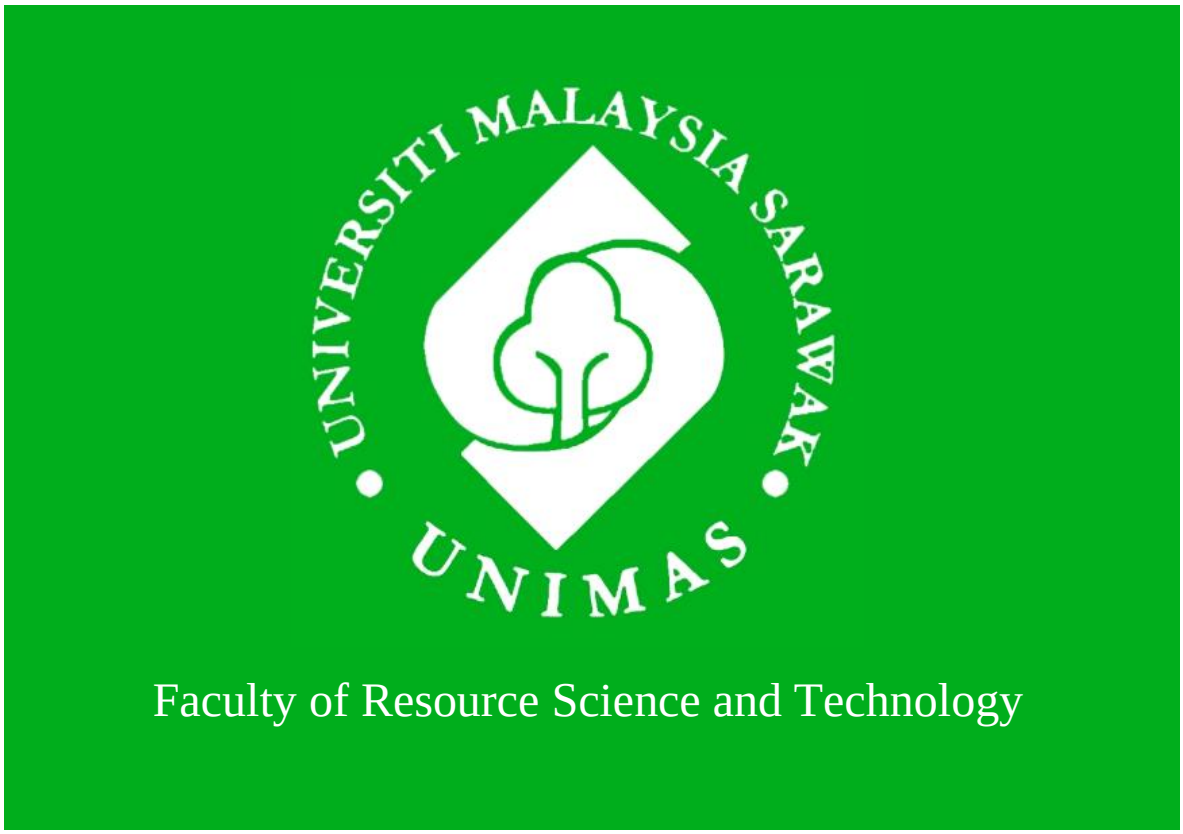


# IDENTIFICATION AND CHARACTERISATION OF FUNGI ASSOCIATED WITH KEDONDONG (*Spondias dulcis*) DISEASES IN SARAWAK

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Bachelor of Science with Honours
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# Identification and Characterisation of Fungi Associated with Kedondong (*Spondias dulcis)* Diseases in Sarawak

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**2015**

## DECLARATION

I hereby declare that the thesis is based on my original work. All the quotations and citations have been duly acknowledge. No portion of the work referred to this dissertation has been previously or concurrently submitted for any other degree programs in UNIMAS or other institutions of higher learning.

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# Identification and Characterisation of Fungi Associated with Kedondong (*Spondias dulcis)* Diseases in Sarawak

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## ABSTRACT

This study was done for the purposes to identify fungi associated with kedondong diseases based on morphological characteristics and molecular analyses and to study the physiological characteristics the effect of different temperature and pH on the growth of isolated fungi. Infected fruits, leaves and petioles of kedondong were taken from Bintulu, Batu Kawa, Kuching and also brought from the Stutong market. Diseases found on kedondong were greyish-black fruit spots, greyish-brown fruit spots, brown fruit spots, black fruit spots, brown fruit rot, dry fruit rot, black leaf spots, brown lesions leaf spots, leaf tip lesion and shiny grey symptom petioles. Infected parts were inoculated onto Potato Dextrose Agar (PDA) media. The fungi identified associated with the diseases were *Phomopsis phyllanthicola*, *Pestalotiopsis microspora*, *Colletotrichum* sp., *Epicoccum sorghinum*, *Botryodiplodia* sp., *Fusarium* sp., *Xylaria* sp. *Aspergillus flavus*, *Curvularia* sp. and two unidentified fungi. The molecular identification of three fungal isolates (*P. phyllanthicola*, *P. microspora* and *E. sorghinum)* was supported by ITS sequence analysis. The growth of the fungi at the different temperature showed that most of the fungi grew significantly faster at temperature 25 $^{o}$C. Effect of pH was varied depending on the fungal isolates. All the fungi were able to grow in the range of pH 3 to pH 8.

Key words: Kedondong, disease, molecular, temperature, pH

## ABSTRAK

*Kajian ini dijalankan untuk tujuan-tujuan mengenalpastikan kulat yang menyebabkan penyakit pada kedondong dengan berdasarkan ciri-ciri morfologi dan analisis molekul, dan untuk mengenalpastikan ciri-ciri fisiologi kulat pada suhu dan tahap pH yang berbeza. Sampel kedodong diperolehi dari* Bintulu, Batu Kawa, Kuching dan dibeli dari pasar Stutong. Bahagian yang dijangkiti diinokulasikan pada media Agar Dektrose Kentang (PDA). Kulat yang telah dikenalpastikan berkaitan dengan penyakit tersebut ialah *Phomopsis phyllanthicola*, *Pestalotiopsis microspora*, *Colletotrichum* sp., *Epicoccum sorghinum*, *Botryodiplodia* sp., *Fusarium* sp., *Xylaria* sp. *Aspergillus flavus*, *Curvularia* sp., Species 10 dan Species 11. Pengenalpastian tiga pencilan kulat (*P. phyllanthicola*, *P. microspora* dan *E. sorghinum)* juga disokong oeh kerja molekul. Pertumbuhan kulat pada suhu berbeza menunjukkan kebanyakan kulat meningkat dengan ketara lebih cepat pada suhu 25 $^{o}$C. Kesan pH ke atas pertumuhan berubah bergantung kepada jenis pencilan kulat. Semua kulat mampu hidup pada jula pH 3 hingga pH 8.

*Kata kunci: Kedondong, penyakit, molekul, suhu, pH*

## 1.0 Research background

### 1.1 Introduction

Fruits are among the most significant and remarkable food crops that are cultivated around the world (Ploetz, 2008). Tropical fruits are those that have their origin in the tropics areas and require a rather tropical or subtropical climate to grow (Bijlmakers, 2009). As in any other tropical fruit crops, kedondong is prone to diseases.

Kedondong belongs to the family Anacardiaceae. It scientifically named as *Spondias dulcis* Forst. or *Spondias cytherea* Sonn. The common names are kedondong, ambarella, golden apple, hog plum, Polynesian plum and Otaheite apple. Kedondong is native to Polynesia (Morton, 1987). It has been introduced into tropical regions such as Malaysia, Indonesia, Thailand, Cambodia, Vietnam, Costa Rica, Columbia, Brazil, Tahiti and from Puerto Rico to Trinidad (Morton, 1987).

Kedondong is an erect fast-growing tree, bearing fruits in about three years from seed propagation. There are two varieties of kedondong tree which are dwarf variety and tall variety (Daulmerie, 1994). Dwarf variety kedondong tree can grow up to two meters height while tall variety kedondong tree can grow up to 10-12 meters height. The kedondong fruits are oblong in shape. Kedondong is a type of edible tropical fruit. The color of kedondong fruits turning from bright green to yellowish with a lot of greyish brown freckles during ripening (Chin & Yong, 1980). The fruit flesh is white and crunchy when immature, becomes fibrous on ripening. Inside each kedondong fruit is a large fibrous seed. The flowers are tiny and greenish white in colour, grouped together as a panicle (Chin & Yong, 1980). The leaves are very aromatic after being crushed.

Different types of fungi, occurring mainly during rainy season can be observed on fruits (Geurts et al., 1986). Therefore, various types of pathogenic fungi associated with diseases of tropical fruits have been reported. Pathogenic fungi are absorptive heterotrophs which obtain their nutrients by decaying organic matters and causing diseases (Fungus, 2013). Disease is the most important constraints to the production of these fruits crop. Most of tropical fruit trees suffer from a number of serious fungal pathogens attack. This is due to fungi favor to develop under warm and humid climate that prevalent in Malaysia for most of the year.

## 1.2 Problem Statement

In order to develop the cultivation of kedondong, the fungal diseases that affect kedondong need to be identified for controlling diseases of kedondong. Following this, there is little doubt to produce the good quality of kedondong fruits for consumption and multiplication purposes within the country as well as export to oversea.

## 1.3 Objectives

a) To identify fungi associated with kedondong diseases based on morphological characteristics and molecular analyses.
b) To study the physiological characteristics:
    - effect of temperature on fungal growth
    - effect of pH on fungal growth.

## 2.0 Literature review

### 2.1 Importance of kedondong

Kedondong tree is grown for their edible fruits. The fruits can be eaten raw or made into pickles, jam and refreshing juice. It contains a lot of nutritious values. In per 100 g of edible portion it contains 0.2 g protein, 12.4 g carbohydrates, 0.1 g fat, 56.0 mg calcium, 67.0 mg phosphorus, 0.3 mg iron, 205.0 ug carotene, 50.0 ug thiamine, 20.0 ug riboflavin, 36.0 mg vitamin C and 46.0 k cal energy value. For medicinal importance, it is useful in diabetes mellitus, indigestion, urinary tract infection, hypertension, and hemorrhoids (Department of Agriculture, Sri Lanka, 2006).

### 2.2 Worldwide importance and economic value of kedondong

Previously the kedondong was generally not grown as a commercial crop and exporters were cautious about marketing such an obscure fruit (Bauer *et al*., 1993). However, extra-regional export markets became established in the late 1980s and the large type fruit is now exported from several Caribbean countries including Trinidad and Tobago, Grenada, St Vincent, Guyana, Surinam, Jamaica, the Dominican Republic and Dominica as a fresh fruit while mature-green to North America and European countries (Daulmerie, 1994). According to Bauer *et al*. (1993), Grenada was able to penetrate the lucrative fresh-fruit market of the United States in the 1980s because of its fruit fly-free status. A number of value-added products made from kedondong such as chutney, pickles and juices are also in demand in the ethnic markets in Canada, the United States and the United Kingdom.

According to Graham *et al.* (2004), the miniature or dwarf golden apple fruit remains unexploited although it has great potential for domestic utilization and foreign trade because of several advantages it has over the large fruit type. Among the main advantages

are that the miniature fruit is available throughout the year while the large fruit type is seasonal in nature. Moreover, fruits from the dwarf trees are less cumbersome to harvest due to the significant difference in tree height. Furthermore, the miniature plants can be established at very high densities giving high yields per unit area compared to the large tree type. Also, the miniature fruit lends itself more easily to certain types of processing.

### 2.3 Pathological disorders of kedondong

Diverse pathogenic fungi have potential to cause diseases on tropical fruits. The common fungi associated with the tropical fruits diseases are *Ceratocysis* spp., *Botryosphaeri*a spp., *Fusarium* spp., *Glomerella* spp., *Mycosphaerell*a spp., *Rosellinia* spp., *Armillaria* spp., *Ganoderma* spp., *Rigidoporus* spp. and *Erythricum salmonicolor* (Agrios, 1988; Ploetz, 2008). These pathogenic fungi cause diseases which can be the limiting factors in the production and marketing of those tropical fruits.

In many other countries, the kedondong tree is subject to gummosis and is consequently short-lived (Geurts *et al.*, 1986). Various cankers also cause problems, including a resinous canker caused by *Lasiodiplodia* sp. (Ponte *et al.*, 1988). A bacterial canker caused by a pathogenic form of *Xanthomonas campestris* pv. *Magiferae indica* (Morton, 1987). Kedondong fruit has a high rejection rate, which can be attributed to a large extent to a lack of control of disease and pests (Bauer *et al.*, 1993).

As the kedondong is not a major crop in the countries in which it was found, there is a lack of attention to its pathological disorders and their control (Geurts *et al.*, 1986). Kedondong fruits of both genetic lines grown in regions of high humidity experience problems associated with anthracnose. Trees cultivated in areas where the average rainfall is 2000

mm and altitude 150 m above sea level are generally smaller in size but experience fewer disease problems (Winsborrow, 1994). Different types of fungi, occurring mainly during the wet season in the Caribbean, are observed on kedondong fruits of both genetic lines.

Bauer *et al.* (1993) reported the occurrence of small (8 mm in diameter) round black lesions on green fruit with gumming developing slowly on the fruit as well as other large black spots of 1.5 cm of diameter. These lesions usually remain superficial (3 mm deep) and do not cause fruit rotting or softening. When fruits ripen the infected area remains pale green and softens. The spotting incidence increased during the rainy season from June to November and were caused by different types of fungi, such as *Guldnardia* spp., *Asteromella* spp., and *Colletotrichum* spp. (Bauer *et al.*, 1993). Brown lesions with no gumming can be observed on ripe fruits, causing rotting, and are identified as *Colletotrichum gloeosporioides*. A stem rot caused by a bacterium also occurs on ripe fruits (Bauer *et al.*, 1993). Sooty mould due to *Tripospermum* sp. is common on the fruit skin (Bauer *et al.*, 1993). A fungus *Sphacelema spondias* was reported to cause round spots on the leaves and fruits in Florida and Brazil (Geurts *et al*., 1986).

**2.4 Effect of temperature and pH on fungal growth**

Different range of temperature and pH affect the growth rate of fungi. Fungal plant pathogens mostly favor warm temperature conditions. They normally known as mesophiles fungi which are growing at temperature ranges of 5$^{o}$C to 35$^{o}$C and being optimum in the range of 20$^{o}$C to 30$^{o}$C. For example, *Botryodiplodia theobromae* grew and sporulated at temperature ranges from 10$^{o}$C to 40$^{o}$C, the optimum being 25$^{o}$C to 30$^{o}$C (Alam et al., 2001). This fungal pathogen is the causal organism of crown rot disease of banana (*Musa sepientum*) and also an important pathogen of mango and other tropical fruits. Besides that,

leaf blight of noni (*Morinda citrifoliawas*) is caused by *Alternaria alternate* grow at the optimum temperature range of 25ºC to 30°C (Hubballi et al., 2010).

Most fungi grow well on the optimum pH between pH 4 and pH 9. For example, clubroot of conifers caused by *Plasmodiophora brassicae* is most severe at about pH 5.7 but its development is arrested by increasing the pH to above 6.0 (Alford, 2000). *Alternaria alternate* causing leaf blight of noni (*Morinda citrifoliawas*) grows at maximum in pH range of 6.00-6.50 (Hubballi et al., 2010).

### 2.5 Disease control

Pathogenic fungi that cause diseases can be controlled by utilizing fungicides or through biological control.

#### 2.5.1 Fungicides

Utilization of fungicides is important to control the diseases caused by pathogenic fungi. The toxicity of the fungicides may be assessed by determining the percentage of inhibition of germination of spores and retardation of mycelia growth in media amended with different concentration of test fungicides (Narayanasamy, 2006). Proper selection of fungicides and the concentration used can retard or terminate fungal growth and development. The most three common systematic fungicides used in agricultural sectors are benzimidazoles, triazoles and strobilurins (Ploetz, 2007). Fungicides favor the production of high quality and aesthetically pleasing fruit crops.

Benzimidazoles fungicide is useful in against anthracnose and fruit rot diseases caused by *Colletotrichum* spp. and *Botryodiplodia* spp (Narayanasamy, 2006). Application of

benzimidazoles fungicide during flowering of fruit crops can reduce the stem-end rot that caused by *Phomopsis* sp., *Botryodiplodia* sp. and *Colletotrichum* sp. (Waller et al., 2002). Triazoles fungicicide is reported to be effective against the fruit diseases of apple, pear, peach and grapes (Angelini, 1996). Triazoles fungicide in the last three weeks before harvesting can control the brown rot disease of peaches and nectarines caused by *Monilinia laxa* (Angelini, 1996). Strobilurins are natural fungicides that can prepare from the Basidiomycete fungi *Strobilurus tenacellus* and *Oudemansiella mucida* (Narayanasamy, 2006). Strobilurins fungicide derived from *Oudemansiella mucida* can be used against a wide fungal pathogens belonging to Oomycetes, Ascomycotina, Basidiomycotina and Deuteromycotin (Narayanasamy, 2006).

### 2.5.2 Bio-control agent

The disease control by using bio-control agents has been encouraged to reduce the side effect of fungicides to the environment. *Trichoderma* sp. is the widest commercially used fungus as bio-control agent (Waller et al., 2002). The antifungal abilities of these beneficial fungi have been known since the 1930s and were used for plant disease control. *Trichoderma* sp. is used as bio-control agent because it is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, affects a wide range of plant pathogens, rarely pathogentic on higher plants, acts as a mycoparasite, competes well for food and site, produces antibiotics and has an enzyme system capable to attack a wide range of plant pathogen (Wells, 1988).

## 3.0 Materials and methods

### 3.1 Samples collection

Samples of disease infected fruits, leaves and petioles of kedondong were collected from homegarden located at Bintulu, Batu Kawa and Kuching. Besides that, samples of diseased kedondong fruits also were purchased from Stutong market in Kuching.

### 3.2 Description of disease symptoms

Symptoms of disease infected fruits, leaves and petioles of kedondong were recognized and described. Digital photographs were taken of each disease symptom.

### 3.3 Fungi isolation

Fungi associated with the diseases of kedondong were isolated from the lesions tissue segments of fruits, leaves and petioles of kedondong. Those lesion tissue segments were cut into 1 mm x 1 mm by using sterilized forceps and scalpel. Following this, lesion tissue segments were agitated in 10% Chlorox for five minutes and were rinsed with three changes sterile distilled water. Then, lesion tissue segments were blotted dry using sterilized filter papers and were inoculated onto already prepared sterile PDA Petri dishes. Each Petri dish were plated with five tissue segments. The Petri dishes were incubated at room temperature and were observed daily until no appearance of new fungi. The percentage of occurrence of fungi associated with those lesion tissue segments were recorded and calculated by using the following formula.

$$\text{Percentage of occurrence (\%)} = \frac{\text{Number of pieces colonized by a pathogen}}{\text{Total number of pieces}} \times 100\%$$

After that, the fungi were isolated and transferred separately onto new PDA in Petri dish. The cultures were incubated for about one week at room to obtain pure culture.

### 3.4 Fungi identification

The identification of fungi were based on morphological characteristics and ITS sequence analysis.

#### 3.4.1 Morphological study

The fungal isolates were identified based on the macroscopic and microscopic characteristics. The macroscopic characteristics examined were mycelium, pigmentation and growth rate. For microscopic characteristics, the structure of conidiogenous cells and the shapes and sizes of conidia were observed. Microscopic examination was carried out by observing the fungal culture under dissecting microscope. A heat-sterilized inoculation needle were used to isolate the fungi spores and hyphae to prepare microscope slides. Then, the microscope slides were dropped with lactophenol blue stain and covered with cover slips for further observation by using compound microscope. Heat was applied if air bubbles were present on the slide.

#### 3.4.2 Molecular study

##### 3.4.2.1 Genomic DNA isolation

The mycelium were scrapped off from the culture (5-7 days) into 1.5 µl centrifuge tube. 500 µl of CTAB and 140 µl 5M NaCl were added into the centrifuge tube. Then, the mycelium were grinded using pestle. The mixture was then incubated at 65$^{o}$C. After that, 500 µl of the CIA was added and incubated in 0 $^{o}$C for 30 minutes. The mixture was then centrifuged at 14000 rpm for 15 minutes. The supernatant was transferred into a new 1.5 µl centrifuge tube. Following this, 0.5 v/v of 5M ammonium acetate was added and incubated in ice for 50 minutes. After that, the mixture was centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred into the new 1.5 µl centrifuge tube. Then, 0.55 v/v of

isopropanol was immediately added and centrifuged at 14000 rpm for 30 minutes. The supernatant was then discarded and remained only the DA pellet on the bottom of the centrifuge tube. The pellet was washed in 200 µl 70% ethanol for two times and centrifuged at 1300 rpm. The supernatant was discarded and dried the pellet at laminar flow cabinet. Lastly, 20 µl of 1x TE buffer wes added and stored it on 0-20 $^{o}$C.

### 3.4.2.2 PCR amplification

12.5 µl PCR master mix were prepared and added it into a PCR tube. Then, 2 µl DNA were added to the master mix. After that, 8 µl of water was added into the tube. 1.25 µl forward primer ITS 5 (5' GGA AGT AAA AGT CGT AAC AAG 3') and 1.25 µl reverse primer ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3') were used. After that, the samples was run in PCR cycles for 2 hours 30 minutes. After done the PCR, the PCR products were loaded into the 1.0% agarose gel well and run under 90V for 45 minutes. Then, the agarose gel was stained with the ethidium bromide (EtBr) solution for 10 minutes and destained in distilled water for 5 minutes. Finally, the gel was viewed under UV transmillunator and photograph was taken.

### 3.4.2.3 ITS sequence analysis

The PCR products were sent to the 1$^{st}$ BASE company for sequencing. Then, the sequence information was blasted agains to sequences in genebank of the National Centre for Biotechnology Information (NCBI) to identify the closest species of fungus to the fungus isolates strain.

### 3.5 Physiological study

The physiological study was performed to identify the optimum temperature and also pH level for the fungal growth. Different temperature and pH levels had been tested on the selected fungi.

#### 3.5.1 Fungal growth at different temperatures

Agar block containing mycelia was cut by using a sterilized 5x5 $mm^2$ diameter size cork borer from four to seven day old culture of the isolated fungi. The agar block was then inoculated on the PDA Petri dishes and incubated at 15ºC, 20ºC, 25ºC, 30ºC and 35ºC. Three replicates were be prepared for each temperature. The average colony diameter was obtained by measuring two perpendicular diameters of the fungus colony daily for seven days or until the mycelium reached the edges of Petri dish. The average colony diameter was calculated using the following formula.

$$\text{Average colony diameter (D)} = \frac{x + y}{2}$$

x = colony diameter at x-axis of petri dish

y = colony diameter at y-axis of petri dish

Then, the average growth rate of tested fungi was calculated by using following formula.

$$\text{Average growth rate} = \frac{(D_2 - D_1) + (D_3 - D_2) + (D_4 - D_3) + (D_5 - D_4) + (D_6 - D_5) + (D_7 - D_6)}{N - 1}$$

$D_n$ = average colony diameter for $n^{th}$ day

N = total number of days

### 3.5.2 Fungal growth at different pH

Potato Dextrose Broth (PDB) was added with 37% HCI or 1M NaOH to adjust the pH levels of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. Three replicates were prepared for each pH level. Then, the PDB was autoclaved at 121$^{o}$C for 15 minutes. After autoclave process, 50ml of PDB was poured into 100ml autoclaved conical flask. Following this, agar block containing mycelia was cut by using a sterilized 5x5 $mm^2$ cork borer from four to seven day old colony of the tested fungi. Then, the agar block was inoculated in the PDB flask. Those inoculated PDB flasks were incubated at room temperature in darkness for seven days. After that, mycelia were filtered using known weight of filter paper and oven-dried at 60$^{o}$C for at least two days. The average dry weight of the mycelia was calculated by using the following formula.

Dry weight of mycelia = A - B

A = weight of mycelia + filter paper after oven-dried

B = weight of filter paper before oven-dried

### 3.6 Statistical analysis

The differences within average growth rate of fungi on temperature and mycelial dry weight on pH level of tested fungi was carried out through one-way Analysis of Variance (ANOVA) by using IBM SPSS Statistics 22 version. Tukey's test at $p<0.05$ was employed for mean comparisons.

## 4.0 Results

### 4.1 Disease symptoms of kedondong

Based on the observation of symptoms on the fruits, leaves and stems of kedondong, 10 disease symptoms were identified. Recognition and description of disease symptoms of kedondong were given (Table 1).

Table 1: Description for disease symptoms of kedondong

| Type of Diseases | Symptoms |
|---|---|
| 1. Greyish-black spots  | The disease was seen on young fruit. Numerous tiny black spots were merged within the greyish spots area on fruits. The disease only appeared on the outer skin. The flesh of fruits were not affected. |
| 2. Greyish-brown spots  | The disease was seen on young fruit. Numerous of tiny brown spots were merged within the greyish spots area on fruit. The disease only appeared on the outer skin. The flesh of fruits were not affected. |