UNIVERSITI PUTRA MALAYSIA

IN VITRO PRODUCTION AND INFECTIVITY OF ONCOBASIDIUM THEOBROMAE TALBOT & KEANE BASIDIOSPORES

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IN VITRO PRODUCTION AND INFECTIVITY OF *ONCOBASIDIUM THEOBROMAE* TALBOT & KEANE BASIDIOSPORES

By

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Agriculture Science in the Faculty of Agriculture Universiti Putra Malaysia

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DEDICATED TO :

MY PARENTS,

BROTHERS,

SISTERS,

BROTHER-IN-LAW,

AND FRIENDS

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Agriculture Science.

IN VITRO PRODUCTION AND INFECTIVITY OF _ONCOBASIDIUM THEOBROMAE_ TALBOT & KEANE BASIDIOSPORES

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Chairman : Zainal Abidin Mior Ahmad, Ph.D.

Faculty : Agriculture

In vitro sporulation of *Oncobasidium theobromae* Talbot & Keane was induced using a modified version of the system by Shari Fudin (1995) by exposing 14 day old cultures to saturated moist air from a humidifier. Basidiospore production was able to be repeated consistently using the new system where a black perspex cabinet was used as an enclosure but the number of spores produced was low (not exceeding 36,300 spores per ml). Sporulation occurred when cultures were exposed to a mean relative humidity above 90% and low mean temperature (< 26°C). Duration of exposure to saturated moist air was vital for *in vitro* sporulation. *O. theobromae* cultures produced spores when exposed to continuous saturated moist air for more than 240 mins/day but significantly higher counts and faster formation of spores were achieved at exposures of 480 mins/day to saturated moist air. 14 days old cultures gave high spore counts.

Observations indicated that the presence of monilioid hyphae was necessary for sporulation of *O. theobromae* since the basidium arises from it, but the formation did not appear to be influenced by factors of sporulation like saturated

moist air. There was no significant difference in the formation of monilioid hyphae when the duration of exposure of cultures to saturated moist air was varied. Basidia only formed when the cultures were exposed to more than 240 mins/day of saturated moist air but the duration of exposure to saturated moist air did not appear to influence the quantity of basidia formed in cultures. Sporulation was improved by maintaining the modified system under controlled environmental conditions with the temperatures between 21°C-23°C and the mean relative humidity above 90%. Cultures were exposed to continuous saturated moist air for 480 mins/day. The spore production under these conditions was consistent with mean spore counts ranging from 62,300 spores/ml to 120,000 spores/ml. The spores produced were viable as confirmed by an average spore germination of 46% and 57.5% after 12 hours and 24 hours incubation in the laboratory.

Infectivity of *in vitro*-produced basidiospores of *O. theobromae* on cocoa seedlings was achieved. Two inocula, basidiospore suspensions and agar plugs were tested at two venues, glasshouse and laboratory. Inoculations conducted in the laboratory were successful while inoculations carried out in the glasshouse gave negative results. Careful post-inoculation maintenance in the laboratory by keeping the inoculated seedlings in the dark at 25 $\pm$ 2 °C for three days in the laboratory and covering the seedlings with wetted polythene bags proved vital for infectivity of the *in vitro*-produced basidiospores. Agar plugs were more successful in inducing infectivity than spore suspensions. Symptoms of VSD were observed 2-5 months after inoculation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian.

PENGHASILAN SPORA ONCOBASIDIUM THEOBROMAE TALBOT & KEANE SECARA *IN VITRO* DAN PENJANGKITANNYA

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Penghasilan basidiospora *Oncobasidium theobromae* Talbot & Keane secara *in vitro* telah dilakukan dengan mengubahsuai kaedah oleh Shari Fudin (1995) di mana kultur yang berumur 14 hari didedahkan kepada udara lembap tepu. Basidiospora telah berjaya dihasilkan dan diulangi secara konsisten dengan menggunakan sistem baru ini di mana sebuah kabinet perspek hitam telah digunakan sebagai penghadang tetapi kuantiti spora yang didapati masih lagi rendah (tidak melebihi 36,300 spora/ml). Pensporulan didapati berlaku apabila min kelembapan bandingan melebihi 90% dan min suhu yang rendah (< 26°C). Jangkamasa pendedahan kultur kepada udara lembap tepu adalah penting dalam pensporulan secara *in vitro*. Kultur *O. theobromae* menghasilkan spora apabila didedahkan kepada udara lembap tepu secara berterusan melebihi 240 min/hari tetapi kuantiti spora yang lebih tinggi serta penghasilan spora lebih awal yang bererti didapati apabila didedahkan kepada udara lembap tepu selama 480 min/ hari secara berterusan. Kultur berumur 14 hari didapati menghasilkan kuantiti spora yang lebih tinggi.

Pemerhatian ke atas kehadiran hifa monilioid menunjukkan bahawa ia diperlukan dalam proses pensporulan oleh kerana basidium terbentuk daripada hifa tersebut, tetapi pembentukan hifa ini didapati tidak bergantung kepada faktor pensporulan seperti udara lembap tepu. Tiada perbezaan yang bererti dalam pembentukan hifa monilioid ini apabila jangkamasa pendedahan kultur kepada udara lembap tepu itu dipelbagaikan. Basidium terbentuk apabila kultur didedahkan kepada udara lembap tepu melebihi 240 min/ hari tetapi jangkamasa pendedahan kepada udara lembap tepu tidak mempengaruhi kuantiti basidium yang terbentuk dalam kultur. Pensporulan *O. theobromae* dapat ditingkatkan dengan mengendalikan sistem yang telah diubahsuai ini dalam keadaan persekitaran yang terkawal di mana suhu adalah tetap antara julat 21 °C - 23 °C dan kelembapan bandingan melebihi 90%. Kultur didedahkan kepada udara lembap tepu selama 480 min/ hari secara berterusan. Penghasilan spora didapati lebih konsisten di mana purata bilangan spora yang dihasilkan adalah daripada 62,300 spora per ml hingga 120,000 spora per ml. Kebolehidupan spora yang dihasilkan juga ditentukan di mana purata percambahan spora adalah 46% dan 57.5% masing-masing selepas pengeraman di dalam makmal selama 12 dan 24 jam.

Keupayaan menjangkiti anak benih koko dengan menggunakan basidiospora *O. theobromae* yang dihasilkan secara *in vitro* telah dicapai. Dua jenis inokulum, ampaian basidiospora serta plak agar telah diuji di dua tempat berasingan iaitu di rumah kaca dan makmal. Inokulasi yang dilakukan di makmal berjaya menghasilkan jangkitan manakala inokulasi di rumah kaca memberi keputusan negatif. Keadaan pos-inokulasi di makmal yang dikawal rapi dengan meletakkan anak pokok yang diinokulat dalam gelap pada 25 $\pm$ 2 °C selama 3 hari serta menutup anak pokok

tersebut dengan beg plastik yang dibasahkan didapati penting untuk basidiospora *in vitro* untuk menjangkiti anak benih koko. Plak agar didapati lebih berjaya dalam jangkitan jika dibandingkan dengan ampaian basidiospora. Simtom penyakit VSD pula dapat diperhatikan 2-5 bulan selepas inokulasi.

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

PAGE

PAGE

LIST OF PLATES

PAGE

CHAPTER 1

INTRODUCTION

Cocoa (*Theobroma cacao L.*) is one of the major agricultural commodities of Malaysia. Cocoa industry in Malaysia has played an important role in agricultural diversification and is now the third most important crop after oil palm and rubber. Malaysia is ranked the fifth largest producer of cocoa in the world. In terms of export earnings, cocoa and cocoa products contributed about RM 672.8 million or 0.5% of total export earnings for the year 1993 (Ministry of Primary Industry, 1998). However , the total area cultivated with cocoa and the nett production in Malaysia has been declining due to prolonged low prices and serious pest problems. It was estimated that land hectarage under cocoa will be further reduced from 271,339 hectares in 1994 to 156,738 hectares in 1997 (Malaysian Cocoa Board, 1998). In terms of yield, cocoa bean production in 1997 was 106,027 tonnes, a drop of 20% from 1995. The recent recovery of cocoa prices could revive or improve the cocoa industry in this region especially in Malaysia. Nevertheless, cocoa production is also dependent on effective pest and disease management to ensure high yield.

One of the major diseases of cocoa is vascular-streak dieback (VSD). It is considered the most threatening disease of cocoa in Malaysia and in neighbouring

cocoa producing countries in Asia (Varghese, 1985). It is a systemic disease caused by *Oncobasidium theobromae* Talbot & Keane, a highly specialised, ecologically-obligate parasite of cocoa (Keane *et al.*, 1972; Prior, 1978; Keane & Prior, 1991). The disease spreads through the air borne basidiospores, which is the only known infective propagule of the pathogen. The spores penetrate young unhardened flushes of cocoa and grow in the xylem tissues of the leaves, branches and stem (Keane *et al.*, 1972; Prior, 1979). Extensive infections debilitates the growth of mature trees and causes death of seedlings.

VSD has very characteristic symptoms which are similar whether on seedlings or in mature trees. Paling of leaves, normally in the hardened flush occurs during the early stages of the symptom development, followed by chlorosis and falling of leaves in the advanced stages. The fungus also causes brown streaking in the vascular tissues (Keane *et al.*, 1972; Prior, 1979; Zainal Abidin *et al.*, 1981).

The occurrence of VSD in epidemic proportions have been reported in Malaysia especially in Sabah. Mortality of more than 70% of immature cocoa was common in Sabah prompting to repeated plantings (Varghese *et al.*, 1987). Apart from this, significant debilitation and dieback of mature plantings can also lead to serious yield losses. Byrne (1976) estimated production losses of 25 - 40% due to VSD incidence.

The fungus sporulates on the abscised leaf scars during the wet weather and the spread of the disease coincides with periods of heavy rainfall (Keane *et al.*, 1972; Keane, 1981; Zainal Abidin, 1982). Sporulation does not occur in axenic

cultures under normal laboratory conditions. Therefore, spores are not readily available for use as inocula especially if required for inoculation purposes such as in work to test disease resistance against VSD.

In depth research into the host-parasite relationship had been carried out but progress in other areas such as disease resistance screening were slow due to irregular and sparse availability of basidiospores under natural conditions. Although Lam (1988) was able to induce sporulation in axenic cultures, the inconsistent production of spores hampered further investigations of the fungus especially studies on its pathogenicity. To date, pathogenicity tests using basidiospores collected from the field have been successful only when inoculated cocoa plants were exposed to natural conditions at night. It appears that, as with sporulation, infectivity requires very specific conditions too.

The scope of work encompassed in the present study emphasizes on *in vitro* sporulation and the infectivity of *in vitro*-produced basidiospores and has the following objectives :

1) to study the production of basidiospores of *O. theobromae* in axenic cultures by examining the influence of two factors, saturated moist air and age of culture.
2) to study the spore morphology and the viability of the *in vitro*-produced basidiospores.
3) to confirm the infectivity of *in vitro*-produced basidiospores on cocoa seedlings.

CHAPTER 2

LITERATURE REVIEW

Vascular-Streak Dieback (VSD) Disease Of Cocoa

A destructive dieback disease of cocoa, distinguishable from the type of dieback induced by environmental factors and insect attack, was recognised in Papua New Guinea in the early 1960s by Shaw (1962) and Bridgland *et al.* (1966a,b, 1967). Then, simply called 'cocoa dieback', it was thought that the disease was associated with *Botryodiplodia theobromae* which was invariably isolated from dead or dying branches (Shaw, 1962). Later, evidence was obtained that the disease was in fact caused by an unknown aggressive pathogen that proved to be a new and primary pathogen specific to cocoa which invaded healthy cocoa well before secondary invaders such as *Botryodiplodia theobromae* (Keane *et al.*, 1972).

This disease was referred to as 'vascular-streak dieback' (VSD), to distinguish it from other types of dieback. It was then identified as the most serious disease of cocoa in Asia and in Papua New Guinea but was unknown in South America and Africa (Keane & Prior, 1991). Keane *et al.* (1972) and Prior (1978)

confirmed that the disease was caused by a new genus and species of fungus, given the name *Oncobasidium theobromae* by Talbot & Keane (1971).

The disease was first described from Papua New Guinea by Keane *et al.* (1972) as a dieback disease which could be distinguished from other forms of cocoa dieback. Keane (1981) and Prior (1985) observed that in Papua New Guinea, VSD was confined to all the major cocoa growing areas in the mainland of Papua New Guinea and the island of New Britain but was not known to occur on the outlying islands of Manus and the North Solomons despite widespread cocoa planting activities there.

VSD was first reported in West Malaysia by Keane & Turner (1972) by which time it had spread widely in new and well established cocoa plantations in many states here. Chan & Lee (1973) later reported occurrence of the disease in five main cocoa growing areas of West Malaysia in Perak (Bagan Datoh and Teluk Intan), isolated patches in Selangor, Trengganu (Jerangau), Johor and Pahang. In 1981, VSD was considered to be the most serious disease of cocoa in Malaysia and the presence of VSD was confirmed in all major cocoa growing areas in Perak, Negeri Sembilan, Malacca, Trengganu, Johor and Pahang (Zainal Abidin *et al.*, 1981). Disease incidence was estimated to average between 10 - 35% in Jerangau (Chan & Syed, 1976).

VSD was reported to be present in Sabah by Williams & Liu (1976). However, it was believed to have occurred in Sabah since the 1960's and became widespread in 1970's (Sidhu, 1987). Varghese (1980) and Bong *et al.* (1983)

confirmed infections of VSD in Tawau, Sandakan, Lahad Datu and Giram. Sidhu (1987) observed a particular severe and widespread epidemic of VSD on cocoa in Sabah following very wet weather in 1983 where seedling mortality in nurseries and immature field plantings was between 56 - 100%. Meanwhile, the occurrence of VSD in Sarawak was first reported by Tiong & Kueh (1986).

Taxonomic studies have confirmed that Malaysian isolates of *O. theobromae* were morphologically identical to the isolates studied in Papua New Guinea (Varghese *et al.*, 1981). Although parallel comparisons were not attempted, the Malaysian isolates were reported to grow consistently in culture (Varghese *et al.*, 1981) and sporulate in axenic culture (Lam *et al.*, 1988). On the other hand, *in vitro* sporulation of isolates from Papua New Guinea had not been reported. Keane & Prior (1991) suggested the possibility of physiological differences existing between isolates from different geographical locations.

The disease had spread in several cocoa growing countries in the Asian region. According to a review by Mainstone *et al.* (1983), VSD was also reported in the Philippines, Indonesia, Thailand, India and China. The first record on Hainan Island in China was reported by Turner and Keane (1982). The disease was reported as being highly destructive in 5 year old cocoa blocks in Kottayam District of Kerala state in India (Abraham, 1981) and also in Southern India (Prior, 1986).

The first official record of VSD in Indonesia was in 1982 from Sebatik Island off the coast of East Kalimantan (Anon, 1985). It was believed that infections had spread due to the close proximity to the already infected cocoa in