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CHITINASES AND OTHER FACTORS AFFECTING INFECTION OF KIWIFRUIT BY *BOTRYTIS CINEREA*.

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Science at Massey University Palmerston North New Zealand.

Kirstin V. Wurms
October 1996
Frontispiece: An infected kiwifruit pedicel with sporulation of *Botrytis cinerea*.

This thesis is dedicated to the three most inspirational women in my life, Una Blair Wurms, Winifred McDonald and Edith May McGillan, and to my partner and best friend, Darryl Wayne Marshall Cook.
This thesis examines the role of kiwifruit host resistance and, in particular, of kiwifruit chitinases, in preventing infection by *Botrytis cinerea*. The effects of various host and pathogen factors on disease incidence were studied in artificial inoculation trials. High inoculum loads and addition of yeast extract to spore suspensions significantly increased infection, and most rots were visible within 6-8 weeks of harvest. In contrast, the average time taken for symptoms to appear increased (8-12 weeks), and total infection decreased when fruit were harvested later in the season or exposed to a curing treatment (6-24 h at 20°C) following pedicel removal. These findings indicate that kiwifruit can develop postharvest resistance to *B. cinerea*.

A range of chitinase assays, including four colorimetric, two fluorometric, a viscometric and a radiometric assay, were evaluated and adapted for use in the kiwifruit-*B. cinerea* system. Most published methods proved too insensitive to quantify the levels of chitinase in this system (160-6400 ng solubilized substrate/minute/g of stem plug tissue). However, a radioassay resolved two-fold concentration differences and distinguished ng/min/g amounts of activity in plant extracts. For detection of chitinase activity in gels after isoelectric focusing, a highly sensitive gel overlay assay was used. This assay was also adapted for use in petri dishes to facilitate rapid, qualitative screening of large numbers of fractions generated in the process of protein purification. Exochitinase activity was assessed using *p*-nitrophenyl-β-D-N,N’-diacetylchitobiose as a substrate in a colorimetric assay.

Differences between plant and fungal chitinases were evaluated by measuring exo- and endochitinase activities in healthy and diseased regions on live and autoclaved leaves. Endochitinase activity was associated with the plant, since it was found in both healthy and diseased areas on leaves, but was absent in autoclaved tissue which had been subsequently inoculated with the fungus. Conversely, equivalent amounts of exochitinase activity were present in diseased lesions on live and autoclaved leaves, but were absent from uninfected areas, showing that all exochitinase activity was of fungal origin.
Enzyme activity was measured in the stem plug (picking scar wound and the underlying sclerified tissue), because this area was found to have higher chitinase and lower protease activity than the main body of the fruit. The initial level of endochitinase activity at harvest was not affected by fruit maturity, but subsequent increases in activity during coolstorage were most marked in later harvested, more mature fruit. Levels of chitinase in the stored fruit from four different harvests correlated with resistance to *B. cinerea*. Curing treatments (1-7 days at 20°C prior to coolstorage) significantly reduced infection and induced activity of a single constitutive basic (pI≈9) 30 kDa protein with putative chitinase activity, but did not significantly increase total chitinase activity. At least one basic and two acidic isoforms were present in uncured, uninoculated healthy tissue, and inoculation with spores of *B. cinerea* appeared to induce new basic and acidic isoforms.

Application of chitosan was evaluated as a potential technique for controlling stem end rot. Solubilization of chitosan required an acidic solvent, but use of this solvent without pH adjustment predisposed host tissue to disease. No chitosan treatment significantly decreased infection below the level found in the inoculated control, hence chitosan is considered unlikely to have commercial application.

Cation exchange and gel filtration chromatography were used to purify to apparent homogeneity a protein with associated chitinase activity from cured kiwifruit stem plugs. The N-terminal sequence of this protein did not resemble any known chitinases, but exhibited 65-72% amino acid identity with thaumatin-like (TL) proteins in barley and tobacco and 66% with zeamatin in maize. This represents the first record of a TL protein in kiwifruit. Further analysis of the extract by Western blotting indicated that the previously ascribed chitinase activity was most probably due to small levels of contaminant chitinases. Properties of TL proteins include enzyme inhibition and membrane permeabilization of fungal hyphae. In addition, some thaumatins are sweet tasting. Further investigation is required to determine whether this compound influences resistance and taste in kiwifruit.

Overall, the results from this study support the theory that chitinases are involved in kiwifruit resistance against *B. cinerea*, although the low level of induction relative to
other crops and slowness of the response suggest that they are not the primary defence mechanism.
ACKNOWLEDGEMENTS

This project has been possible only through the help and support of many people. I am extremely grateful to my competent and positive supervisors Drs. Peter Long, Siva Ganesh, Keith Sharrock and David Greenwood. Dr. Long has been an excellent chief supervisor and friend, who provided both invaluable advice through the course of my studies and a great sense of humour to help me through the tough times. Dr. Siva Ganesh performed miracles by translating statistics into English. Very special thanks to Dr. Sharrock for generous use of resources, equipment and lab space at HortResearch and for excellent editorial comments. Dr. Greenwood’s enthusiasm for science and willingness to help at any time has also been inspirational. Thanks also to all the staff at HortResearch, Mt Albert, especially Martin Heiffer for skilful photography. I extend my gratitude to the staff in the Plant Science Department at Massey University, particularly Hugh Nielsen, Lorraine Davis, Lois Mather and the secretaries for providing a helpful and friendly work environment.

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  - iii. Suitability of selected conditions for measuring low and high chitinase activity

- **B** Quench curve correction

#### 5.3.2 Materials And Methods

- **A** Assay optimisation
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Chitinase activity (log$_e$ transformed) of fruit, as influenced by inoculation, solvent and/or chitosan application, and after 0, 1, 2 or 3 days "curing" exposure to A) 0°C or B) 20°C. Stem plugs were extracted from the fruit 0-42 days after harvest. Only data from healthy stem plug extracts are presented. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. ↓ = end of curing period.

Percent infection (raw data) of fruit assessed after 12 wk storage at 0 ± 0.3°C in 1994, as influenced by A) solvent type (averaged over solvent pH adjustment and chitosan application), B) solvent pH adjustment and chitosan application (averaged over solvent type). a,b,c,d represent significant differences in Duncan’s Multiple Range test (α=0.05).

Chitinase activity (raw data) in cured, uninoculated, healthy 94/C/U/H kiwifruit stem plug extracts as influenced by A) storage duration (averaged over storage temperature), B) storage temperature (averaged over storage duration), assessed in 1994/1995. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. LSD = least significant difference.

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Total proteins and corresponding chitinase activities of cured and uncured crude kiwifruit stem plug extracts as detected by silver staining on a pH 3-10 isoelectrofocusing polyacrylamide gel (A) and Calcofluor staining of the associated overlay gel with a glycol chitin substrate (B). Lanes 1-2, pI standards; lanes 3-4, replicate uncured 94/N/U/H extracts; lanes 5-6, replicate cured 94/C/U/H extracts.

Silver stained pH 3-10 IEF gel of total proteins in Rotofor fractions of diseased 92/N/I/D kiwifruit stem plug extract. Std, pI standards (1 µl); C, crude unfocused 92/N/I/D extract; 1, 2, 4 ... 18, 19, 20, fractions collected after electrofocusing in the Rotofor, where fraction 1 was closest to the Rotofor anode, through to fraction 20 which was adjacent to the cathode. Rotofor fractions were applied as 10 µl aliquots to each well.

Total protein and corresponding chitinase activities of cured 94/C/U/H kiwifruit stem plug extract after fractionation during protein purification. Proteins on a pH 3-10 IEF polyacrylamide gel were silver stained (A) and chitinase activity in the glycol chitin overlay gel was detected by Calcofluor staining (B). Lane 1, pI standards (1 µl); lane 2, crude cured 94/C/U/H extract; lanes 3-6, 4 µl samples of concentrated 94/C/U/H extract after separation on cation exchange and gel filtration columns; lane 3, unbound chitinase; lane 4, bound chitinase fraction CHa (ii); lane 5, bound chitinase fraction CHb (i); lane 6, bound chitinase fraction CHb (ii).
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Silver stained SDS-PAGE of total proteins in crude and purified cured 94/C/U/H kiwifruit stem plug extract. Lane 1, molecular weight standards; lane 2, crude extract; lane 3, ~5 μg of fraction CHb (ii) recovered from the central peak (14 min) of gel filtration chromatography on a SEC-S3000 HPLC column following adsorption to an Econo-Pac S cation exchange column.

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LIST OF ABBREVIATIONS

aa               amino acid
ANOVA            analysis of variance
AR               analytical reagent
BCAs             biological control agents
CRD              completely randomized design
DMAB             dimethylaminobenzaldehyde
DTT              dithiothreitol
ELISA            enzyme linked immunosorbent assay
GlcNAc           N-acetylglucosamine
GRPs             glycine-rich proteins
HPLC             high performance liquid chromatography
HRGPs            hydroxyproline-rich glycoproteins
IEF              isoelectric focusing
LR               laboratory reagent
LSD              Fisher’s least significant difference
LSMeans          least squares means
MES              2-(N-morpholino)ethanesulphonic acid
MWCO             molecular weight cut off
NaOAc            sodium acetate
NaTT             sodium tetrathionate
PAGE             polyacrylamide gel electrophoresis
PAL              phenylalanine ammonia lyase
6-PAP            6-pentyl-α-pyrene
PBS-Tween        phosphate buffered saline-Tween 20
PGIPs            polygalacturonase-inhibitor proteins
PR               pathogenesis-related
PVP              polyvinylpyrrolidone
PVPP             polyvinylpolypyrrolidone
RBD              randomized block design
rh               relative humidity
<table>
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<tr>
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<tr>
<td>RO</td>
<td>reverse osmosis</td>
</tr>
<tr>
<td>SA</td>
<td>sulfosalicylic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TL</td>
<td>thaumatin-like</td>
</tr>
<tr>
<td>TMV</td>
<td>tobacco mosaic virus</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSS</td>
<td>total soluble solids</td>
</tr>
<tr>
<td>UDA</td>
<td><em>Urtica dioica</em> agglutinin</td>
</tr>
<tr>
<td>VP</td>
<td>vine position</td>
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<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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