



**IDENTIFICATION OF GENES ASSOCIATED WITH
TOLERANCE IN THE CAVENDISH BANANA
SELECTION, GCTCV-218,
AGAINST
FUSARIUM OXYSPORUM F. SP. *CUBENSE*,
'SUBTROPICAL' RACE 4**

Noëlani van den Berg

**Submitted in partial fulfilment of the requirements
for the degree of**

**Philosophiae Doctor
(Plant Pathology)**

**In the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria
March 2006**

Supervisor: Dr. Altus Viljoen
Co-supervisors: Prof. Dave K. Berger
Prof. Paul R.J. Birch
Prof. Michael J. Wingfield



DECLARATION

I hereby certify that this research, unless specifically indicated to the contrary in the text, is the result of my own investigation and that no part of this thesis has been submitted to any other university.

Noëlani van den Berg

TABLE OF CONTENTS

Acknowledgements	i
Preface	iii
Abbreviations and symbols	v
CHAPTER 1 Resistance to Fusarium wilt in banana: A Review.....	1
INTRODUCTION	2
FUSARIUM WILT OF BANANA (PANAMA DISEASE)	3
THE HOST: BANANA	5
RESISTANCE IN PLANTS TO PATHOGENS	7
Terminology.....	7
The Constitutive Defence Response	8
The Induced Defence Response.....	9
Recognition	9
Hypersensitive response.....	9
Oxidative burst.....	11
Ion fluxes	12
Cell wall strengthening and vascular occlusion.....	12
Lignification.....	12
Callose deposition.....	13
Phenolic compounds	14
Indole acetic acid	15
Other Defence Responses	16
Pathogenesis-related proteins (PR proteins)	16
Peroxidases	16
Phytoalexins.....	18
Signal Transduction	18
Salicylic acid.....	19
Jasmonic acid.....	20
Ethylene	20
Interaction between the SA, JA and ET pathways.....	21
Systemic resistance	21
COMPONENTS OF PLANT DEFENCE MECHANISMS AGAINST FUSARIUM WILT	23
Structural Defence	23

Biochemical Defence	25
IMPROVEMENT OF BANANA FOR FUSARIUM WILT RESISTANCE.....	27
Genes Associated with Resistance to Fusarium Wilt	27
Conventional Breeding	29
Unconventional Improvement	30
Somaclonal variation	30
Induced mutations.....	30
Protoplast fusion	31
Genetic modification.....	31
CONCLUSION.....	32
REFERENCES	35
CHAPTER 2 Evaluation of the Cavendish banana GCTCV-218 for tolerance to <i>Fusarium Oxysporum</i> f. sp. <i>ubense</i> ‘subtropical’ race 4 (VCG 0120)	65
INTRODUCTION	67
MATERIALS AND METHODS	69
Greenhouse Trials	69
Plant material	69
Inoculation	69
Disease rating.....	70
Field Trial.....	70
Plant material	70
Disease rating.....	70
Phenolic Assays	71
Plant material	71
Extraction of phenolics	71
Total soluble phenolic acids.....	72
Non-conjugated phenolic acids (Free acids).....	72
Glycoside-bound phenolics.....	72
Ester-bound phenolics.....	72
Cell wall-bound phenolics	73
RESULTS	73
Greenhouse Trials	73
Field Trials.....	74
Phenolic Assays	74

Total soluble phenolics	74
Non-conjugated phenolic acids (Free acids).....	74
Glycoside-bound phenolics.....	74
Ester-bound phenolics.....	74
Cell wall-bound phenolics	75
DISCUSSION	75
REFERENCES	78
CHAPTER 3 Construction of a cDNA library with genes associated with tolerance to <i>Fusarium oxysporum</i> f. sp. <i>ubense</i> in Cavendish bananas.....	91
ABSTRACT	92
INTRODUCTION	93
INTRODUCTION	93
MATERIALS AND METHODS	96
Inoculation of Banana Plants with <i>Foc</i>	96
Plant material and growth conditions	96
Preparation of inoculum.....	96
Inoculation and sample collection	97
Total RNA isolation.....	97
cDNA synthesis	97
Suppression Subtractive Hybridisation (SSH).....	98
Monitoring SSH efficiency	99
Southern analysis	100
Subtracted library construction	101
Colony PCR and sequencing of selected clones	101
RESULTS	103
Inoculation of Banana Plants with <i>Foc</i>	103
RNA isolation	103
cDNA synthesis	103
Suppression Subtractive Hybridisation (SSH).....	103
Southern Blot analysis	104
Subtracted library construction	104
Colony PCR and sequencing	105
DISCUSSION	105
REFERENCES	109

CHAPTER 4	High-throughput screening of a Banana cDNA library using DNA microarray analysis.....	125
	ABSTRACT	126
	INTRODUCTION	127
	MATERIALS AND METHODS	128
	cDNA Microarray Analysis of the SSH Library.....	128
	Preparation of SSH clones and slide spotting.....	128
	Probe preparation.....	128
	Hybridization and washing	129
	cDNA Microarray screening of the SSH library.....	130
	Inverse Northern Dot Blots.....	131
	Membrane preparation.....	131
	Probe preparation, hybridisation and detection	131
	RESULTS AND DISCUSSION.....	132
	cDNA Microarray Screening of the SSH Library.....	132
	Inverse Northern Blot Analysis of Selected Transcripts.....	133
	REFERENCES	136
CHAPTER 5	Identification of genes associated with tolerance to <i>Fusarium oxysporum f.sp cubense</i> in Cavendish bananas	141
	ABSTRACT	142
	INTRODUCTION	143
	MATERIALS AND METHODS	144
	Sequencing and Analysis of cDNA Clones	144
	Real Time Reverse Transcriptase-PCR.....	145
	Template preparation	145
	Primer design	145
	RT-PCR optimisation.....	146
	Quantitative Expression Assays.....	147
	Data analysis	148
	RESULTS	149
	Sequencing and Analysis of cDNA Clones	149
	Real-Time Reverse Transcriptase PCR	151
	Primer design and RT-PCR optimisation	151
	Quantitative Expression Assays.....	152



DISCUSSION	154
REFERENCES	160
SUMMARY	200

ANNEXURE: High-throughput screening of suppression subtractive hybridization cDNA libraries using DNA microarray analysis. Noëlani van den Berg, Bridget G. Crampton, Ingo Hein, Paul R.J. Birch, and Dave K. Berger.

ACKNOWLEDGEMENTS

I would like to express my appreciation and thanks to the following people and institutes:

Dr. Altus Viljoen, Prof. Dave Berger, Dr. Paul Birch and Prof. Mike Wingfield. Thank you for the guidance, advice and patience during the preparation of this thesis.

The National Research Foundation (NRF), the Banana Growers Association of South Africa (BGASA), the Technology and Human Resources for Industry Programme (THRIP) and The Mellon Foundation for financial assistance.

My friends and fellow students in the Banana Research Programme for their support and friendship.

Du Roi Laboratories for providing tissue cultured plants.

Dr. Ingo Hein from the Scottisch Crops Research Institute. Thank you for all the endless hours of patience in the laboratory and for all your encouragement, mentoring, and advice.

Anton, you are the love of my life. Thank you for your unconditional love and for always believing in me.

My mother and Pierre for loving me and giving me the opportunities to achieve my goals.

My Saviour and friend, Jesus, Christ, for His love and strength to concur the world and all its challenges.



**People who make their own rules
when they know they're right...
People who get a special pleasure
out of doing something well (even if
only for themselves)...
People who know there's more to
this whole living thing than meets
the eye: they'll be with Jonathan
all the way.**

**Jonathan Livingstone Seagull
(Richard Bach)**

PREFACE

Musa acuminata Colla (banana) is one of the most important food crops in the world and provides a staple food and source of income in many households, especially in Africa. However, bananas worldwide are under serious threat by *Fusarium oxysporum* Schlecht. f.sp. *cubense* (E.F. Smith) Snyder & Hansen (*Foc*). There exists no control strategy against the pathogen and control involves the use of resistant cultivars and cultural practices that prevent the introduction and spread of the disease into disease-free areas. Natural disease resistance exists in wild-type bananas and a few hybrids, but these bananas are not acceptable to the Cavendish market and the search for a new tolerant or resistant Cavendish banana is underway. Conventional breeding strategies are however hindered by the fact that Cavendish bananas are sterile and do not produce seed. Therefore, non-conventional strategies such as transformation are more realistic and could be more successful. Unfortunately, very few banana genes have been isolated and characterised up to date and the banana-*Foc* interaction has not yet been studied extensively, if at all, on the molecular level. This leads to a lack of knowledge in understanding disease resistance mechanisms in banana and complicates the matter of transforming susceptible bananas with resistance genes.

This thesis firstly aims to evaluate the disease tolerance of a Cavendish banana, GCTCV-218, infected with *Foc* and secondly to isolate the disease resistance genes expressed early in the banana-*Foc* interaction. Fusarium wilt is a root pathogen and few molecular studies have been done on the plant response in roots to pathogens. This is, so far, known the first molecular study on the Cavendish banana-*Foc* interaction.

Chapter 1 provides the reader with a short review of banana, and the pathogen, *Foc*. The chapter then gives a broad overview of disease resistance in plants and further provides information on Fusarium resistance in banana and other crops with reference to the type of resistance (i.e. constitutive or actively induced chemical or structural resistance).

The Cavendish banana, GCTCV-218, is a somaclonal variety selected by researchers at the Taiwan Banana Research Institute (TBRI) in Taiwan and showed promising results in disease resistance trials against ‘tropical’ race 4 (VCG 0121). **Chapter 2** evaluates the disease tolerance of GCTCV-218, under South African conditions against *Foc* ‘subtropical’ race 4 (VCG 0120). Literature has reported that phenolic compounds may be involved in resistance against *Foc* and this chapter will also study the different phenolic compounds in GCTCV-218 compared to the susceptible Williams at different time intervals after *Foc* infection.

Chapter 3 describes the construction of a banana cDNA library containing gene fragments that are differentially expressed in GCTCV-218 in response to *Foc* compared to the susceptible Williams cultivar. A highly effective PCR-based technique, termed Suppression Subtractive Hybridisation (SSH), was applied in this chapter.

Chapter 4 reports on the development of a high-throughput screening method of the banana SSH cDNA library using DNA microarray analysis. This is a novel approach in screening SSH libraries for false positives that have escaped the subtraction process and has been published in **Bio Techniques (2004) 37: 818-824**.

Seventy-nine gene fragments were selected for sequencing after screening the library. In **Chapter 5**, the selected gene fragments were sequenced and subjected to BLASTX, BLASTN and DBEST searches. A table containing non-redundant gene fragments was compiled and some of these gene fragments were subjected to alignments with known corresponding genes from the NCBI database. The expression profile of four defence related genes was further investigated by quantitative Reverse Transcriptase-PCR.

ABBREVIATIONS AND SYMBOLS

α	Alpha
AC	Acetate
ADP	Adenosine diphosphate
AFLP	Amplified Fragment Length Polymorphism
AIR	Alcohol insoluble residue
AMV	Avian Myeloblastosis Virus
AOS	Active oxygen species
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Avr	Avirulence
β	Beta
BLAST	Basic Local Alignment Search Tool
BLASTX	BLAST algorithm to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
BLASTN	BLAST algorithm to compare a nucleotide query sequence against a nucleotide sequence database.
bp	base pairs
BTH	benzo-(1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester
Ca ²⁺	Calcium (II) ions
cADPR	Cyclic ADP ribose
CAV	Culture collection, Altus Viljoen
Ca(NO ₃) ₂ .H ₂ O	Calcium nitrate
°C	Degrees Celsius
CDPK	Calcium-dependant protein kinase
cDNA	complementary Deoxyribonucleic Acid
CIRAD-FLHOR	Centre de Coopération Internationale en Recherche Agronomique pour le Développement - Département des productions fruitières et horticoles
cm	centimetres
Ct	Cycle number at which the fluorescence signal crosses a fixed

	threshold
cv	cultivar
DIG-dUTP	Digoxygenin deoxyuridine triphosphate
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dITP	Deoxyinosinetriphosphate
DIG	Digoxygenin
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
EDTA	Ethylenediamine tetraacetic acid
EMBRAPA-CNPMPF	Empresa Brasileira de Pesquisa Agropecuária – Mandioca e Fruticultura Tropical
EF1	Elongation factor 1
ER	Enrichment ratio
EST	Expressed sequence tags
ET	Ethylene
FABI	Forestry and Agricultural Biotechnology Institute
FHIA	Fundación Hondurereña de Investigación Agrícola
f.sp.	Formae speciales
<i>Foc</i>	<i>Fusarium oxysporum</i> f. sp. <i>ubense</i>
<i>Fod</i>	<i>Fusarium oxysporum</i> f. sp. <i>dianthi</i>
<i>Fol</i>	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i>
g	gram
g/l	gram per litre
GMP	Guanosine monophosphate
GSII	1,3-β-glucan synthase
h	Hour
HCl	Hydrochloric acid

hpi	Hours post inoculation
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
HR	Hypersensitive Response
H	Hour
hrs	Hours
H ⁺	Hydrogen
IAA	Indole acetic acid
IAEA	International Agricultural Exchange Association
IITA	International Institute for Tropical Agriculture
IMP	Inosine 5' monophosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Induced resistance
ISR	Induced systemic resistance
ITS	Internal transcribed spaces
JA	Jasmonic acid
JME	Jasmonic methyl ester
K ⁺	Potassium
KCl	Potassium chloride
LAR	Local acquired resistance
LB	Luria-Bertani
LMP	Low melting point
LRR	Leucine-rich repeats
M	Molarity
MAPK	Mitogen-activated protein kinases
MeJA	Methyl jasmonate
MeOH	Methyl hydroxide
min	Minutes
mg	Milligrams
MgCl ₂	Magnesium Chloride
ml	Millilitres
ml ⁻¹	Per millilitre
ml/l	millilitre per litre

mm	Millimetres
mM	millimolar
mRNA	messenger Ribonucleic Acid
N ₂	Nitrogen
NaCO ₃	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NBS	Nucleotide-Binding Site
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	nanometre
No.	Number
NO	Nitric oxide
NOS	NO synthase
O ₂ ⁻	Super oxide
OH	Hydroxyl radical
P	Proline
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
pH	Log hydrogen ion concentration
pI	Isoelectric point
POD	Peroxidase
PPO	Polyphenol oxidase
PR	Pathogenesis-related
<i>R</i>	Resistance
R ²	Correlation coefficient
RDA	Representational difference analysis
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid

RT	Reverse transcriptase
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SD	Standard Deviation
SAR	Systemic acquired resistance
SDS	Sodium Dodecyl Sulfate
SDW	Sterile distilled water
SNP	Sodium nitroprusside
spp	species
SSC	Sodium Saline Citrate
SSH	Suppression Subtractive Hybridisation
ST	Subtracted tester
TAE	Tris-acetate tetraacetic acid
TBRI	Taiwan Banana Research Institute
TFA	Trifluoroacetic acid
T _m	Melting temperature
TMV	Tobacco mosaic virus
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol chloride
tRNA	Total ribonucleic acid
UD	Unsubtracted driver
UDP	Uridine diphosphate
µg	Micrograms
µl	Micro litre
µM	Micro molar
UT	Unsubtracted tester
UV	Ultra violet
VCG	Vegetative Compatibility Group
v/v	Volume per volume
v/v/v	Volume per volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
λ	Lambda (wavelength)
%	Percentage



Σ

Sum of