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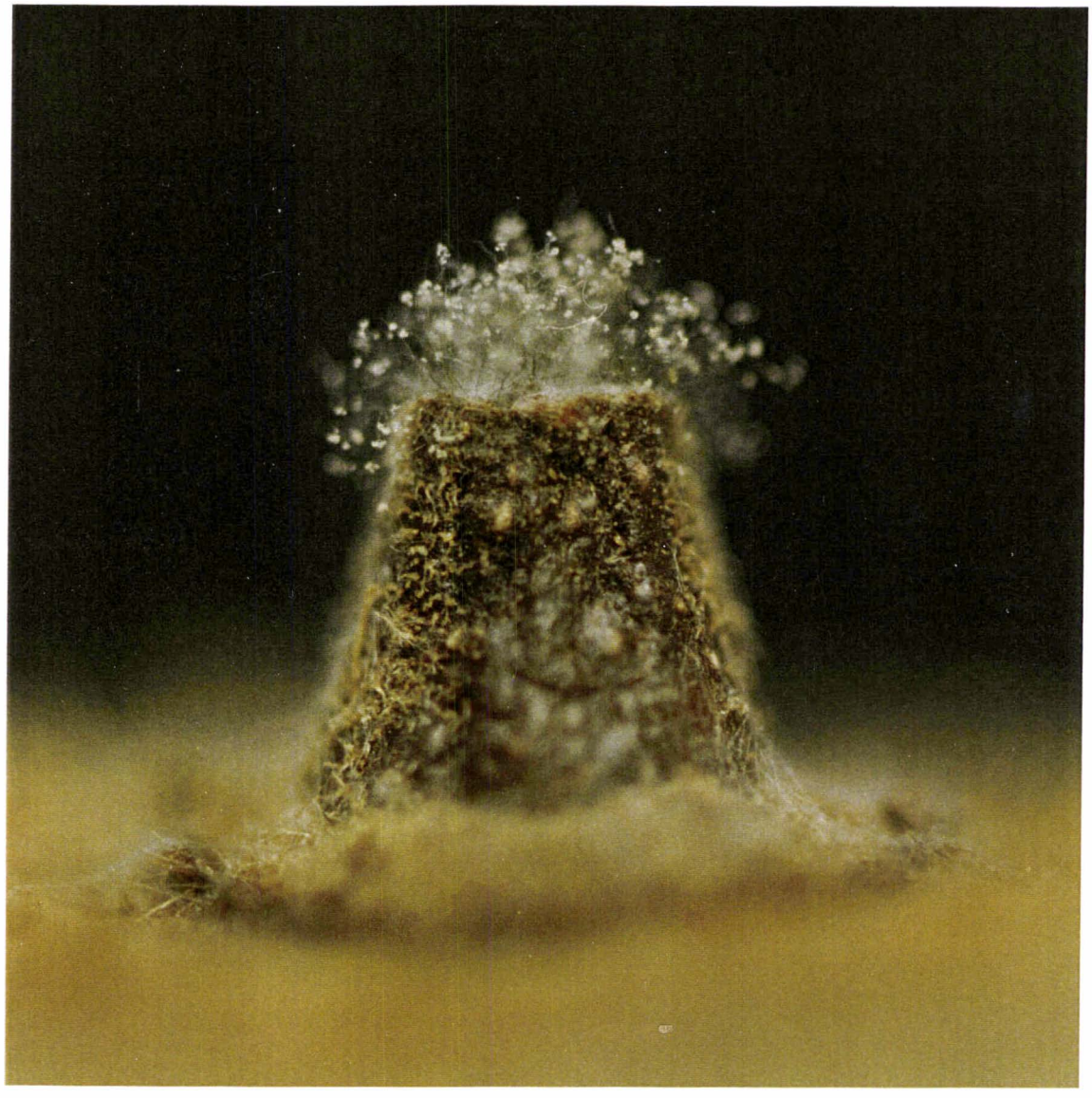
CHITINASES AND OTHER FACTORS AFFECTING INFECTION OF KIWIFRUIT BY *BOTRYTIS CINEREA*.

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.

ABSTRACT

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.

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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-pyrone
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

RO	reverse osmosis
SA	sulfosalicylic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TEMED	N,N,N′,N′-tetramethyl-ethylenediamine
TCA	trichloroacetic acid
TL	thaumatin-like
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
TSS	total soluble solids
UDA	*Urtica dioica* agglutinin
VP	vine position
WGA	wheat germ agglutinin