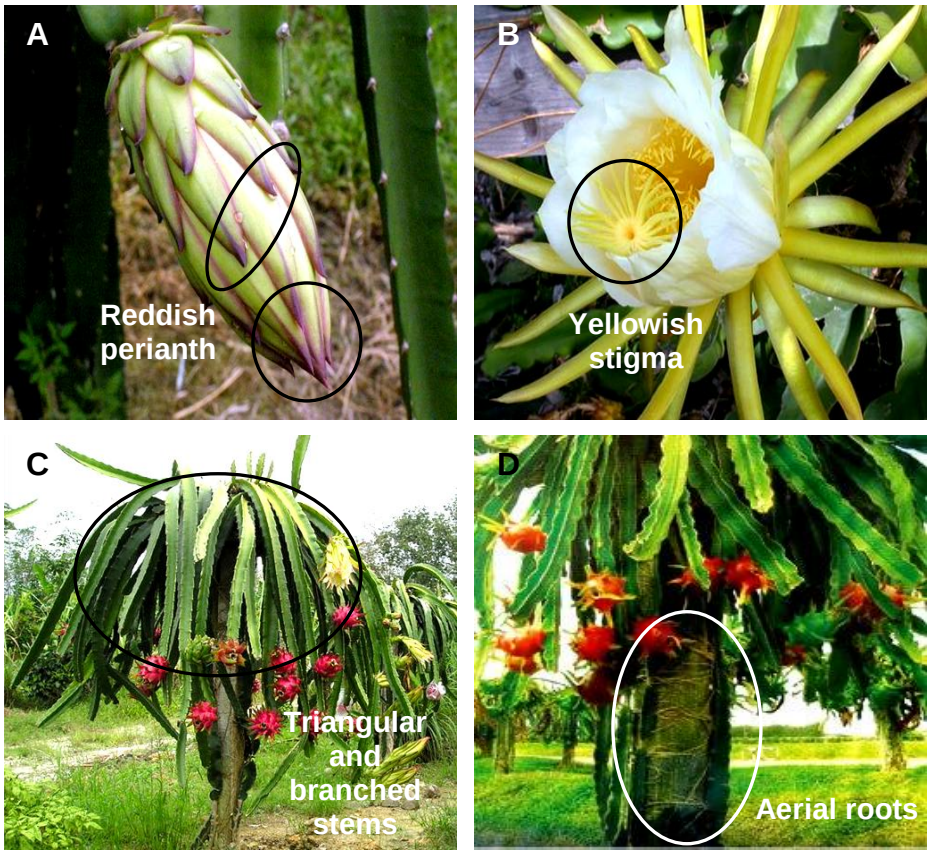

Figure 2.3: Morphology of *H. polyrhizus* plant. (A) Reddish perianth of flower; (B) Yellow stigma; (C) Triangular and branched stems; (D) Aerial roots

2.1.4 Nutritional values, health benefits and products

The nutritional values and health benefits of dragon fruit have been well-documented and being promoted all over the world. The red pigments of *H. polyrhizus* comprise betanin, betacyanin and lycopene (Stintzing *et al.*, 2001; Wybraniec *et al.*, 2001; MoBhammer *et al.*, 2005; Vaillant *et al.*, 2005; Wu *et al.*, 2005; Herbach *et al.*, 2006), collectively known as anthocyanins which are antioxidants and good for the body metabolisme. Betanin contains nitrogen and constitutes the principal pigment of garden beets *(Beta vulgaris)*. It is a red glycosidic food dye obtained from beetroot and degrades when subjected to light, heat, and oxygen (Strack *et al.*, 1993). Betacyanin is the phytochemical in beet that gives it rich 'amethyst' color that significantly reduces homocysteine levels in our body (Wybraniec *et al.*, 2001). Lycopene is a red, fat-soluble pigment found in vegetables, particularly tomatoes and red-coloured fruits. It is one of a family of pigments called carotenoids. Lycopene (as well as other carotenoids such as lutein and beta-carotene) may help in prevention of macular degenerative disease and the leading cause of blindness in people over the age of 65. Lycopene is the only micronutrient in a body whose serum level was shown to be inversely related to the risk of age-related macular degeneration (Armstrong and Hearst, 1996). Besides that, this natural antioxidant also is known to fight cancer, cure heart disease and lower blood pressure (Khan *et al.*, 2008).

Several studies on phytochemistry of *H. polyrhizus* mentioned that this fruit increases the immune systems, and helps in digestion and blood circulation.

In addition, it showed a positive respond in controlling the emotional pressure and neutralized toxins in the body. It also can reduce the cholesterol level in the blood (Ching and Yusof, 2005). In summary, each of *H. polyrhizus* fruits contain protein, fat, fiber, carotene, calcium, phosphorus, iron and vitamins which are able to maintain and promote a healthy body (Morton, 1987; Mahani and Halimi, 2007; Ariffin *et al.*, 2009) (Table 2.1). The nutritional values of *H. polyrhizus* are not only limited on its fruit, but including the whole plant.

Table 2.1: Nutritional information of *H. polyrhizus* (Morton, 1987; Mahani and Halimi, 2007)

Nutritional value (unit/100g)	Range
Water (g)	82.5-83.0
Protein (g)	0.159-0.229
Fat (g)	0.21-0.61
Fiber (g)	0.7-0.9
Carotene (mg)	0.005-0.012
Calcium (mg)	6.3-8.8
Phosphorus (mg)	30.2-36.1
Iron (mg)	0.55-0.65
Vitamin B1 (mg)	0.028-0.043
Vitamin B2 (mg)	0.043-0.045
Vitamin B3 (mg)	0.297-0.43
Vitamin C (mg)	8.0-9.0
Thiamine (mg)	0.28-0.30
Riboflavin (mg)	0.043-0.044
Niacin (mg)	1.297-1.3
Other (g)	0.54-0.68

Besides being consumed fresh, the red-fleshed dragon fruit can be processed into cordial, yoghurt, ice cream, jelly, candy, pastry, dried fruit, jam, wine and other products. The seeds of dragon fruit contain oils that are a mild laxative (Ariffin *et al.*, 2009). There are reports that the pulp and the skin of dragon fruit can become natural food colouring (Harivaindaran *et al.*, 2008) and

cosmetic such as lipsticks (Mahani and Halimi, 2007). This natural food colouring is safe to be used because it does not have any side effect and harmless to our health. Young shoots can be cooked and the stem can be another source of vegetable with medicinal values in our diet. In South America, the stems of dragon fruit are crushed and stored for almost 2 months before being used as livestock's foods which can increase milk production. The dry flowers can be processed to make tea (Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007).

2.1.5 Diseases and pests

Like many other crops, dragon fruit is also attacked by several of economically important diseases. The most serious disease on dragon fruit is bacterial diseases (Figure 2.4A). Literatures recorded that *Xanthomonas compestris* causes a severe stem rot on dragon fruit (Barbeau, 1990; N'Guyen, 1996; Jacobs, 1999; Luders, 1999; Zee *et al.*, 2004; Crane and Balerdi, 2005; Le Bellec *et al.*, 2006; Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007; Paull, 2007). Similar symptoms but different species of bacteria i.e. *Erwinia caratovora* was also reported to cause a serious bacterial disease on dragon fruit with a general water soaked lesion and subsequently becoming a soft rot (Barbeau, 1990; N'Guyen, 1996; Luders, 1999; Kostov and Ye, 2006; Le Bellec *et al.*, 2006; Cheah and Zulkarnain, 2008).

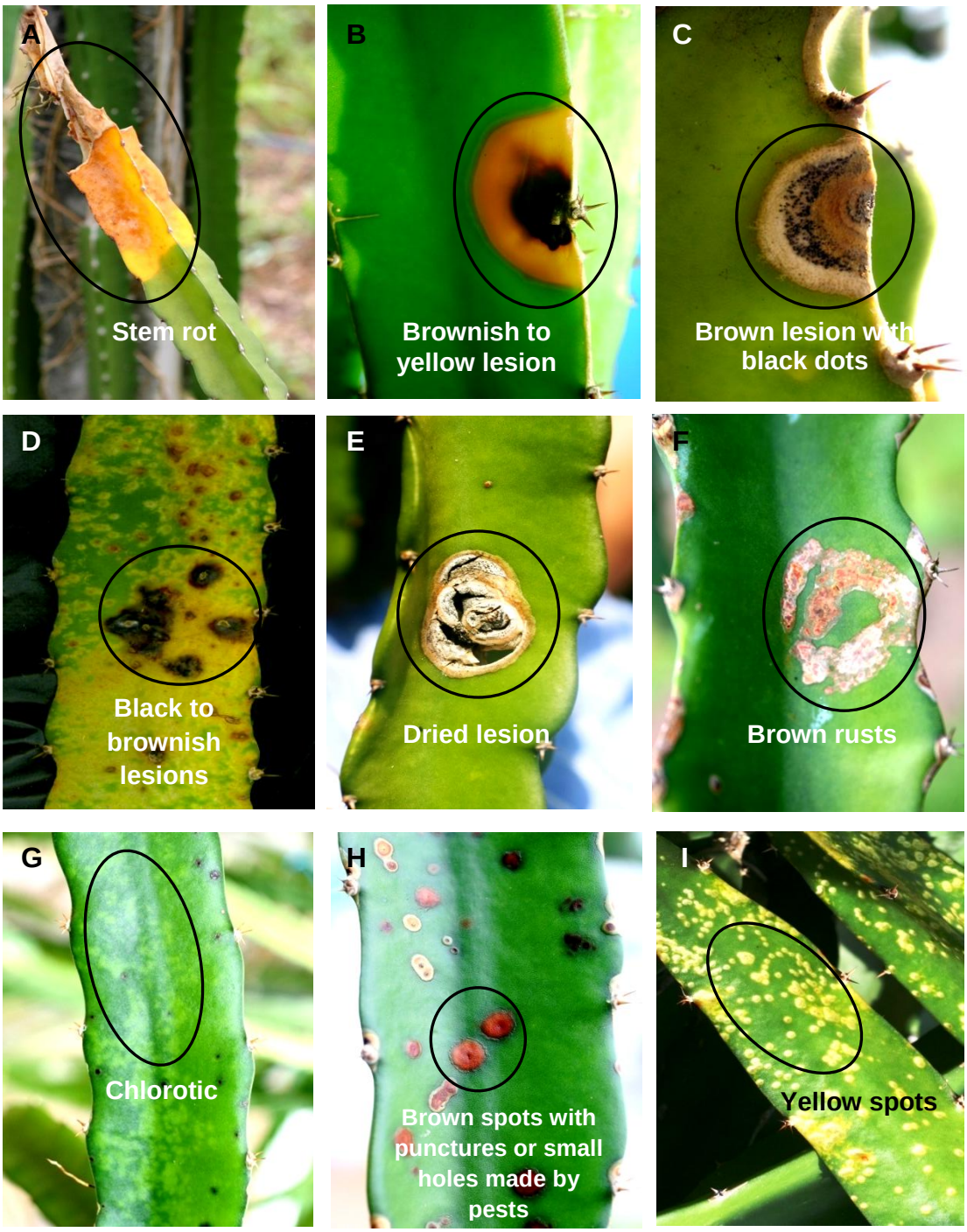

Figure 2.4: Symptoms of dragon fruit diseases caused by bacteria, fungi, virus and pests (A) Bacterial disease; (B-C) Anthracnose disease; (D) Symptoms caused by *F. proliferatum*; (E) Symptoms caused by *G. candidum*; (F) Symptom caused by *A. alternata*; (G) Viral disease; (H-I) Symptoms caused by pests

Besides bacterial diseases, dragon fruit is also infected by several pathogenic fungi. The most severe disease caused by fungi is anthracnose. Lau *et al.* (2003), Mahani and Halimi (2007), Masratul Hawa *et al.* (2008a) and Masyahit *et al.* (2009) reported the occurrence of anthracnose disease caused by *Colletotrichum gloeosporioides* in Malaysia. Similar disease also was observed in Florida (Crane and Balerdi, 2005; Palmateer *et al.*, 2007), Okinawa (Taba *et al.*, 2006), and Brazil (Takahashi *et al.*, 2008). Anthracnose disease is characterized by brownish to yellowish lesions with chlorotic haloes and the formation of conidia in ascervuli (Figures 2.4B and 2.4C).

Masratul Hawa *et al.* (2008b) reported another new disease on *H. polyrhizus* caused by *Fusarium proliferatum*. This pathogen causes black to brownish lesions on stems of *H. polyrhizus* (Figure 2.4D). Besides *F. proliferatum*, *F. oxysporum* caused basal rot of dragon fruit (Lau *et al.*, 2003; Crane and Balerdi, 2005; Kostov and Ye, 2006; Wright *et al.*, 2007). Other species of *Fusarium* that has been associated with diseased dragon fruit are *F. semitectum, F. merismoides, F. compactum, F. solani, F. chlamydosporum, F. dimerum* (Hew *et al.*, 2008; Masratul Hawa *et al.*, 2008a, b) and *F. lateritium* (Le *et al.*, 2000; Paull, 2007). Several literatures documented that *Dothiorella* caused brown spots on stems and fruits of dragon fruit (Jacobs, 1999; Zee *et al.*, 2004; Crane and Balerdi, 2005; Le Bellec *et al.*, 2006; Hamidah and Zainuddin, 2007; Mahani and Halimi, 2007). Various other fungi that caused diseases on dragon fruit are *Phytopthora* spp., *Aspergillus niger*, and *A. flavus* (Le *et al.*, 2000; Kostov and Ye, 2006), *Botryosphaeria dothidea* (Valencia-Botin *et al.*,

2003), *Helminthosporium* spp. (Lau *et al.*, 2003), *Gloeosporium agaves* and *Macssonina agaves* (Le Bellec *et al.*, 2006), *Phomopsis* spp., *Oidium* spp. (Hamidah and Zainuddin, 2007), *Geotrichum candidum* (Figure 2.4E), *Alternaria alternata* (Figure 2.4F), and *Curvularia lunata* (Hew *et al.*, 2008).

On the other hand, viral disease is also encountered in dragon fruit. A handful of literatures recorded that *Cactus Virus X* (CVX) causes chlorotic symptoms to dragon fruit stems (Boyle *et al.*, 1997; Liou *et al.*, 2001, 2004) (Figure 2.4G).

Few pests have been observed on dragon fruit. Ants belonging to the genera *Atta* (Barbeau, 1990) and *Solenopsis* (N'Guyen, 1996; Le Bellec, 2003) cause major damage to the plants as well as to the flowers and fruits. *Cotinus mutabilis* perforates the stem and *Leptoglossus zonatus* sucks the sap, leaving stains and some degree of deformation (Barbeau, 1990) (Figures 2.4H and 2.4I). Different species of aphids and scale insects also have been observed on fruits and flowers. Rats and birds caused serious damages, mainly to flowers (Le Bellec, 2003) and also to young and ripe fruits (N'Guyen, 1996). The activity of bees (*Apis mellifera*) may cause manual pollination difficult, but it must nevertheless be accomplished (Le Bellec, 2004). In fact, bees can be extremely efficient and after only a few hours of activity, they will have harvested all the pollen. The pollen must thus be collected before the bees arrive and manual pollination carried out the next morning as soon as the bees have left the plantation. Other serious pests taken for granted but not seriously controlled are snails and slugs.

2.2 Parasitism, Endophytism and Pathogenicity

An organism that grows, feeds, and sheltered on or in a different organism while contributing nothing to the survival of its host is called parasite (Price, 1980). The removal of foods by a parasite from its host is called parasitism (Agrios, 2005). The removal of nutrients and water from the host by the parasite usually reduces efficiency in the normal growth of the plant and becomes detrimental to further development and reproduction of the plant. In some other cases of parasitism, an organism lives on or in other organism and both obtain the benefit from the association. This phenomenon is known as symbiosis. In most plant diseases, the amount of damage caused to the plants is often greater than would be expected from the mere removal of nutrients by the parasite.

Endophyte is an organism, especially a fungus that lives inside a plant in a parasitic or mutualistic relationship (Cheplick and Faeth, 2009). Endophytes are ubiquitous and have been found in all species of plants studied to date. However, most of the manners in which the endophytes interact with their host are not well understood. Endophytes may be transmitted either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual). Vertically transmitted fungal endophytes are asexual and transmitted via fungal hyphae penetrating the host. Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind and/or insect vectors. Since they spread

is in a similar way to pathogens, horizontally transmitted endophytes are often closely related to pathogenic fungi, though they are not pathogenic themselves (Cheplick and Faeth, 2009).

Some endophytes are likely to be host specific, while some are known to colonize multiple species of plants. Endophytic species are very diverse; it is thought that only a small minority of all existing endophytes have been characterized (Schmidt, 1994). Endophytes may benefit their host plants by preventing pathogenic organisms from colonizing the plants. Extensive colonization of the plant tissues by endophytes creates a 'barrier effect', where the local endophytes outcompete and prevent pathogenic organisms from taking hold of the host plants. Endophytes may also utilize chemicals which inhibit the growth of in-coming competitors, including pathogenic organisms (Funk *et al.*, 1994). Endophytes, therefore are also being investigated for their roles in agriculture as biological control agents. Inoculating crop plants with certain endophytes may provide increased disease or parasite resistance. It is speculated that there may be thousands of endophytes useful to mankind but unfortunately only a few scientists all over the world are working in this field (Guo *et al.*, 1992).

Pathogenicity is the ability of the parasite to interfere with one or more of the essential functions of the host plant and consequently to cause disease (Agrios, 2005; Talaro and Kathleen, 2008). Moss and Smith (1984) defined pathogenicity as the outcome of a complex interaction in time between a host and a pathogen, each potentially variable in a changing environment.

Nevertheless, it is convenient to distinguish between the host specificity of the pathogen and the severity of disease which it provokes in a single host or in a number of similar ones.

Fusarium is a genus of phytopathogenic fungi reported to have increased in the virulence and importance in causing plant disease in the tropics (Salleh, 2007). Some *Fusarium* species are wholly saprophytic while others, in addition to their saprophytic potential, also range from being widely to highly pathogenic and non-pathogenic; however, some are obligate parasites. Furthermore, they may be pathogenic in one environment and saprophytic in another. The terms 'physiological races' and formae speciales (f. sp.) are used to describe the degree of host specificity of the pathogen. Some progress has been made in the genetic analysis of the origin and status of members of these categories, which may differ within and between species of *Fusarium* (Moss and Smith, 1984).

2.3 Disease Cycle

In each of the infectious diseases, a series of events occurs in succession and leads to the development and perpetuation of the disease and the pathogen. This series of events is called a disease cycle (Figure 2.5). A disease cycle sometimes corresponds fairly and closely to the life cycle of the pathogen, but it refers primarily to the appearance, development, and perpetuation of the disease as a function of the pathogen rather than to the pathogen itself. The disease cycle involves the changes in the plant and its