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MORPHOLOGICAL, BIOCHEMICAL AND DNA STUDIES FOR THE DEVELOPMENT OF MARKERS FOR THE SELECTION OF *Theobroma cacao* L. CLONES RESISTANT TO VASCULAR STREAK DIEBACK DISEASE

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By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Science and Environmental Studies Universiti Putra Malaysia

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Dedicated to my family

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

AFLP	=	Amplified Fragment Length Polymorphism
BAL	=	Borneo Abaca Limited
BSA	=	Bovine Serum Albumin
CBD	=	Coomassie Brilliant Blue Dye
CCM	=	Corticium Culture Medium
DMRT	=	Duncan's Multiple Range Test
CPB	=	Cocoa Pod Borer
IPM	=	Integrated Pest Management
MARDI	=	Malaysian Agricultural Research and Development Institute
PCR	=	Polymerase Chain Reaction
PPD-PC	=	p-phenylenediamine-pyrocatechol
PR	=	Pathogenesis-related
RAPD	=	Random Amplified Polymorphic DNA
RFLP	=	Restriction Fragment Length Polymorphism
SAS	=	Statistical Analysis System
SEM	=	Scanning Electron Microscope
SRFA	=	Selective Restriction Fragment Amplification
SSRs	=	Simple-sequence-repeats
STS	=	Sequence-tagged-site
TEM	=	Transmission Electron Microscope
UPGMA	=	Unweighted Pair Group Method with Arithmetic Averaging
UV	=	Ultra-violet
VSD	=	Vascular Streak Dieback
YAC	=	Yeast Artificial Chromosome
μm	=	micrometer
μg	=	microgram
μl	=	microliter
kbp	=	kilo base pair
mbp	=	mega base pair
kDa	=	kilo Dalton
mm	=	milimeter
nm	=	nanometer
mg	=	miligram
ml	=	mililiter
pg	=	picogram

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

MORPHOLOGICAL, BIOCHEMICAL AND DNA STUDIES FOR THE DEVELOPMENT OF MARKERS FOR THE SELECTION OF *Theobroma cacao* L. CLONES RESISTANT TO VASCULAR STREAK DIEBACK DISEASE

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October 1999

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Vascular streak dieback (VSD) is a serious disease of *Theobroma cacao* L. (cocoa) plant and it is attributed to the fungal pathogen *Oncobasidium theobromae*. Control of the disease is largely dependent on the use of systemic fungicides. Resistance to the pathogen had been found in the cocoa planting materials but as yet there was no marker to select for the resistant clones. In relation to this, this study was conducted to develop suitable markers for selection of cocoa clones resistant to a VSD disease. These include morphological, biochemical, enzyme activity, isozyme pattern, light and electron microscopy, and DNA markers.

The cocoa clones chosen represented three levels of resistance to VSD disease. The reported resistant clones were MHP80, SCA6, AMA15-15, ICS95, PA13, KKM25 and SCA9; moderately resistant clones were PBC140, KKM22 and MHP136, while the susceptible clones were MJS21, ICS84, NA32, NA33, UA13, MHP14, MHP37, PA7, IMC67 and EET 399.

The results showed that the morphological characteristics of leaf (shape, color and size), pod (color, shape and surface) and bean (shape, size and and cotyledon color) could not be used to distinguish between the cocoa

clones resistant and susceptible to VSD disease.

The results on the biochemical and enzyme parameters indicated that the activity of polyphenol oxidase and concentration of chitinase in cocoa leaf tissues were related to resistance to VSD disease. The level of both enzymes were significantly higher ($P \leq 0.05$) in the resistant cocoa clones compared to the susceptible clones. Others biochemical parameters such as the concentration of total soluble phenol and β-1,3-glucanase, activity of peroxidase, proteins and isozyme patterns could not distinguish the resistance of cocoa clones to VSD disease.

Studies on light microscopy showed that the growth of *Oncobasidium theobromae* in the leaves of resistant clones resulted in less hyphae compared to those from the susceptible clones. However, the results from the electron microscope studies showed that the infected tissues of both resistant and susceptible cocoa clones showed similar damage symptoms such as cell shrinkage and plasmolysis.

The results on Amplified Fragment Length Polymorphism (AFLP) technique to distinguish between cocoa clones that are resistant and susceptible to a VSD disease using selected primer combinations showed polymorphisms among cocoa clones. However, the similarity index and cluster analysis obtained from the data could not classify the cocoa clones as being resistant or susceptible to VSD disease. Meanwhile, a cloned unique fragment of resistant cocoa clone obtained from an AFLP analysis was able to differentiate between resistant (ICS95, KKM25, MHP80, AMA15-15) and susceptible (NA32, PA7, MHP14, MHP37) cocoa clones. This DNA marker was named VSDr1.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAJIAN MORFOLOGI, BIOKIMIA DAN DNA UNTUK MEWUJUDKAN PENANDA-PENANDA BAGI MEMILIH KLON *Theobroma cacao* L. YANG RINTANG TERHADAP PENYAKIT MATIROSOT JEJALUR VASKULAR

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Penyakit matirosot jejalur vaskular (VSD) ialah penyakit yang serius pada tanaman *Theobroma cacao* L. (koko) dan ia disebabkan oleh sejenis kulat patogen, *Oncobasidium theobromae*. Kawalan utamanya ialah dengan penggunaan racun kulat sistemik. Bahan tanaman koko yang rintang terhadap patogen telah dikenalpasti tetapi sehingga kini belum ada penanda untuk memilih klon yang rintang. Sehubungan dengan itu, kajian ini telah dijalankan untuk mewujudkan penanda yang sesuai bagi memilih klon koko yang rintang terhadap penyakit VSD. Ini termasuklah kajian morfologi, biokimia, aktiviti enzim, corak enzim, mikroskop biasa dan elektron dan penanda DNA.

Klon-klon koko yang dipilih adalah mewakili tiga aras kerintangan terhadap penyakit VSD. Klon yang dilaporkan rintang ialah MHP80, SCA6, AMA15-15, ICS95, PA13, KKM25 dan SCA9; klon sederhana rintang ialah PBC140, KKM22 dan MHP136, sementara klon rentan ialah MJS21, ICS84, NA32, NA33, UA13, MHP14, MHP37, PA7, IMC67 dan EET399.

Keputusan kajian mendapati bahawa ciri morfologi daun (bentuk, warna dan saiz), buah (warna, bentuk atau kelicinan permukaan) dan biji (bentuk,

saiz atau warna kotiledon) tidak dapat membezakan klon koko yang rintang dan rentan kepada penyakit VSD.

Keputusan mengenai parameter biokimia dan enzim pula menunjukkan aktiviti polifenol oksidase dan kepekatan kitinase di dalam daun koko mempunyai kaitan kerintangan terhadap penyakit VSD. Kedua-dua aras enzim tersebut adalah tinggi ($P \leq 0.05$) pada klon koko yang rintang berbanding klon yang rentan. Parameter biokimia lain seperti kepekatan jumlah fenol terlarut dan β-1,3-glukanase, aktiviti peroksidase, corak protein dan isozim didapati tidak boleh membezakan kerintangan klon koko terhadap penyakit VSD.

Kajian mikroskop biasa menunjukkan pertumbuhan hifa *Oncobasidium theobromae* pada daun koko yang rintang adalah sedikit berbanding dengan klon yang rentan. Walau bagaimanapun, keputusan dari kajian mikroskop elektron mendapati tisu yang dijangkiti pada kedua-dua jenis klon tersebut menunjukkan simtom kerosakan yang sama seperti pengecutan dan plasmolisis.

Keputusan kajian menggunakan teknik `Amplified Fragment Length Polymorphism' (AFLP) untuk membezakan klon koko yang rintang dan rentan menunjukkan polimorfisma di antara klon-klon koko dengan menggunakan kombinasi primer pilihan. Walau bagaimanapun, indeks kesamaan dan analisis kluster daripada data yang diperolehi tidak dapat mengkelaskan klon koko kepada rintang dan rentan kepada penyakit VSD. Sementara itu, fragmen unik DNA daripada analisis AFLP yang telah diklonkan didapati dapat membezakan di antara klon yang rintang (ICS95, KKM25, MHP80, AMA15-15) dan rentan (NA32, PA7, MHP14, MHP37). Penanda ini dinamakan VSDr1.

CHAPTER I

INTRODUCTION

Vascular streak dieback (VSD) disease, attributed to the fungal pathogen, *Oncobasidium theobromae,* is a serious disease in cocoa plants (Keane and Turner, 1972; Turner and Shepherd, 1978). The fungal basidiospores firstly attaches itself to the leaves and then, progresses through the leaf petiole towards the stem attacking the xylem vessels (Keane *et al.*, 1972; Tey *et al.*, 1985). The current practice of identifying diseased plants is through observing the symptoms. The first symptoms of infection are shown by chlorosis of one leaf usually on the second or third flush behind the tip, enlarged lenticels are usually evident on the stem immediately below the petiole of this leaf (Keane *et al.*, 1972). In the diseased region, the cambium turns rusty-brown rapidly when exposed to air by stripping the bark (Prior, 1981; Tey *et al.*, 1985). The disease progression on a mature tree usually takes about five months from the initial leaf infection to death of a branch up to one meter long (Keane *et al.*, 1972). A young cocoa seedling, however, would succumb in a few weeks.

The control of VSD disease in the past is mainly dependent on the application of systemic fungicides. However, these chemicals usually lose their efficacy due to the evolution of the pathogen. Meanwhile, some cocoa clones have been found to be resistant to the disease (Bong and Phua, 1984; Tey and Musa, 1984), and thus present an alternative approach in dealing with the

disease. The conventional method to evaluate resistance has been the selection for resistant plants after exposure to the natural inoculum of the pathogen (Tey *et al.,* 1987b). Resistant clones were classified based on the percentage of the plants not infected after 14 months of the trial period. This method, however, is time consuming. Thus, it hypothesized that other techniques could be used to identify the cocoa clones resistant to a VSD disease.

An alternative method is to expose cocoa leaves to basiodiospore suspension of *Oncobasidium theobromae* for a certain period and then observing the development of the fungal pathogen *in vitro.* However, this method has not been tested yet.

Several reports on the involvement of lytic enzymes (chitinase, ß-1,3-glucanase) and enzymes that metabolize phenols (polyphenol oxidase, peroxidase, phenylalanine ammonia-lyase, ß-glycosidase) during plant defense against pathogens and parasites have been published (Van Loon, 1986; Barz *et al.,* 1990; Hoagland, 1990). Increase in peroxidase, polyphenol oxidase, chitinase and ß-1,3-glucanase activities have been associated with immunization in several plant-pathogen systems (Hammerschmidt *et al.,* 1982; Bajaj *et al.,* 1986; Chai and Doke, 1987; Dalisay and Kuc, 1988; Metraux and Boller, 1988; Metraux *et al.,* 1988; Tezun *et al.,* 1988; Pan *et al.,* 1991). Although the enzyme activities were usually increased in the infected tissues, this does not prove that they were involved in the disease resistance

(Lazarovits and Ward, 1982). However, a strong correlation between the activity of an enzyme before infection and certain disease resistance may indicate such a role. A strict relationship between the enzyme levels of plants and susceptibility to VSD disease in cocoa is yet to be established. In addition, less attention has been paid to the variation in activity of these enzymes in plants prior to infection and its role in resistance.

The availability of molecular techniques has opened new ways to characterize germplasm, to determine genetic diversity and to establish gene linkages of agronomic traits to molecular markers. Amplified Fragment Length Polymorphism (AFLP) technique has been used to assess genetic relationships, to quantify levels of genetic diversity and to identify cultivars of agriculturally important plants (Zabeau and Vos, 1993; Vos *et al.,* 1995; Hill *et al.,* 1996; Maughn e*t al.,* 1996; Powell *et al*., 1996; Sharma *et al.,* 1996; Donini *et al.* 1997; Qi and Lindhout, 1997; Van Toai *et al.,* 1997). Unique DNA fragments that are obtained in AFLP may correspond to unique locations in the genome and, if associated with a particular phenotype, may be used as a selection marker.

Objectives of Study

The objectives of this study are to develop markers suitable for the selection of resistant cocoa clones to VSD disease. This involves a multi-parameter approach as follows:

1. To study the morphology of leaf (shape, color and size), pod (color, shape and surface) or bean (shape, size and cotyledon color) of various cocoa clones and their relationships to VSD disease susceptibility.
2. To determine the variations in total soluble phenol, chitinase, ß-1,3-glucanase, peroxidase and polyphenol oxidase activity in the leaves of several cocoa clones and their relationships to VSD disease susceptibility.
3. To study the growth of *Oncobasidium theobromae* on the leaf of resistant and susceptible cocoa clones after exposure to the fungal pathogen.
4. To determine whether the cocoa clones used that are known to be resistant or susceptible to VSD disease could be differentiated using the AFLP technique.
5. To clone a unique DNA fragment obtained by AFLP and to investigate its use as a DNA probe for the selection of VSD resistance in cocoa.

CHAPTER II

LITERATURE REVIEW

Economic Importance of Cocoa

Theobroma cacao L. (Sterculiaceae), an important tropical rain forest species, is grown for its oil-rich seed, to produce cocoa and cocoa butter (Figueira *et al.,* 1993). Cocoa has a high food value, containing as much as 20 percent protein, 40 percent carbohydrate, and 40 percent fat (Whistler *et al.,* 1956). It is also mildly stimulating because of the presence of theobromine, an alkaloid that is closely related to caffeine. The pulverized residue, which is called cocoa, is used in beverages and as a flavoring. Chocolate is also derived from cocoa beans.

Cocoa seeds are a major cash crop of the tropical world, but prices fluctuate widely and economic hardships occur when prices are low. Despite this, only about 10% by fresh weight of the fruit is commercialized, although several promising commercial products could be obtained from the fruit (Greenwood-Barton, 1965). Unutilized portions of cocoa pods contain many potential new products that could provide extra income for cocoa growers. The most promising products appear to be cocoa pulp and the gums from pod husks (Figueira *et al.,* 1993). The high proportion of fat in the bean kernels (cocoa butter) is used in medications, cosmetics, and soaps.

Cocoa pod husks contain 3 to 4% of potassium on a dry basis (Wood and Lass 1985). Pod husk ash has been used to make soap in Ghana and Nigeria (Oduwole and Arueya 1990; Arueya 1991).

A cocoa husk extract called cocoa pigment, which is a mixture of condensed or polymerized flavonoids (such as anthocyanidins, catechins, leukoanthycyanidin), sometimes linked with glucose, has been utilized in Japanese food industries (Kimura, 1979). Recently this extract has been shown to inhibit cytopathic effects of HIV in cell culture (Unten *et al.,* 1991). The anti-HIV activity was attributable to interference with the virus adsorption, rather than inhibition of the virus replication after adsorption.

In cocoa, lysigenous cavities filled with mucilaginous substances occur in roots, stems, flowers, and leaves (Brook and Guard 1952) as well as fruit husks (Figueira *et al.,* 1991). Krishna Moorthy and Subba Rhao (1976, 1978, 1980) also isolated gums from the seed pulp. Polysaccharides of cocoa were first characterized by Whistler *et al.* (1956), who found differences in hot-water-soluble polysaccharides between seed and pod husks. Blakemore *et al.* (1966) examined the hot-water-soluble fraction of husk polysaccharide and concluded that the major part of this fraction was a pectic material. Cocoa pod husks were examined as a source of pectin by mild acid extraction by Adomako (1972) and Berbert (1972), but yields were low and the pectin was inferior to apple or citrus pectin in gel-forming ability. Krishna Moorthy and Subba Rhao (1978, 1980) found that gums from seed pulp were effective in

low concentrations as a binder for pharmaceutical pills, and reported that suspending properties were superior to tragacanth, sodium alginate, sodium carboxy-methyl cellulose, and methyl cellulose. Figueira *et al.* (1991). have recently characterized cocoa gums from pod husks and stems to evaluate their potential as a replacement for gum karaya or as a new commercial product.

Cocoa Industry in Malaysia

The first commercial cocoa planting in Malaysia started in 1950s in Jerangau, Terengganu (Anon, 1991). This was followed by the Borneo Abaca Limited (BAL Plantations Sdn Bhd) in Sabah. The initial planting material was a mixture of Amelonado and Trinitario types. The programs on varietal improvement have produced superior planting material, which are more vigorous and tolerant to diseases. This led to the expansion in planting areas. Availability of land and the high price of cocoa beans in the late 1970s and early 1980s further boosted the expansion of cocoa cultivation throughout the country. The introduction of clonal planting material and rehabilitation programs also contributed markedly to this expansion. Some problems as described below which can pose as threats to this industry have been identified and steps towards solving them have been actively undertaken by both the public and private sectors.

Unstable and low prices of cocoa dry bean, low productivity, pest and disease problem, shortage and high cost of labor and competition for land use from other crop have been identified as the major factors affecting the growth

of the cocoa planting industry. Whereas, the quality of cocoa beans and cocoa products, limited cocoa product range and uses, and lack of processing technology and facility are the major issues faced by the cocoa grinding and manufacturing industry. These factors will influence the growth of the cocoa planting and downstream industry in Malaysia.

As a result of the prolong low cocoa bean prices which are below production costs to many growers, more cocoa areas have been either abandoned or chopped down. In 1992, the total cultivated areas was estimated at 388 700 ha, declined from about 405 000 ha in 1987 (Anon, 1992). The cultivated area was declined further in 1997 to about 156 682 ha (Anon, 1997). For 1998, the reduction is marginal estimated at about 5% from 1997 to around 150 000 ha (Anon, 1998). With the recovery in prices, it is expected that the cultivated cocoa areas will be stabilized at this level. The practical solution to low prices of cocoa bean is to reduce the cost of production through increase in productivity and efficiency, which can be within reach of the cocoa growers. Development of new technology specifically to address the various constrains would further enhance the competitive edge of the cocoa industry that can generate profit even at low prices.

Low productivity in the small holder sector has been identified as a problem. One of the causes is the premature senescence of young cocoa fruit (cherelle wilt) that lead to sub-optimal productivity, particularly when losses are already high due to fungal diseases and insect infestation. Lack of

input, inappropriate application of technology and improper management are the major factors attributed to low productivity. The productivity of the small holder sector could be increased through modification in the planting system, use of high yielding cocoa clones and economical shade trees, proper fertilizer application and more efficient pest and disease management. In most cases, the plants and/or the area need to be rehabilitated. Mature budding constitutes the main technique of rehabilitation for these uneconomic farms besides pruning and other cultural practices (Mohd Jelani, 1985; 1991). The suitability and improvement of various economic shade trees such as durian, rubber, oil palm and bread fruit in order to increase the productivity are being extensively evaluated (Denamany *et al.,* 1992; Mohd Jelani *et al.,* 1992; Sapiyah *et al.,* 1992; Nawi, 1992a,b).

High density planting of cocoa has been introduced in recent decades in Malaysia, and the prospect of higher early yield was anticipated (David and Pang, 1989; Ab. Kahar *et al.,* 1991; Lam and Lim, 1991). It is important to identify certain morphological traits in cocoa clones that are suitable to high density, pest and disease management. The suitability of clones for high density planting has been examined by Kasran *et al.* (1993) and they suggested that slow growing planting materials with high productivity to avoid self-shading should be used. Many attempts have been made actively to reduce tree vigor by utilizing dwarfing rootstocks to obtain slow growing planting materials suitable for high density planting. However, the effect of dwarfing rootstock on scion growth have not been detected thus far in cocoa