Early diagnosis and detection of eutypa dieback of grapevines

Richard Lardner

Thesis submitted for the degree of Doctor of Philosophy at The University of Adelaide

Discipline of Plant and Pest Science
Faculty of Sciences

November 2003

Table of contents

Abstract i
Declaration iii
Acknowledgments iv
Abbreviations v
Chapter 1 General Introduction 1
1.1 Introduction 1
1.2 History of Eutypa as a pathogen 2
1.3 Eutypa dieback of grapevines 3
1.3.1 Host Pathogen Interactions 3
1.3.1.1 Disease cycle 3
1.3.2 Symptoms of eutypa dieback 5
1.3.2.1 Woody tissue symptoms 5
1.3.2.2 Foliar symptoms 6
1.3.3 Relative susceptibility of V. vinifera cultivars to E. lata 7
1.4 Control of eutypa dieback 7
1.5 Significance of the pathogen to the grape growing industry 8
1.6 Production of secondary metabolites by E. lata 10
1.7 Diagnosis of eutypa dieback 12
1.8 Early diagnosis of fungal plant pathogens 13
1.8.1 Immunological techniques 14
1.8.2 Isozyme analysis 15
1.8.3 Nucleic acid-based diagnosis 15
1.8.3.1 RFLP-based techniques 16
1.8.3.2 PCR-based generation of markers 17
1.8.3.3 Generation of species-specific primers for diagnostic purposes 19
1.8.4 Molecular analysis of E. lata 20
1.9 Spectroscopy 21
1.10 Summary and objectives 22
Chapter 2 General materials and methods 23
2.1 Collection and maintenance of E. lata isolates 23
2.2 Fungal isolates 24
2.3 Growth of isolates for DNA extraction 26
2.4 DNA extraction from fungal mycelium 26
2.5 Southern hybridisation 27
2.5.1 Southern transfer of genomic DNA to nylon membranes 27
2.5.2 Preparation and digestion of plasmid DNA 27
2.5.3 DNA hybridisation techniques 28
Chapter 3 Development of SCAR markers specific to E. lata 29
3.1 Introduction 29
3.2 Methods 31
3.2.1 RAPD amplification and primer selection 31
3.2.2 Cloning and characterisation of RAPD fragments 32
3.2.3 Primer design 34
3.2.4 Validation of SCAR markers 34
3.2.5 Assessment of SCAR and ITS markers developed in France and California 34
3.2.6 Genetic variation within E. lata 35

		3.2.6.1 Analysis of banding patterns	36
3.3	Results		36
	3.3.1	RAPD primer screening	36
	3.3.2	Cloning and sequencing of PCR products	39
	3.3.3	Sequence analysis	39
	3.3.4	Primer design	40
	3.3.5	Validation of SCAR markers	42
	3.3.6	Assessment of specificity of French PCR primers towards Australian isolates of E. lata	45
	3.3.7	Assessment of specificity of Californian ITS primers towards Australian isolates of E. lata	46
	3.3.8	Genetic variation within E. lata	48
3.4	Discussion		51
Chapter 4	Development of RFLP markers specific to E. lata.		55
4.1	Introduction		55
4.2	Materials and Methods		56
	4.2.1	Construction of genomic DNA library of E. lata	56
		4.2.1.1 Preparation of insert and vector DNA	56
		4.2.1.2 Ligation and transformation reactions	57
		4.2.1.3 Analysis of recombinant colonies for specificity to E. lata	58
		4.2.1.4 Storage of recombinant colonies	60
	4.2.2	Genetic variation within E. lata	60
		4.2.2.1 Data analysis	60
4.3	Results		60
	4.3.1	Analysis of E. lata genomic DNA library	60
	4.3.2	Analysis of recombinant colonies for specificity to E. lata	61
	4.3.3	Genetic variation within E. lata	65
4.4	Discussion		75
Chapter 5	Detection of E. lata in infected grapevine wood		78
5.1	Introduction		78
5.2	Methods		79
	5.2.1	PCR-based detection of E. lata	79
		5.2.1.1 PCR amplification conditions	79
		5.2.1.2 DNA extraction protocols	80
	5.2.2	Southern hybridisation-based detection of E. lata	84
		5.2.2.1 Slot blot transfer	84
5.3	Results		85
	5.3.1	Rapid extraction protocol of Lecomte et al. (2000)	85
	5.3.2	Rapid extraction protocol of Irelan et al. (1999)	87
	5.3.3	SEAPS extraction protocol	88
	5.3.4	CTAB-based extraction protocol	88
	5.3.5	Qiagen DNeasy kit	89
	5.3.6	Modified DNeasy extraction protocol	89
	5.3.7	Bio-101 soil DNA extraction kit	89
	5.3.8	Silica-based extraction protocol	90
	5.3.9	Southern hybridisation-based detection of E. lata	91
5.4	Discussion		95
Chapter 6	Production of secondary metabolites by E. lata on artificial media		100

6.1	Introduction	100
6.2	Methods	101
	6.2.1 Fungal isolates	101
	6.2.2 Growth media	101
	6.2.3 Isolation of metabolites	102
	6.2.3.1 Liquid media	102
	6.2.3.2 XAD®-amended media	102
	6.2.3.3 Agar media	102
	6.2.4 HPLC analysis	103
	6.2.5 Toxicity of secondary metabolites to grapevine leaf discs	103
	6.2.6 Data analysis	104
6.3	Results	104
	6.3.1 Production of secondary metabolites on liquid media	104
	6.3.1.1 Variation in secondary metabolite production between isolates grown on liquid media	112
	6.3.2 Solid media	118
	6.3.3 Toxicity of secondary metabolites towards grapevine leaf discs	121
	6.3.4 Data analysis	122
6.4	Discussion	123
Chapter 7	Production of secondary metabolites by E. lata on grapevine cane extracts	129
7.1	Introduction	129
7.2	Methods	130
	7.2.1 Fungal growth media	130
	7.2.2 Isolation of metabolites	130
	7.2.2.1 Ground grapevine cane with water	130
	7.2.2.2 Liquid grapevine cane extract	131
	7.2.2.3 Grapevine cane extract agar	131
	7.2.3 Toxicity of secondary metabolites towards grapevine leaf discs	131
	7.2.4 Data analysis	131
7.3	Results	131
	7.3.1 Ground grapevine cane with water	137
	7.3.1.1 Ground grapevine cane (20 days)	137
	7.3.1.2 Ground grapevine cane (30 days)	137
	7.3.2 Liquid cane extract (30 days)	140
	7.3.3 Grapevine extract agar (30 days)	140
	7.3.4 Toxicity of secondary metabolites towards grapevine leaf discs	141
	7.3.5 Data analysis	144
7.4	Discussion	146
Chapter 8	General Discussion	151
Appendix A	Buffers and Reagents	160
Appendix B	Fungal growth media	164
Appendix C	HPLC Chromatograms following growth of isolates on artificial media	167
Appendix D	HPLC Chromatograms following growth of isolates on artificial media	177
References		181

Abstract

Eutypa dieback of grapevines, caused by *Eutypa lata*, is a major cause of reduced longevity in vineyards worldwide. The fungus grows in the woody tissue of infected vines, producing translocatable toxins that cause foliar symptoms of the disease. By the time foliar symptoms are evident the pathogen may have become well established in the vine. One aim of this study was to develop DNA markers to allow rapid reliable identification of *E. lata* and to detect the pathogen in infected wood. The second aim was to analyse secondary metabolite production by *E. lata* in order to gain information on the compounds responsible for the foliar symptoms of the disease and to identify metabolites which could be used as markers to detect the early stages of the disease prior to the expression of foliar symptoms. In addition, genetic variation of the pathogen was assessed using RFLP and RAPD analysis.

Two techniques were used to develop DNA markers; first, SCAR markers derived from RAPD fragments were developed and, second, an *E. lata* genomic DNA library was constructed, from which DNA fragments specific to *E. lata* were identified. These markers were used in either PCR- or Southern hybridisation-based assays to detect the pathogen in infected wood. PCR-based detection of the pathogen in infected wood was prone to inhibition by phenolic compounds, however, Southern hybridisation techniques were capable of detecting *E. lata* in wood. Genetic variation among 38 isolates of *E. lata* was assessed using six randomly selected clones from the genomic DNA library. A subset of 11 isolates was subjected to RAPD analysis using 10 random primers. Considerable genetic diversity, in terms of RFLP and RAPD profiles, was observed among isolates. There was no apparent correlation between grouping of isolates following neighbour joining analysis and either host species or geographic origin of

isolates. The RAPD and RFLP profiles of two isolates differed significantly from the majority of the other isolates. These isolates, which were morphologically similar to all other isolates, were subsequently found not to be *E. lata*.

Secondary metabolite production of 11 isolates was analysed by HPLC following growth on a range of media. A wider range of secondary metabolites was detected in *E. lata* than has previously been reported. Two of the secondary metabolites, eutypine and an unidentified compound with a retention time of 19.6 min, were produced by eight of nine isolates of *E. lata*. Neither of the non-*E. lata* isolates produced these compounds. It was concluded that the remaining isolate of *E. lata* may have lost the ability to produce these compounds following storage. Whilst a wider range of isolates needs to be screened before a candidate marker can be selected, these results suggest that certain compounds are present in the majority of *E. lata* isolates and, hence, may prove suitable markers for the detection of the pathogen prior to the expression of foliar symptoms.

The molecular probes developed in this study will allow the rapid and reliable identification and detection of *E. lata* in grapevine cane or wood. These probes also have the potential to be used as a research tool to gather information on the epidemiology of the disease and to assess the efficacy of potential control agents against *E. lata*. Suitable control measures could then be applied to vines which have been shown by the use of chemical markers to have latent infection. Used in combination, therefore, the DNA and biochemical markers could facilitate improved management of eutypa dieback.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for photocopying and loan when deposited in the University Library.

Signed: Date:

Acknowledgements

First I would like to thank my supervisors, Drs Eileen Scott, Belinda Stummer, Trevor Wicks and Mary Cole. In particular, I thank Eileen and Belinda, both for editing the manuscript and for constant support and enthusiasm over the last 3 ½ years.

This research was supported by the Commonwealth Cooperative Research Centres Program and conducted by the CRC for Viticulture with support from Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Federal government. Thanks also to the American Vineyard Foundation, the CRCV visiting scholars program and the GWRDC for financial support which allowed me to spend 4 months at the USDA in California as part of my studies. Sincere thanks to Dr Russell Molyneux and Noreen Mahoney for hosting my visit to California and teaching me the techniques required for the analysis of secondary metabolites. In particular, I thank Russell for assistance in editing the appropriate sections of my thesis and Noreen for assistance with HPLC analysis and extractions.

I also thank Drs Nancy Irelan and Richard DeScenzo, E. & J. Gallo Winery, for providing Gallo-proprietary PCR diagnostic primers for the detection of *E. lata* along with a research protocol for using the primers to detect *E. lata* in grape tissue, and for advice regarding the use of these primers. These primers are the subject of pending and allowed patents in various countries, including the United States and Australia.

Thank you to Dr Mette Creaser and Sharmini John for providing grapevine cane samples and fungal isolates, and to Drs Belinda Stummer, Belinda Rawnsley, Peter Long, Rosie Bradshaw and Mary Cole for providing fungal isolates. I also thank Dr Maurice Carter, whose collection of *E. lata* isolates was used extensively in this study. I also thank the staff and students in labs N105 and N107, in particular Belinda Rawnsley, Suzanne Colmagro and Tim Zanker for their friendship and support over the past 3 ½ years. In addition I thank the administrative staff, in particular Gary Taylor and Heather Fraser for assistance throughout the course of my candidature.

Finally, I would like to thank both my parents, without whose efforts this thesis could never have been written.

Abbreviations

˚C	degree Celsius
AFLP	amplified fragment length polymorphism
amp	ampicillin
ANGIS	Australian National Genomic Information Service
ATP	adenosine 5'-triphosphate
bp	base pair
CIAP	calf intestinal alkaline phosphatase
CTAB	hexadecyltrimethylammonium bromide
cv	cultivar
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dd H_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide triphosphatases
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediamine *tetra* acetic acid
g, mg, µg, ng	gram, milligram, microgram, nanogram
h	hours
HPLC	high performance liquid chromatography
IPTG	isopropyl-ß-D-thiogalactoside
ITS	internal transcribed spacer
kb	kilo base
l, ml, µl	litre, millilitre, microlitre
M, mM	molar, millimolar
mAU	milli absorbance units
min	minute
MYB	malt yeast broth
NaCl	sodium chloride
NaOH	sodium hydroxide
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
PVP-10	polyvinylpyrrolidone, molecular weight 10,000
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNAse A	ribonuclease A
RO	reverse osmosis
rpm	revolutions per minute
Rt	retention time
SCAR	sequence characterised amplified region
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TES	Tris-EDTA-SDS
UV	ultra violet
V	volts
vol	volume(s)
X-Gal	5-bromo-4-chloro-3-indolyl-galactopyranoside