# PATHOGENICITY AND AETHIOLOGY OF *FUSARIUM* SPECIES ASSOCIATED WITH POKKAH BOENG DISEASE ON SUGARCANE

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**by**

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| | |
|---|---|
| µm | Micrometer |
| ANOVA | Analysis of variance |
| $CO_2$ | Carbon dioxide |
| C | Pahang state |
| CLA | Carnation Leaf-piece agar |
| cm | centimeter |
| D | Kelantan state |
| DSI | Disease Severity Index |
| dai | Day after inoculation |
| f. sp. | Forma specialis |
| g | gram |
| GPT | Gula Padang Terap |
| H | Hour |
| HX | Hypoxanthine |
| HC | Heterokaryon Compatible |
| HSC | Heterokaryon Self-compatible |
| HSI | Heterokaryon Self-incompatible |
| I | Indonesia |
| J | Johor state |
| K | Kedah state |
| Kg | Kilogram |
| KGFP | Kilang Gula Felda Perlis |
| KCIA | Potassium chloride Agar |
| L | Liter |
| min | Minute |
| ml | Mililiter |
| mm | milimeter |
| MMC | Minimal Medium Chlorate |
| $NH_4$ | Ammonium medium |
| $NO_2$ | Nitrite medium |
| $NO_3$ | Nitrate medium |
| *nit* | nitrate non-utilizing mutants |
| NaOCl | Sodium hypochlorite |
| $^0C$ | Degree Centigrade |
| P | Penang state |
| PDA | Potato Dextrose Agar |
| PDC | Potato Dextrose Chlorate |
| PPA | Peptone Pentachloronitrobenzene Agar |
| R | Perlis state |
| spp. | Species |
| SPSS | Statistical Package for Social Science |
| T | Terengganu state |
| U | Sugarcane |
| USM | Universiti Sains Malaysia |
| UV | Ultraviolet light |
| VCGs | Vegetative Compatibility Groups |
| VC | Vegetative Compatibility |
| VIC | Vegetative Incompatibility |
| W | Watt |
| WA | Water Agar |

**KEPATOGENAN DAN ETIOLOGI *FUSARIUM* SPESIES YANG BERASOSIASI DENGAN PENYAKIT POKKAH BOENG PADA TEBU**

## ABSTRAK

Kehadiran penyakit pokkah boeng pada tebu di dapati terdapat di hampir kesemua negara yang menanam tebu secara komersial. Tinjauan di jalankan di ladang tebu, kebun kecil dan perkarangan rumah yang menanam tebu di Semenanjung Malaysia (Kedah, Perlis, Pulau Pinang, Kelantan, Terengganu, Pahang dan Johor ) dan Jawa Timur (Indonesia). Di dapati gejala awal pokkah boeng adalah klorosis dan kekuningan pada daun yang muda dan gejala akhir selalunya daun tidak terbentuk dengan baik dan bahagian pucuk daun mengherot. Sebanyak 133 isolat *Fusarium* telah dipencilkan daripada sampel yang di dapati sepanjang tinjauan. Agar - agar daun teluki (CLA) dan agar - agar kentang dekstros (PDA) digunakan sebagai media untuk mengidentifikasi *Fusarium* ke peringkat spesies berdasarkan ciri - ciri morfologi koloni, kadar pertumbuhan, bentuk dan saiz makrokonidia dan mikrokonidia, sel konidiogenous dan klamidospora. Sebanyak 73% (98 isolat) diklasifikasi sebagai spesies *Fusarium* dalam seksyen Liseola (*F. proliferatum*, *F. subglutinans*, *F. sacchari*) dan 27% lagi merupakan spesies yang umum (*F. semitectum*, *F. equiseti* dan *F. solani*). Ujian kepatogenan telah dijalankan di rumah tanaman dengan menggunakan dua varieti tebu iaitu rentan (PS-81-362) dan rintang (83-R-310) yang telah diinokulasi dengan teknik suntikan dan rendaman ampaian spora ($2x10^6$ konidia/ml) menggunakan pencilan *F. proliferatum*, *F. subglutinans* dan *F. sacchari* yang terpilih. Kesemua strain *F. sacchari* yang diuji adalah patogenik terhadap tebu dengan indeks keparahan penyakit (DSI) berbeza-beza dari 0.33 hingga 5.00. Bagi kedua-dua teknik inokulasi, tiada perbezaan bererti ($p>0.05$) terhadap DSI yang di sebabkan oleh *F. sacchari* pada varieti PS-81-362. DSI 0 menunjukkan tiada simptom yang dapat dilihat dan 5 untuk daun menunjukkan gejala berpintal, kedut dan terbantut atau mati. Sebanyak 98 strain spesies *Fusarium* yang telah diidentifikasi daripada seksyen

Liseola digunakan untuk ujian keserasian vegetatif (VC) dengan menghasilkan mutan pereduksi nitrat (*nit*) sebagai sektor rintang klorat di atas media minimum (MMC) dan agar-agar kentang dektrosa (PDC) yang ditambah dengan 1.5 % , 2.0 % , 2.5 % , 3.0 % dan 3.5 % $KClO^3$. Mutan *nit* yang dijana telah digunakan untuk mengetahui kumpulan keserasian vegetatif (VCG) di dalam setiap populasi. Sebanyak 51 strain *F. sacchari*, 18 strain *F. subglutinans* dan 15 strain *F. proliferatum* menunjukkan keserasian heterokarion sendiri (HSC) dan digunakan untuk ujian pasangan dengan *nit* mutan yang lain. Pertumbuhan heterokarion adalah lebih cepat dan lebat apabila NitM dipasangkan dengan *nit*1 berbanding *nit*1 dengan *nit*3. Sebanyak 13, 5 dan 8 VCG masing-masing dikenalpasti untuk *F. sacchari*, *F. subglutinans* dan *F. proliferatum*. Berdasarkan keputusan yang diperolehi melalui ciri - ciri morfologi, ujian kepatogenan dan ujian keserasian, kesimpulannya penyakit pokkah boeng di Semenanjung Malaysia di sebabkan oleh *F. sacchari*.

**PATHOGENICITY AND AETHIOLOGY OF *FUSARIUM* SPECIES ASSOCIATED WITH POKKAH BOENG DISEASE ON SUGARCANE**

## ABSTRACT

Pokkah boeng disease on sugarcane has been recorded in almost all countries where sugarcane is grown commercially. In our survey throughout sugarcane plantations, small holders and household compounds within Peninsular Malaysia (Kedah, Perlis, Penang, Kelantan, Terengganu, Pahang and Johor) and East Java (Indonesia), the first visible symptoms of pokkah boeng were chlorosis and yellowing of young leaves and the final results were usually a malformed and distorted top. A total of 133 isolates of *Fusarium* species were isolated from samples collected during the survey. For identification of *Fusarium* species from pokkah boeng disease, carnation leaves agar (CLA) and potato dextrose agar (PDA) media were used which emphasized on colony features, growth rates, shapes and sizes of macroconidia and microconidia, conidiogeneous cells and chlamydospores. About 73% (98 isolates) of the isolates were identified as three *Fusarium* species in the Section Liseola (*F. proliferatum*, *F. subglutinans*, *F. sacchari*) and the other 27% belong to common species of *F. semitectum*, *F. equiseti* and *F. solani*. In plant house pathogenicity tests, two sugarcane cultivars i.e. susceptible (PS-81-362) and resistant (83-R-310) to pokkah boeng disease were inoculated by injection and soaking techniques with $2x10^6$ conidia/ml of selected strains of *F. proliferatum*, *F. subglutinans* and *F. sacchari*. All strains of *F. sacchari* tested were pathogenic to sugarcane plants with DSI varied from 0.33 to 5.00. There were no significant ($p>0.05$) differences in disease severity index (DSI) caused by strains of *F. sacchari* on variety PS-81-362 for both inoculation techniques. The DSI varied where 0 for no visible symptoms and 5 for plant with symptoms of twisted, wrinkled and shortened leaves or death. A total of 98 strains of *Fusarium* species has been identified within the Section Liseola were used in vegetative compatibility (VC) studies by generating *nit* mutants as chlorate-resistant sectors on minimal chlorate (MMC) and potato dextrose chlorate (PDC) media that

were supplemented with 1.5%, 2.0%, 2.5%, 3.0% and 3.5% $KClO_3$. Recovered *nit* mutants were used to study vegetative compatibility groups (VCGs) within each population. Fifty-one strains of *F. sacchari,* 20 strains of *F. subglutinans* and 17 strains of *F. proliferatum* were heterokaryon self-compatible and used in pairings with other *nit* mutants. The growth of heterokaryon was more vigorous and robust in pairings of NitM with *nit*1 than those in pairings of *nit*1 with *nit*3. A total of 13, 5 and 8 VCGs were identified among the populations of *F. sacchari*, *F. subglutinans* and *F. proliferatum* respectively. Based on the results from morphological characteristics, pathogenicity and compatibility tests, it can be concluded that pokkah boeng disease in Peninsular Malaysia is caused by *F. sacchari*.

# CHAPTER ONE
# INTRODUCTION

Humans are dependent upon plants for their very existence and most of the human food supply worldwide is derived from the following 20 crops: banana, barley, cassava, citrus, coconut, corn, oats, peanut, pineapple, potato, pulses (beans, peas), rice, rye, sorghum, soybean, sugar beet, sugarcane, sweet potato, wheat and yam (George *et al.*, 1985). Plants not only provide food for humans but also beautify the surrounding, purify the air and protect our natural resources. However, plants also suffer from pests and diseases that cause losses in yield and in turns could lead to human suffering.

## 1.1 Sugarcane

Sugarcane (*Saccharum officinarum* L.) is a monocotyledonous plant from the family Gramineae of the subfamily Andropogoneae (Charrier, 2001) and considered as one of the oldest crops cultivated by man (Peng, 1984) in nearly 60 countries as a commercial crop with Brazil, Cuba, Fiji, India, West Indies Islands, Mauritius and U.S.A as major sugarcane growing nations (Naik, 2001). At the beginning of 6000 BC, it was dispersed through the Philippines, Borneo, Java, Malaya and Burma to India (Blackburn, 1984). Sugarcane is basically $C_4$ plants that exploit solar energy through photosynthesis that fixes $CO_2$ by going through $C_4$ metabolic pathway (Naik, 2001). The yield ultimately depends on the size and efficiency of this photosynthesis system (Naik, 2001).

There are six species listed in the genus; *Saccharum officinarum* L., *S. spontaneum* L., *S. robustum* Brandes and Jesweit ex Grassl, *S. barberi* Jesweit, *S. sinense* Hassk and *S. edule* Hassk (Tan, 1989). The cultivated species are *S. officinarum*, *S. sinense* and *S. barberi* that belong to two main groups which consist a thin, hardy north Indian types *S. barberi,* thick and juicy noble canes *S. officinarum* and *S. spontaneum* L. where both are wild sugarcanes of Southeastern Asia (Naik, 2001).

*S. officinarum* is the species usually referred to when we speak of sugarcane. It has broad spreading leaves and thick stems ranging in colour from yellow, green to red and glossy black (Williams *et al.*, 1980). It was referred to as 'noble canes' due to their excellent quality with thick, juicy, low-fibred canes of high sucrose content (Purseglove, 1979). In 14$^{th}$ century, the black–stemmed 'noble canes' was a traded item with the Portuguese in Malacca (Williams *et al.*, 1980) and probably was domesticated from the wild species *S. robustum* in New Guinea and then spread rapidly to India through Java and Malaysia (Yayock *et al.*, 1988). It was *S. officinarum* which caused the sugar industry to spread throughout the tropics and subtropics (Blackburn, 1984). Now, they are still widely grown throughout the tropical world for juice production, chewing and the manufacturer of brown unrefined sugar (Williams *et al.*, 1980). In many countries, sugarcane is an important cash crop as well as an important source of foreign exchange.

Sugarcane in Malaysia was revived in the 1960's when the Malaysian government introduced its agricultural diversification programme to overcome the country over-dependence on rubber. There are many varieties of commercially grown sugarcanes in Malaysia and about 240 foreign sugarcanes varieties as well as 146 clones exist in Malaysia (Tan, 1989). The crop is produce in a large plantation for commercial production and canes from small farmers are meant for fresh juice consumption. In Malaysia, the sugarcane varieties for fresh juice consumption are Tebu Betong, Tebu Hitam, Tebu Jalur, Tebu Kapur, Tebu Kuku, Tebu Kerbau, Tebu Kuning and Tebu Merah while F148, F172, and Ragnar are some of the sugarcane varieties for commercial production (Tan, 1989). Currently the two biggest sugarcane plantations in the country are located in Padang Terap Plantation, Kedah and Kilang Gula Felda Perlis.

The sugarcane plants comprised of water and solids where soluble solids consist of 75 - 92% sugars, 3 - 7% salts, and other free organic acids and organic non-

sugars. The basic source of sugar is sucrose as a primary sugar carried in the phloem (Escalona, 1952). Sucrose is a disaccharide (glucose and fructose) and a very important component in food industries because it reacts as a sweetening agent in food and drinks we take (Simpson and Ogorzaly, 2001). It supplies about 13% of all energy that is derived from foods (Escalona, 1952). Ripe sugarcane of 12 months age will have around 16% fiber, 80% absolute juice, ash and other colloids in small proportions. About two thirds of the world productions of sugar come from cane and the reminder from beets (Ochse *et al.*, 1961). The demand for sugar is increasing especially in developing countries such as Malaysia.

Besides the production of sugar, there is a byproduct of the manufacturing sugarcane i.e. molasses. The molasses is often used as a fertilizer for cane soils, as a stock feed and also to produce ethyl alcohol (ethanol) for main uses in cosmetics, pharmaceutical, cleaning preparation, solvents and coatings. Other products produced from molasses are butyl alcohol, lactic acid, citric acid, and glycerin (Paturau, 1982; Harris and Staples, 1998). Another useful byproduct of sugar production is known as pulp or sugarcane bagasse, the main source of fuel (Harris and Staples, 1998) in sugar factories and also being used in paper making, cardboard, fiber board and wall board (Purseglove, 1979). In Malaysia, Kilang Gula Felda Perlis also produce mud–cake other than molasses and bagasse that were used as organic fertilizer that is a rich source of macronutrients and micronutrients. It shows that sugarcane plays an important role as a very useful crop worldwide. Therefore, there is a great demand for sugarcane in the economy because of the requirement for the sugar and its byproducts. However, sugarcane can be susceptible to many diseases and pests that lead to shortages of this sweet substance. Only if science could keep on researching and improve the methods of cultural practices and pest control, the shortage could probably be averted.

## 1.2 Sugarcane Plantation in Malaysia

In 1980s, the total area planted with sugarcane in Malaysia is around 17, 000 ha, confined mainly to areas in Kedah and Perlis where the climate is most suitable (Tan, 1989). The two largest sugarcane plantations are situated in the northern area of Kedah and Perlis i.e Gula Padang Terap, Kedah (GPT) and Kilang Gula Felda Perlis (KGFP) for local consumption. In Malaysia the yield of sugarcanes and other sugar crops increased from 60.26 tonnes/ha in 1991 to 75 tonnes/ha in 2004 based on the Food and Agriculture Organization of the United Nations (FAO) statistics (Figure 1.1). These plantation can only supply sugar for locals demand but still Malaysia have to import sugar from other countries especially from Fiji, Tasmania (Australia) and Hawaii (Tan, 1989; Peng, 1984) to meet the requirement. The imported quantity of sugarcane and other sugar crops has significantly increased from 5,304.48 tonnes in 1990 to 10,491.73 tonnes in 2004 (Figure 1.2).

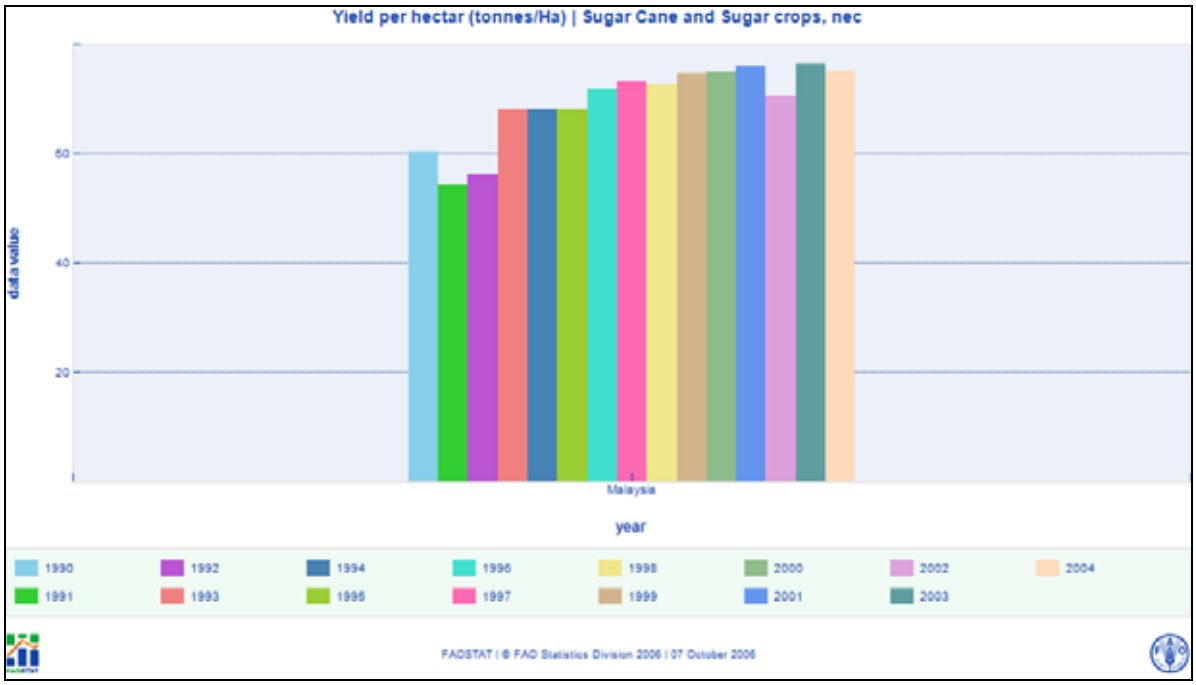


Figure 1.1: Yield per hectar (tonnes/Ha) of sugarcane and other sugar crops (Source: Food and Agriculture Organization of the United Nations Statistics)

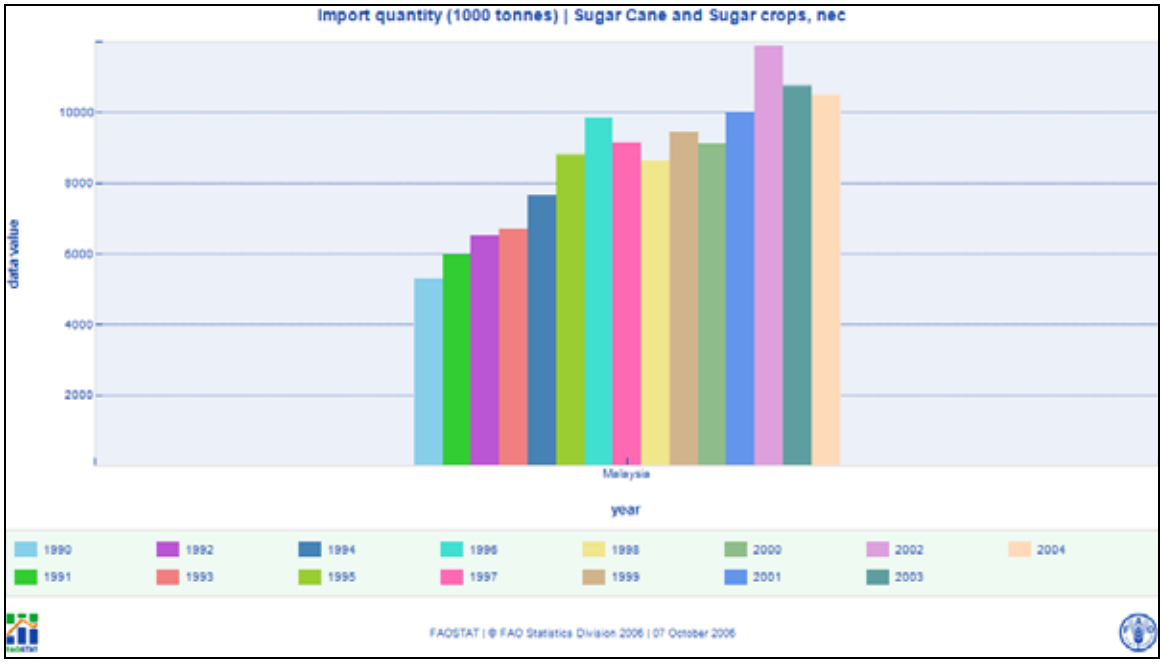


Figure 1.2: Import quantity (1000 tonnes) of sugarcane and other sugar crops (Source: Food and Agriculture Organization of the United Nations Statistics)

In both plantations, sugarcane is planted by adopting ridge and furrows system by using stem cuttings known as "setts". The land was prepared where under these conditions deep ploughing has to be resorted so that infiltration of water leading to adequate availability of oxygen to plants. The ridges and furrows are formed using tractors by following the contour. Setts are planted end to end untill the furrows are covered with 5-6 cm soil, leaving upper portion of the furrows unfilled. Immediately after covering the setts, water is let into the furrows. The ridge-furrow system is the most ideal system of planting under highly irrigated sugarcane cultivation because it provides good soil aeration and solid support to the plants.

Other method of growing sugarcane is ratooning where after harvest time, buds on the leftover underground stubbles germinate again and give rise to another crop, hence called rotoon crop. The frequency of ratooning depends on the sugarcane variety as these can be as productive and healthy as the plant crop. Until third to fourth ratoons can be grown successfully throughout the cane growing areas in Kedah and Perlis. The ratoon crop is more profitable based on the fact that expenditure on preparation of the field, cost of seed cane and planting. The ratoon cane is replanted if the sugarcane yields keep on reducing because of diseases and pests or new variety that is more stable and profitable is found. The problem of ratoon crop is it carries some inoculums of pathogens or eggs of insect pests. After multiplication it might produce more severe diseases and pests in the next crop. In this manner, multiplication of diseases and pests could take an epidemic turn some time in subsequent years. Proper care must be taken to keep the crop free from diseases and pests.

### 1.3 Diseases of Sugarcane

The sugarcane is affected by numerous pathogens and Tan (1989) had listed 42 cane diseases in Malaysia are caused by 22 fungi, 4 bacterial, 3 viral and the rest are disorder of various types (physiological, mechanical and genetics). Barnes (1974) showed that bacteria, fungi and viruses are the major causal organisms (Edgerton,

1955; Martin *et al*., 1961; Sharma, 2006) but most of the diseases are caused by fungal infections (Blackburn, 1984). This crop has an unrivalled record of coping with new diseases with a few have caused major losses or more widespread. The increased in land used for sugarcane as a commercial crop is to be expected will bring more disease and pest problems in Malaysia (Geh, 1973).

The losses due to these diseases may vary from place to place and depending upon the crop variety. Therefore, the diseases could not be ignored and neglected because of their effects on the quality and/or quantity of sugarcane. All parts of sugarcane plant can be infected to diseases and one or more diseases can occur on virtually every plant and in every field (Barnes, 1974; Hideo, 1988).

The specific diseases that usually occur in practically every sugar producing country with the potential for economic damage are red rot (*Glomerella tucumanensis*), smut (*Ustilago scitaminea*), pineapple disease (*Ceratocystistis paradoxa*), root rot (*Pythium arrhenomanes*), gumming disease (*Xanthomonas vasculorum*) and Fiji disease cause by a virus (Edgerton, 1955; Martin *et al*., 1961; Hideo, 1988; Sharma, 2006).

In Malaysia, Geh (1973) reported the diseases found in major sugarcane plantations, experiment stations and small holders caused by fungi were red rot (*Glomerella tucumanensis*), smut (*Ustilago scitaminea*), pokkah boeng (*Fusarium moniliforme* var. *subglutinans*), yellow spot (*Cercospora koepkii*), eye spot (*Drechslera sacchari*), ring spot (*Leptorsphaeria sacchari*), brown stripe (*Helminthosporium stenospilum*), tar spot (*Phyllachora sacchari*), white rash (*Elsinoe sacchari*) and sooty mould (*Caldariomyces* sp.), whereas viral diseases include ratoon stunting, sugarcane mosaic virus and Fiji disease. The smut disease, yellow spot, Fiji disease and pokkah boeng were found in Gula Padang Terap (GPT), Kedah (Idwan, 2005).

## 1.4 Objectives of Study

The pokkah boeng disease was known in Malaysia and Indonesia but studies on aethiology i.e. characterization of the causal organism have not been attempted. Therefore, the objectives of the studies were:

1. To study the pokkah boeng symptoms and syndromes on sugarcane in the field,
2. To isolate and identify the causal organisms by using morphological characteristics,
3. To ascertain the pathogenicity of the organisms based on Koch's postulate, and
4. To study the genetic diversity of the causal organisms using Vegetative Compatibility Groups analysis.

These studies are expected to give scientific information on intensity and situation of pokkah boeng disease on sugarcane in Malaysia and Indonesia and to the identity of the pathogen.

# CHAPTER TWO
# LITEREATURE REVIEW

## 2.1 Sugarcane disease; Pokkah boeng

Walker and Went (1896) were the first who described the pokkah boeng disease on sugarcane and it was then observed and studied in Java (Martin *et al.*, 1961; Roger, 1968; Babu, 1979). Since then, the pokkah boeng disease has been recorded in almost all countries where sugarcane is grown commercially (Norman *et al.*, 1953; Martin *et al.*, 1961; Babu, 1979; Tan, 1989; Raid and Lentini, 1991). Geh (1973) first reported the presence of the disease in Malaysia. Although pokkah boeng has been recorded in almost all cane growing countries but it only caused severe damage in Java where the widely grown variety, POJ 2878 was particularly very susceptible to the disease (Edgerton, 1955; Blackburn, 1984). The variety, POJ 2878 was an excellent breed produced in Java and hence was important for the sugarcane plantation (Blackburn, 1984). However, the variety was grown in a climate where hot and dry season is followed by a wet season that was conducive for the spread of pokkah boeng (Roger, 1968; Babu, 1979). Usually 3 - 7 months old sugarcane are attacked with pokkah boeng disease (Edgerton, 1955) when the plants are growing rapidly and more susceptible to infection rather than older cane (Martin *et al*., 1961; Raid and Lentini, 1991).

Barnes (1974) reported that pokkah boeng was one of the serious diseases of sugarcane and farmers often worry by its sudden spectacular appearance in their fields (Norman *et al.*, 1953). It may cause considerable damage to the crop (Ochse, 1961) but the damage is not severe except in very susceptible varieties (Dickson, 1956). In some cane growing countries, the disease has been and is of little effect on economic importance in which their presence has been recorded but sudden outbreak of the disease can be very costly to control the disease.

## 2.2 Disease Symptoms

Description of the disease symptoms in Javanese term “pokkah boeng” means “malformed and twisted top” was given by Dillewijn (1950). The symptom is easy to recognize, since it attack the top parts of a plant and young leaves start to become chlorosis (Humbert, 1968). The early stages of infection were typified by chlorosis which appears on the basal areas of young leaves as they emerge from the spindle (Geh, 1973; Edgerton, 1955). The infected leaves become crumpled and the twisted leaves unfold normally and the leaves shortened (Edgerton, 1955; Leslie and Frederiksen, 1995). Later, irregular reddish stripes and specks develop within the chlorotic parts into lens or rhomboid–shaped holes (Martin *et al*., 1961). These symptoms also occur on the stem as dark reddish streaks and fine lines in the nodes. In the internodes, the symptoms were characterized by long lesions that give an external and internal ladder-like appearance due to rupturing of the diseased cells which cannot keep up with growth of the healthy tissues (Raid and Lentini, 1991). If infection is limited to the leaves, the plant usually recovers, if not, internal ladder–like lesion develops in the stem (Blackburn, 1984). During wet weather, a soft rot develops in the affected areas (Edgerton, 1955). The most serious injury is when the fungus penetrated the growing points that caused the entire top of the plant dies and this is referred to as top rot (Martin *et al*., 1961; Raid and Lentini, 1991). Heavily infected plants showed a malformed or damaged top and stalk (Martin *et al.*, 1961; Hideo, 1988). The malformation and death of the top parts of the plant may occur in highly susceptible varieties.

## 2.3 Causal Organisms

Bolle (1927) in Java was the first to isolate and inoculate pokkah boeng pathogen and found out that the disease was caused by *Fusarium moniliforme* Sheldon. The species was the only member of Section Liseola (Booth, 1971) and the species name was later abandoned (Egan *et al*. 1997; Nirenbergh and O'Donnell,

1998; Leslie *et al*., 2005). *Fusarium* is a genus of deuteromycetous fungi with various pathogenic species that cause a wide range of important plant diseases (Nelson *et al.,* 1981). *Fusarium* species can affects many agricultural and horticultural crops and produces a range of toxic compounds that contaminate food and can adversely affect livestock and humans. The *Fusarium* spp. in Section Liseola is common on maize, sorghum, rice and sugarcane, where they cause diseases and also may produce mycotoxins such as fumonisins, moniliformin and beauvericin (Booth, 1971, Summerell *et al*., 2001). All the crops mentioned are in family Gramineae and *F. moniliforme* had been reported from 31 other families of plants (Booth, 1971). The pokkah boeng pathogen also attacks sorghum and had been reported that the disease was caused by *F. moniliforme* (teleomorph *Gibberella fujikuroi*) (Leslie and Frederiksen, 1995). The causal organism can reduce the quality of the harvested crop (Dohare *et al*., 2003) and mainly among varieties with high sugar yields (Duttamajumder *et al*., 2004). Approximately 40.8 - 64.5% sugars can be reduced from sugarcanes infected by *Fusarium moniliforme* var. *subglutinans*, depending upon the cultivars (Dohare *et al*., 2003).

Diseases of sugarcane in which species of *Fusarium* are involved include those listed as pokkah boeng, stalk rots or wilt and seed-cane rots (Blackburn, 1984) but the strains involved might be different (Martin *et al*. 1961). In Malaysia, the causal organisms are more favorable to attack sugarcane leaves rather than other parts (Tan, 1989). It also has the ability to combine with *Colletotrichum falcatum* and cause red rot disease on sugarcane (Humbert, 1968).

In Malaysia the causal organisms for pokkah boeng was known as *F. moniliforme* var. *subglutinans* (Geh, 1973). It has been reported that this pathogen also caused *Fusarium* sett or stem rot, although Martin *et al*. (1961) suggested that the strains involved might be different. The other species that was reported as the causal organism of the disease that belong to the Section Liseola was *F. sacchari* (Egan *et al*.,

1997; Nirenbergh and O'Donnell, 1998), also found on sugarcane in Asia (Leslie *et al*., 2005). In India, *F. sacchari* from sugarcane was first described as *Cephalosporium sacchari* Butler and Hafiz Khan (Butler and Hafiz, 1913). It can cause an important disease of pokkah boeng on sugarcane (Egan *et al*., 1997). The species were also found to be associated with other members in Gramineae family such as sorghum and maize (Leslie *et al*., 2005). Pokkah boeng disease that attacked sorghum was caused by *F. moniliforme* (teleomorph *Gibberella fujikuroi*) (Leslie and Frederiksen, 1995).

In Indonesia, Semangun (1992) listed several *Fusarium* species that were isolated from pokkah boeng disease of sugarcane i.e. *F. anguioides* Sherb., *F. bulbigenum* Cke. and Mass. var. *tracheiphilum* (E. Sm.) Wr., *F. moniliforme* Sheld., *F. moniliforme* Sheld. var. *subglutinans* Wr. and Rkg. [*Gibberella fujikuroi* (Saw.) Ito ap. Ito and Kamura], *F. moniliforme* Sheld. var. *anthophilum* (A. Br.) Wr., *F. neoceras* Wr. and Rkg., *F. orthoceras* App. and Wr. var. *longius* Wr. and *F. semitectum* B. and Rav. Giatgong (1980) reported that *F. moniliforme* Sheldon and *G. fujikuroi* (Saw.) Wr. were the causal organisms of pokkah boeng on sugarcane in Thailand.

## 2.4 Means of Dispersal

The pathogens of pokkah boeng disease are transmitted by the movement of spores from one locality to another by air currents (Martin *et al.,* 1961; Raid and Lentini, 1991), and will colonize the leaves, flowers and stems of the plant (Burgess, 1981). For spores to take off, it depends on the environmental situation (windy day, rainy day or dry day) that require different strategies to disperse (Deacon, 2006). Fungal that dispersed by rainsplash are based on the ”puff” and “tap” mechanisms (Figure 2.1) that will cause the dry spores to become airborne and usually the spores are curved like *Fusarium* species (Deacon, 2006).

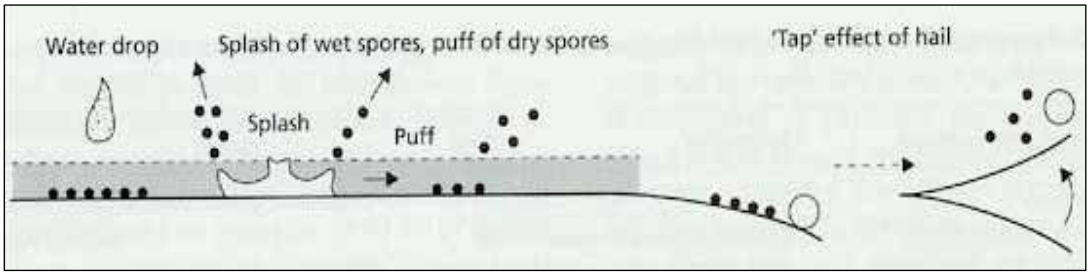


Figure 2.1: Dispersal of spores by rainsplash based on ”puff” and “tap” mechanisms (Source: Deacon, 2006)

Fungi that grow on leaf surfaces and produce chains of spores can be removed by wind, by mist-laden air or by hygroscopic (drying) movements that cause spore to buckle (Figure 2.2) (Deacon, 2006).

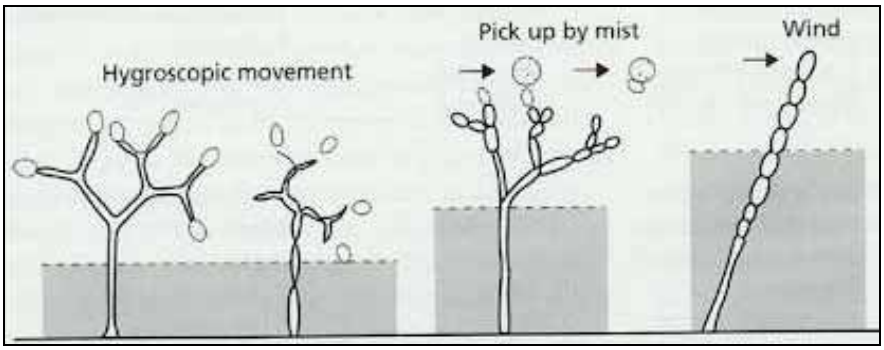


Figure 2.2: The mechanisms of spore liberation from chains by hygroscopic, by mist and by wind (Source: Deacon, 2006)

Hot and dry weather will lead to the opening of leaves between partially unfolded leaves that provide an opportunity for airborne conidia to settle on the leaves (Blackburn, 1984). When the rains start, the conidia are washed down to the susceptible parts of the spindles along the margin of a partially unfolded leaves where they germinate. The conidia germinate and the mycelium can pass through the soft cuticle of young leaves to the inner tissues because the epidermis tissues are still fragile and not protected by the plant system (Dillewijn, 1950; Barnes, 1974). The mycelium spreads to vascular bundles of the immature stem and blocks the vessels

that eventually leads to growth distortions and rupture and the development shows the ladder–like lesions (Holliday, 1980).

Bourne (1953) reported that the pupae and adults of sugarcane stem borers also can spread the fungus. The top borer known as *Chilo* spp. often results in a distortion and shortening of the leaves which is similar to that caused by pokkah boeng disease (Hideo, 1988). Pokkah boeng disease of sugarcane may also spread from seeds contaminated with the fungus (Narendra and Setty, 1979).

## 2.5 Taxonomy of *Fusarium* Species

Taxonomically, *Fusarium* species is an anamorph from the form–class Deutromycetes, in the form–order Moniliales and belonging to the form–Family Tuberculariaceae (Alexpoulos *et al*., 1996). The *Fusarium* taxonomists that involved in classification of this species can be divided into lumpers while some as splitters (Nelson, *et al*., 1994). Therefore, several classification systems were generated with each differ in species concepts. Snyder and Hansen (1940; 1941; 1945) had narrowed the species concepts and proposed a nine species classification system. For that reason they were known as drastic lumpers. Gerlach and Nirenberg (1982) listed many species and varieties and were known as an enormous splitters in which some names were given only based on the host each *Fusarium* was isolated. The classification systems that are too detailed created some difficulties for identification such as the monograph of *Fusarium* species by Wollenweber and Reinking (1935) in which they identified and named approximately 1,000 species of *Fusarium*. With that, efforts have been made by *Fusarium* taxonomists to make the classification system easier to understand and acceptable within many existences of different systems. Some of the classification systems were purposed by Booth (1971) and Nelson *et al*. (1983). They combined their own research with the others classification system to produce a suitable taxonomic system and were recognized as “moderates’” *Fusarium* taxonomists.

In Booth (1971) classification system, he used conidiophores and conidiogenous cells, media usage and standardized incubation conditions for identification that really important criteria in *Fusarium* species taxonomy. The polyphialides and monophialides are important to separate Sections and species within *Fusarium*. Length and shape of microconidiophores were used confidently to separate *F. oxysporum*, *F. solani* and *F. moniliforme*. Booth (1971) also pointed out that perithecia was important as sexual stage of *Fusarium* species and finally separated the genus into Sections. The study on conidiophores and conidiogenous cells was a major contribution in *Fusarium* spp. taxonomy system by Booth. There are 12 sections, 44 species and 7 varieties in the Booths' system. Meanwhile, Nelson *et al.* (1983) separated each of the Section based on the presence or absence of microconidia and chlamydospores (intercalary or terminal) as well as the shape of microconidia and macroconidia (basal cells or foot cells).

The Section Liseola of *Fusarium* species are responsible for many economically important plant diseases and therefore are well-known by all *Fusarium* taxonomists. It is recognized in most morphologically-based classification systems for *Fusarium*. Booth (1971) had characterized members of this Section based on the formation of chains or false heads with microconidia, the shape of microconidia (spindle to ovoid), macroconidia with constricted apical and pedicellate basal cell, chlamydospores absent and cultures brownish white to orange cinnamon. Wollenweber and Reinking (1935) accepted three species and three varieties in the Section Liseola. Booth (1971), Nirenberg (1976), Gerlach and Nirenberg (1982), Nelson *et al*. (1983), Nirenberg and O'Donnell (1998) accepted 2, 10, 10, 4 and 29 species and varieties respectively within the Section Liseola (Leslie and Summerell, 2006). However, *Fusarium* taxonomists still disagree on the number of species within this Section and the appropriate morphological criteria to distinguish them, since the classifications are not universal.

## 2.6 The Identification of *Fusarium* spp.

*Fusarium* species can be recognized and differentiated from one another by using different approaches of identification. Data from morphological characteristics, pathogenicity test and vegetative compatibility groups (VCGs) are useful in relation to distinguish *Fusarium* species within Section Liseola that are pathogenic to sugarcane.

### 2.6.1 Morphological characteristics

The morphological species concepts of *Fusarium* are based on the observable morphological characters e.g. conidia, size and shape, are well described and widely available. The conidial type and morphology are commonly viewed when identifying of *Fusarium* species and the most important data to be collected (Summerell *et al*. 2003). Therefore, it is usefull criteria to be used for initial classification of biodiversity of *Fusarium* (Leslie *et al*., 2001). In identifying *Fusarium* spp. by morphological approach, CLA (3.2.4) and PDA (3.2.1) media were commonly used (Booth, 1971; Fisher *et al*., 1982; Nancy *et al*., 1982). *Fusarium* in the Section Liseola that involved with pokkah boeng disease is the most difficult group to be confidently identified especially by using morphological characteristics. In addition, for this study the pathogenicity test and vegetative compatible groups were also employed to assist the identification by using morphological characteristics.

### 2.6.2 Pathogenicity test

Fungi isolated from plants could be the pathogens that cause disease or saprophytes that can grow in the dysfunction tissues of plants with disease and not pathogenic to healthy plants (Nelson *et al*., 1983; Agrios, 2005). Some pathogens only cause severe diseases in plants which have been subjected to stress (inadequate soil moisture, extremes temperature or herbicides) (Burgess *et al*., 1994). For *Fusarium* spp. that causes pokkah boeng disease of sugarcane, it is still questionable whether *F. subglutinans* and/or other allied *Fusarium* species in Section Liseola are the causal

against of the disease. For that reason, pathogenicity test based on the Koch's postulates were used to prove that the isolated *Fusarium* species from diseased plants are the pathogens causing pokkah boeng disease. Normally plants were inoculated with conidia used as inoculum in the pathogenicity test of *Fusarium* (Burgess *et al*., 1994).

By following the Koch's postulates, firstly the cultivars used in the pathogenicity test should be identical to those on which the disease has been observed and isolated from the field. Then, when the cultures were inoculated into susceptible plants, it must initiate the characteristic disease symptoms. Finally, the organisms were re-isolated in pure culture and re-identified, after which it must be similar to the original organism that had been observed before (Brock and Brock, 1978; Agrios, 2005). Each steps are followed correctly and if produced the identical pathogen after re–isolation, then the pathogenicity test had been succeeded (Agrios, 2005).

### 2.6.3 Vegetative compatibility groups

Vegetative compatibility (VC) is also known as heterokaryon compatibility (HC) is used to strengthen the morphological data in the identification of *Fusarium* spp. VCG is based on genetic studies among strains where numerous underlying genes together produce a single result when two strains are compared (Leslie *et al.*, 1992; Leslie, 1993). VC can be considered as compatible when two hyphae can anastomose and fuse during growth to form a stable heterokaryon (Puhalla and Spieth, 1985; Klittich and Leslie, 1988; Leslie, 1993). Isolates that are vegetatively compatible belong to a common vegetative compatibility group (VCG) (Leslie and Summerell, 2006). However, if hyphae of the two strains do not fuse then the strains are considered to be vegetatively incompatible and are in different VCGs (Summerell *et al.*, 2001; Leslie and Summerell, 2006).

VCG analyses in *Fusarium* were carried out using nitrate non-utilizing (*nit*) mutants to force heterokaryons (Sidhu, 1986; Klittich *et al*., 1986, Sunder and Satyavir,

1998). With this, it is easy to score by using spontaneous *nit* mutants (Puhalla, 1985; Correll *et al*., 1986a; Sidhu, 1986; Bosland and Williams, 1987; Jacobson and Gordon, 1988). The *nit* mutants of *Fusarium* spp. are obtained when isolates are cultured on a medium containing $KClO_3$ and each *nit* mutants were classified as *nit*1, *nit*3 and NitM based on differential growth on media containing different nitrogenous compounds as the sole source of nitrogen. The four phenotyping media are minimal medium (MM) with nitrate, MM with nitrite, MM with hypoxanthine and MM with ammonium that differ in their nitrogen sources (Leslie and Summerell, 2006). All *nit* mutants can be used to force heterokaryons but the mutants in the *crn* class, however, must be discarded (Leslie and Summerell, 2006). Finally, this practice is to make pairings between *nit* mutants derived from different strains.

VCG analysis will provide an identification tool and a way to assess genetic variability in *Fusarium* population. In addition, it increases our understanding of the population biology of the genus (Summerell *et al*., 2001). Data from the morphological characteristics, pathogenicity test on healthy sugarcane and VCG's will form an integrate information to correctly identify the *Fusarium* species causing pokkah boeng disease.

# CHAPTER THREE
# GENERAL MATERIALS AND METHODS

## 3.1 Fungal Sources

A total of 133 strains of *Fusarium* species were isolated from sugarcane with pokkah boeng symptoms. The *Fusarium* strains were systematically numbered based on state locality (C - Pahang, D - Kelantan, J - Johor, K - Kedah, P - Penang, R - Perlis, T - Terengganu and I - Indonesia) and host codes (U - sugarcane).

## 3.2 General Culture Media

The general or standard media that regularly used in this research were potato dextrose agar (PDA) (Booth, 1971) (Burgess *et al*., 1994), water agar (WA) (Burgess *et al*., 1994), peptone pentachloronitrobenzene agar (PPA) (Papavizas, 1967; Nash and Snyder, 1962), carnation leaf-piece agar (CLA) (Fisher *et al*., 1982; Nancy *et al*., 1982), potassium chloride agar (KCIA) (Nelson *et al*., 1983; Burgess *et al*., 1994). Preparation and the ingredients used are presented in the Appendices 1, 2, 3, 4, and 5.

## 3.3 Sterilization

Materials and media were confirmed free from living organisms other than a selected one by using sterile technique procedures. The following techniques were applied, since propagules of bacteria and fungi are ubiquitous:

### 3.3.1 Heat sterilization

There are two types of heat sterilization of media and materials i.e. moist and dry heat.

3.3.1.1 Moist heat sterilization

Autoclave or pressure cooker was used for moist heat sterilization where materials were heated with saturated steam. It is the most reliable method for sterilization with recommended time and temperature depending on types of media and

materials. A temperature 121$^{o}$C with 0.7kg/cm$^{2}$ pressure for 15 min were used to autoclave culture media, soils, distilled water and glycerin, and also to discard living materials (Leslie and Summerell, 2006).

3.3.1.2 Dry heat sterilization

This technique of sterilization was used to sterilize glasswares (test tubes, beakers, glass petri dishes, conical flasks, pipettes, burettes and glass rods), metal instruments (forceps, scalpels and scissors) and heat-stable compounds. Objects that involved were heated to a temperature for a sufficient length of time to destroy contaminants. The temperature used was 160$^{o}$C for 1h depending on the type of materials (Leslie and Summerell, 2006). Glasswares were wrapped in heavy paper to prevent recontamination during cooling, transport or storage. After the sterilization process, the oven and its contents were allowed to reach ambience temperature before opening the doors to prevent breakage and recontamination by rushing cool air.

### 3.3.2 Sterilization by filtration

Additives such as vitamins, antibiotics may be destroyed by heating and therefore should be sterilized by filtration. The membrane filters with pore size 0.45 µm (Whatman®) were used as medium filters. Microorganisms and other large particles are retained on the filter when additives were added into media after autoclaving because the small size of the pores and dry adsorption onto pore walls (Dhingra and Sinclair, 1985).

### 3.3.3 Preparation of sterile media

A suitable sterile media were prepared when pure cultures of pathogens are desired. The sterilization by moist heat (3.3.1.1) dissolved and dispersed the ingredients. To prevent boiling over in the autoclave the flasks or bottles that were used should be no more than half full. After autoclaving, medium was then allowed to

cool slightly and poured into disposable plastic petri dishes to a depth of about 5 mm. The medium was then allowed to cool until it hardened and let the plates for a day or two to ensure that none have been contaminated. For slant agar preparation, dissolved medium were poured into bijou bottles or test tubes plugged with cotton. When sterilization was completed both tubes and bottles were placed in a slanted position until the medium solidified.

### 3..4 Sterile Transfer

All activities involved transferring of pathogen was done in a laminar flow. The transfer needles and loops were dipped into 70% alcohol and flame sterilized along its entire length before contacted with a culture to avoid cross-contamination. The transfer needle was cooled by touching it briefly to the sterile medium to ensure that residual heat in the flamed needle did not kill the sample being transferred. The cap or cotton plug from test tubes, beakers contain medium were remove and sterilized by lightly flame near the mouth that would killed any propagules of microorganisms that were in contact with the glass.

### 3..5 Surface sterilization

Surface sterilization is important to ensure a clean laminar flow chamber where all culturing works of isolates were carried out. It was done by swabbing the surface area before working with liquid disinfectants such as 70% ethanol or 1% sodium hypochlorite (NaOCl). The lamina flow surfaces also were exposed to short wave UV-light for 10 min before used. Trays, benches and other surfaces were sterilized too.

### 3.4 Standard Incubation Conditions

All cultures for identification are incubated in alternating 12 hours photoperiod (Salleh and Sulaiman, 1984) 40 cm below a light bank containing two 40W cool white fluorescent tubes and one black light long-wave (UV light) tube.

### 3.5 Purification of *Fusarium* Cultures

Pure culture is the priority for the identification of microorganism and there are a number of techniques used. For *Fusarium* species identification, a single – spore technique was employed.

#### 3.5.1 Single-spore technique

A suspension of spores was made in 10 ml sterile distilled water in a Bijou bottle from 7 days old *Fusarium* cultures. The culture loop was used to take a small portion of the mycelium and streaking it over thin agar surface in a Petri dish. As the streak progressed the spores became more and more separated till finally individual colonies arising from few or single spore were obtained. A single germinated conidium was removed on a small square of agar by using a transfer needle. Colonies initiated from single conidia were uniform and consistent in appearance and ensured pure cultures. It was also valuable for separating mixed cultures encountered in isolations from diseased plant materials or from soils.

### 3.6 Preservation of *Fusarium* Cultures

There were temporary and permanent preservations of *Fusarium* cultures. Both are necessary application for working cultures and further studies where it is possible to retain them in the condition in which they were at the time of isolation.

### 3.6.1 Temporary Stock Cultures

The stock is considered temporary because only cultures for interest that are maintained in the laboratory for study and reference.

**Agar slants**

The most common way of maintaining stock cultures is on agar slants. WA and half-strength PDA (125g potatoes; 10g dextrose; 20g agar; 1 liter distill water were prepared as slant agar in McCartney bottles. Three replicates for each strain were ready for working cultures. All slant agars were incubated at room temperature for 7 days and kept at $4\pm1^{o}$C.

**Carnation leaf pieces Agar (CLA) (Fisher *et al*., 1982; Nancy *et al*., 1982)** CLA was prepared by placing sterile carnation leaf pieces onto WA. All strains were cultured in CLA and incubated under the standard incubation conditions for about 2 weeks then colonized leaves were taken out and placed in sterile cryules (Wheaton cryule-1.8 ml). During dehydration over silica-gel the cryules were left partly open in a container at room temperature ($28\pm1^{o}$C) for 48 hours. The dehydration of colonized leaf pieces then stored at $4\pm1^{o}$C and can be used as a temporary method of storage. Cultures can be revived by placing the leaf pieces on CLA and restored for every 6 months.

### 3.6.2 Preservation of *Fusarium*

In these procedures, the activity of the *Fusarium* cultures is reduced to a very low level and the organism hence remains viable for long periods of time. Several techniques of preservation of *Fusarium* isolates were employed:

**Storage in soils**

A mixture of loam soil and sand (ration of 7:3) was placed in a Bijou bottle of about 1/3 full and autoclaved three times intermittently at $115^{o}$C with 1.1kg/cm$^2$

pressure for 30 min (Dhingra and Sinclair, 1985). The strains were cultured on PDA and left to grow for 10 days at the standard incubation conditions. Then conidia suspensions were prepared with sterile water and poured in the sterile soils. The bottles were then stored in a refrigerator at 4±1$^{o}$C after 7 days incubation at room temperature. Cultures were revived by sprinkling a few grains of soil onto PDA. Many fungi can survive for long period and remain viable in storage in this condition (Bakerspigel, 1954).

**Storage in deep-freezer**

This method of preservation was based on Hwang (1966) with slight modifications. The protective agent, 15% glycerol (Brock and Brock, 1978) was sterilized for 30 min at 121$^{o}$C 15 psi intermittently. Conidia from 10 day – old cultures were harvested with sterilized 15% glycerol (v/v). The sterile cryule (Wheaton cryule-1.8 ml) were inserted with 1 ml conidial suspension from sterile glycerol and stored in a deep-freezer at -80$^{o}$C. After one month, the viability of each strain was checked.