UNIVERSITI PUTRA MALAYSIA

AZOXYSTROBIN SENSITIVITY, MORPHOLOGICAL AND GENETIC MARKER STUDIES OF *COLLETOTRICHUM GLOEOSPORIOIDES* (PENZ) PENZ. AND SACC. FROM MANGO AND C CAPSICI (H. SYD.) E. BUTL AND BISBY FROM CHILLI

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By

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Chairperson : Dr. Faridah Abdullah

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This project evaluated the sensitivities of *Colletotrichum gloeosporioides* and *C. capsici* towards the fungicide Azoxystrobin. This was complemented by morphological and genetic marker studies of the two species in order to record the natural variation of the two species.

Twenty five cultures of *C. gloeosporioides* were isolated from mangoes (*Mangifera indica*) and 22 of cultures of *C. capsici* isolated from chillies (*Capsicum annum*) were used. They were collected from Malaysia, Indonesia, Thailand, Philippines and Taiwan. Single spore cultures were made to establish the species identity.

Isolates of both species were tested separately for fungicide sensitivity. The spore suspension was optimised to 10^5 spores/ml for bioassays. The assay medium used was a glycerol based Alkyl Ester broth, amended with rates of technical azoxystrobin at 0.0001, 0.001, 0.01 and 0.1ppm with blanks of Alkyl Ester broth as controls.

The tests were divided into statistical validation and actual baseline studies. For validation studies, 6 isolates in 6 replicate tests were used in the calculation of experimental errors. The baseline test consisted of 3 replicate treatments per Azoxystrobin rate per isolate with double controls for 14 isolates of *C. gloeosporioides* and 15 isolates of *C. capsici*. The validation isolates were included together with the baseline isolates during final analysis to give a total of 20 isolates for *C. gloeosporioides* and a total of 21 isolates for *C. capsici* .

The Alkyl Ester broth was inoculated with spore-suspensions of each isolate separately to give a final concentration of 10^5 spores/ml in the mixture. 60 µl droplets of the broth-spore mixtures were incubated in 55 mm petri dishes and covered with cover slips. The reference isolate PML1 was included in all tests to monitor the consistency. Assessment was done by scoring the number of spores out of 50 that showed germ tube elongation to a minimum of twice the spore length.

Dose responses were plotted of log rate against the number of spores germinated to derive 80% effective dose or ED_{80} values for each isolate. This entire experiment was repeated twice and the data analysed to determine the intra and inter experimental variation. Sensitivity values were also calculated by linear regression analysis using arcsine transformation of the data (ACSAPWIN). Results showed that the ED_{80} range for *C. gloeosporioides* was between 0.01 ppm to 0.1 ppm while this was between 0.001 ppm to 0.01 ppm for *C. capsici*.

From cultural studies, *C. capsici* displayed similar characteristics whereas this range was more variable for *C. gloeosporioides*. Morphological studies showed that the growth rates of *C. gloeosporioides* and *C. capsici* were similar with an average growth rate of 7 cm/day for its diameter. The best growth medium was Alkyl Ester Agar followed by Oatmeal Agar, V8 Juice Agar, Potato Dextrose Agar, Malt Agar and lastly, Cooke's medium.

Genetic characterization was done using short primer Random Amplified Polymorphic DNA (RAPD) studies, Long primer-Random Amplified Polymorphic DNA studies (LP-RAPD) and Random Amplified Microsatellite studies (RAMS). The final dendrograms showed general clustering according to countries of origin for *C. gloeosporioides* and *C. capsici*.

Abstract testis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat keperluan untuk ijazah Master Sains.

KAJIAN SENSITIVITI TERHADAP AZOXYSTROBIN DAN JUGA KAJIAN MORFOLOGI DAN 'MARKER' GENETIK UNTUK *COLLETOTRICHUM GLOEOSPORIOIDES* (PENZ) PENZ. AND SACC. DARI MANGA DAN *C. CAPSICI* (H. SYD.) E. BUTL. AND BISBY DARI CILI.

Oleh

QUAH JU LEE

Disember 2000

Penyelia : Dr. Faridah Abdullah

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Projek ini menilai sensitiviti *Colletotrichum gloeosporioides* dan *C.capsici* terhadap fungisid Azoxystrobin. Projek dilengkapi dengan kajian-kajian morfologi dan petanda genetik bagi kedua--dua species dengan tujuan untuk merekodkan maklumat variasi semulajadi bagi kedua-dua spesis.

Dua puluh lima kultur *C. gloeosporioides* yang dipencilkan dari buah mangga dan dua puluh dua kultur *C. capsici* dari buah cili telah digunakan. Kultur telah dikumpulkan dari Malaysia, Indonesia, Thailand, Filipina dan Taiwan. Sampel berpenyakit telah diisolatkan daripada perumahnya dan pemencilan dibuat untuk membolehkan pengenalpastian spesis dibuat seterusnya.

Isolat daripada spesis telah diuji secara berasingan untuk mendapatkan maklumat sensitivity terhadap fungisid. Suspensi isolat telah dioptimumkan pada kepekatan 10^5 spora/ml untuk ujian. Medium ujian yang telah digunakan adalah broth Alkyl Ester

yang berdasarkan gliserol dengan kepekatan Azoxystrobin pada 0.0001 ppm, 0.001 ppm, 0.01 ppm dan 0.1 ppm sementara kawalan yang digunakan adalah broth Alkyl Ester sahaja.

Ujian telah dibahagikan kepada kajian bacaan ralat ujikaji atau 'validation' dan kajian sebenar sensitivity atau 'baseline'. Untuk kajian 'validation'; 6 isolat telah digunakan dalam 6 ujian replikat dan data telah digunakan untuk mengira bacaan ralat eksperimen. Ujian 'baseline' terdiri daripada 3 rawatan replikat untuk setiap kadar bagi setiap isolat dengan dua kali ganda replikat masing-masing untuk rawatan kawalan bagi 14 isolat *C. gloeosporioides* dan 15 isolat *C. capsici*. Isolat dari kajian 'validation' kemudiannya dimasukkan bersama dengan isolat 'baseline' untuk memberikan sejumlah 20 isolat untuk *C. gloeosporioides* dan 21 isolat untuk *C. capsici*.

Broth Alkyl Ester telah diinokulat secara berasingan untuk ampaian spora setiap isolat untuk menghasilkan kepekatan akhir sebanyak 10^5 spora/ml dalam campuran tersebut. Titisan 60 µl dari campuran broth dengan spora itu seterusnya dieramkan dalam piring petri 55 mm dengan ditutup dengan sisip kaca. Isolat rujukan , PML1, telah dimasukkan ke dalam semua ujian untuk memantau keseragaman ujian. Penilaian dilakukan dengan mengira bilangan spora yang menunjukkan percambahan melalui tiub germa sekurang-kurangnya dua kali ganda panjang spora tersebut dari 50 spora setiap plat.

'Dose responses' kemudiannya telah diplot bagi kadar log melawan bilangan spora tercambah untuk menghasilkan nilai-nilai ED_{80} untuk setiap isolat. Keseluruhan

ujian telah dijalankan dua kali untuk menentukan variasi sesama isolat dalam kumpulan atau 'intra' dan diantara isolat atau 'inter' dalam ujian-ujian. Nilai-nilai sensitiviti juga dikira melalui regresi linear dengan menggunakan transformasi arc-sin bagi data tersebut (ACSAPWIN). Keputusan menunjukkan nilai ED_{90} bagi *C. gloeosporioides* adalah diantara 0.01 ppm hingga 0.1 ppm sementara nilai ED_{90} bagi *C. capsici* adalah diantara 0.001 ppm hingga 0.01 ppm.

Dari kajian kultur *C. capsici* menunjukkan ciri-ciri yang hampir serupa sesama sendiri manakala ciri-ciri *C. gloeosporioides* menunjukkan lebih variasi. Kajian morfologi pula menunjukkan bahawa kadar pertumbuhan diameter hampir serupa dengan purata 7 cm/hari. Medium pertumbuhan yang paling baik adalah Agar Alkyl Ester diikuti oleh Agar Oatmeal, Agar Jus V8, Agar Potato Dektrosa, Agar Malt Ektrak dan akhirnya Medium Cooke.

Pencirian genetik telah dilakukan dengan menggunakan kajian primer pendek RAPD, kajian primer panjang LP-RAPD dan kajian RAMS. Keputusan akhir dalam bentuk dendrogram menunjukkan perkelompokan umum mengikut negara asal masing-masing untuk *C. gloeosporioides* dan *C. capsici*.

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Especially to my beloved father, Dr. Quah Soon Cheang, for all his care, love and guidance.

Finally, to my beloved late mother who never failed to keep the faith and the belief that all things are possible with the help of God.

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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Quah Ju Lee

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Chemicals

CTAB	cetyltrimethylammonium bromide
EDTA	Ethylenediamine tetraacetate
KCl	Potassium chloride
$MgCl_2$	Magnesium chloride
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer

Media

AEA	Alkyl Ester Agar
AEB	Alkyl Ester Broth
CB	Cooke's Broth
CDA	Czapek Dox Agar
CMA	Corn Meal Agar
DWA	Distilled Water Agar
LBA	Lima Bean Agar
MA	Malt Agar
MEA	Malt Extract Agar
MEB	Malt Extract Broth
OMA	Oatmeal Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SDA	Sabourand Dextrose Agar
TWA	Tap Water Agar

Units

°C	Celsius
ED	Effective Dose
hr	hour
kb	kilobase
min	minute
ml	mililiter
mM	milimolar
mg	miligram
nmole	nanomole
ppm	part per million
p.s.i.	pound per square inch
sec	second
spores/ml	spores per mililiter of spore suspension
µg	microgram
µl	microliter
µm	micrometre
µM	micromolar
V	volt
w/v	weight / volume

CHAPTER 1

INTRODUCTION

The disease anthracnose is a prevalent post-harvest disease in South East Asia. It is caused by several fungi, the most common of which is *Colletotrichum*. Certain species of the genus are known to have a wide host range while others have specific hosts. Among members of the genus, the most common form is *Colletotrichum gloeosporioides*, which has a wide host range. It causes symptoms of black sunken lesions on surfaces of fruits such as mangoes, papayas and apples (Freeman *et al.*, 1998) which reduces the market value of the otherwise perfect fruits. An equally important species is *C. capsici* which has a narrow host range, infecting only chillies (Misra and Mahmood, 1960). Mangoes and chillies are the major hosts for *C. gloeosporioides* and *C. capsici* respectively. If not controlled, anthracnose is capable of causing huge post-harvest losses. The most common means of control throughout the world is by the use of fungicides.

The use of fungicide depends on the expectation of achieving a yield greater than its input and its application costs. Compared to other methods of increasing yield, fungicides are comparatively more effective and economical in the long term Fungicide-treated plants showed significantly greater yield in weight of marketable fruits (Torgeson, 1967).

A fungicide is a molecule that is able to destroy or halt the development of fungi without killing the host (Torgeson, 1967). In the simplest term, a fungicide is a substance destructive to fungi (Abercrombie *et. al.* 1971). There are two general types of fungicides, namely protectants and systemic. Protectants act by shielding the plant surface whereas systemic fungicides are absorbed and translocated to growing points of the plants. Eradicants are used to control disease spread on plants with pronounced diseases symptoms. Fumigants are volatile compounds that protects the whole plant. Currently, classical protective organic fungicides are commonly used, although the usage of highly active systemic compounds with curative action are increasingly being tested (Torgeson, 1967).

Of the agrochemicals used worldwide, fungicides make up only 19% compared to herbicides and insecticides, which makes up 48% and 28% of total pesticide sales in 1997 respectively. Global sales of pesticides amounted to US $31.25 billion in 1997 (Anon, 1997). Adverse trading conditions during the economic downturn of 1998-1999 have caused a decrease in pesticide sales volume of 5-10%. However, the converse was true for Azoxystrobin where sales grew over [illegible] registration in the United Kingdom, Europe and United States of America in 1997. In 1999, world wide sale of US $415 million was achieved making Azoxystrobin a leading proprietary fungicide in the world (Anon, 1999).

Azoxystrobin, Zeneca Agrochemical's new broad spectrum systemic fungicide from the strobilurin group, originated from natural fungicides, namely oudemansin A and strobilurin A that are produced by a number of edible mushrooms that grow in temperate forests on decaying wood. These natural anti-fungal products helped the

mushroom to compete with other fungi for nutrients. The compounds were tested and found to give promising results of disease control. This lead to a programme of chemical synthesis to design analogues with improved activity and optimized physical properties, such as greater photostability and low volatility. Further research, led to the selection of compounds which showed a high level of fungicidal activity with excellent crop safety in addition to a low mammalian toxicity and benign environmental profile. This compound was identified as Azoxystrobin and was given the registrated trade name of Amistar® (Anon, 1996).

Presently, registrations have already been granted in over 40 countries. In the USA, 'Amistar' has been registered under the EPA's Reduced Risk Pesticide Program. In the European Community, it is the first new active ingredient to be Annex I listed. Registrations have been granted or are pending in several Asian countries with widespread sales anticipated in the region from 1999 onwards (Dale *et al.*, 1999).

In March 2000, Azoxystrobin (Amistar®) was awarded the 'Millennium Product' status by the Design Council of United Kingdom for being a product that challenges existing conventions, is environmentally responsible, and demonstrates the application of a new technology. The product has been approved for use in 61 countries on more than 65 crops (Anon, 2000).

In situations of intensive usage it is important to have appropriate monitoring to detect shifts in sensitivity before the product becomes commercially significant. The basis of monitoring is to ensure product effectiveness with prolonged use. In order to do this, the sensitivity of the pathogenic fungi to Azoxystrobin before product