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

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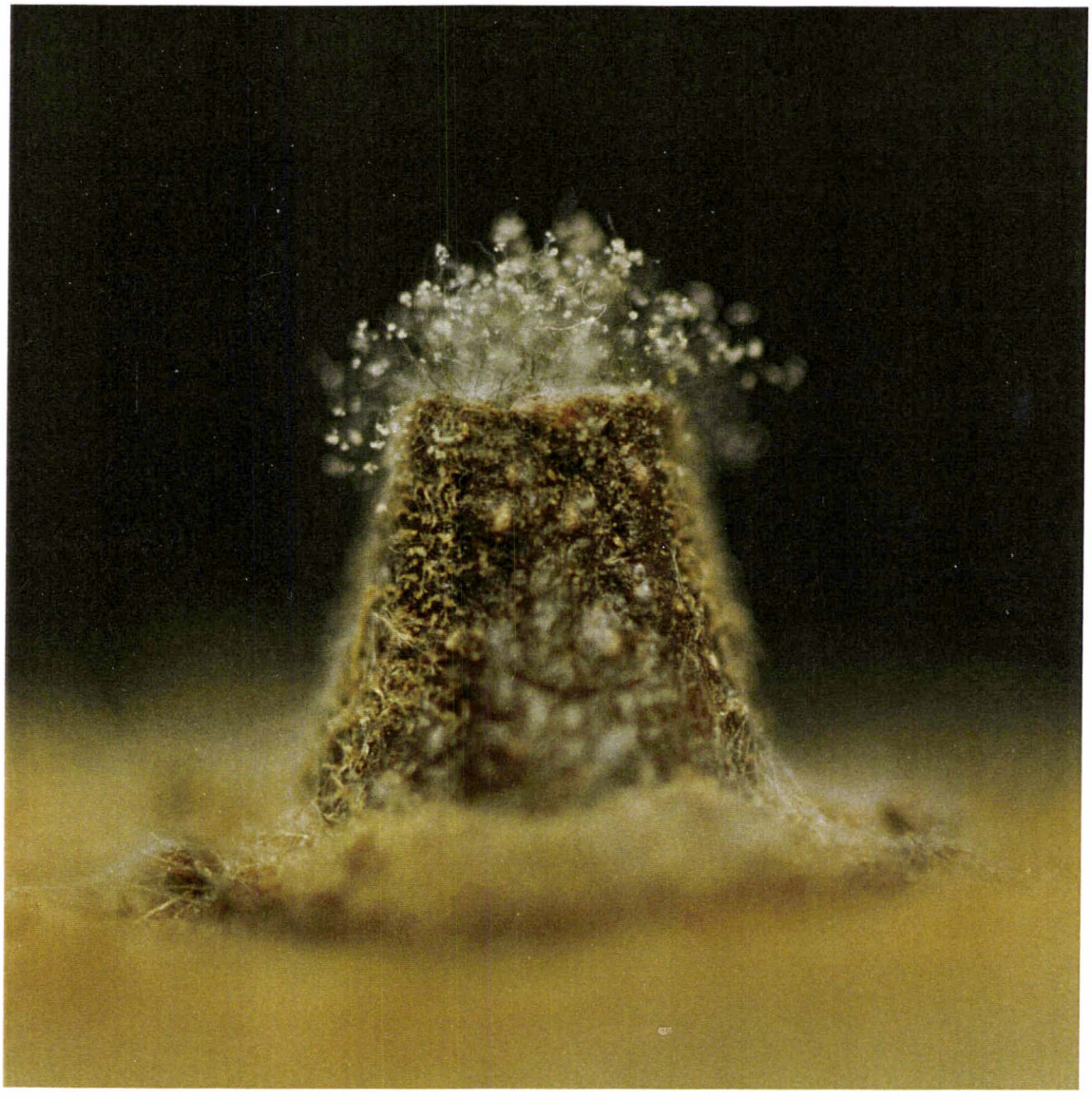

Frontispiece: An infected kiwifruit pedicel with sporulation of *Botrytis cinerea.*

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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This project has been possible only through the help and support of many people. I am extremely to 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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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

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

1 INTRODUCTION

1.1 KIWIFRUIT - BOTANICAL INFORMATION

Domesticated varieties of kiwifruit are large-fruited selections of *Actinidia deliciosa.* There are more than 50 species of *Actinidia*, which are perennial and often straggling plants that mostly originate from the forest margins of warm-temperate south-western China (Ferguson 1990). At present, the desirable storage qualities of *Actinidia deliciosa* var. deliciosa 'Hayward' [A. chev.] Liang and Ferguson, a New Zealand-produced cultivar, have made it the main variety of commerce, both in New Zealand and overseas (Sale 1990). Current research in New Zealand aims to eventually broaden this cultivar base (Beatson 1992; Seal 1992). The Hayward cultivar is a vigorous, deciduous vine with simple, alternate large (up to 20 cm diam.) obovate-shaped leaves. Shoots of the current season develop from axillary buds on canes of the previous season's growth (Ferguson 1990). Large cream-white pistillate and staminate flowers are borne on separate plants. The fruit is oval in shape, with a brown skin covered with short stiff hairs (Beever & Hopkirk 1990). Inside, the soft, bright green juicy flesh contains hundreds of small dark seeds, radially arranged around a creamy-white central core of woodier tissue. This brightly coloured flesh, along with the unique tangy taste of the fruit, are largely responsible for the success of the kiwifruit industry (Ferguson & Bollard 1990; McMath 1992; Perera & Lodge 1992).

1.2 ECONOMIC IMPORTANCE

Although kiwifruit accounts for less than one percent of world fresh fruit consumption, it nevertheless is one of New Zealand's largest horticultural export earners (Evans 1995). The domestication of kiwifruit is a recent event. Fruit was introduced to Europe, the USA and New Zealand at the beginning of this century. Commercial cultivation began in New Zealand in the 1930's, with overseas exports burgeoning in the 1960's (Ferguson

& Bollard 1990). Since then the kiwifruit industry has expanded rapidly. Major producers now include New Zealand, Italy, Japan, U.S.A., France and Chile. Chile is New Zealand's main competitor because of cheaper transport and labour costs associated with production of their southern hemisphere crop. Kiwifruit was the third largest Chilean fruit export in 1990 (Plaza 1992). New Zealand growers currently supply about a quarter of world production. In 1993, 50 million trays of New Zealand kiwifruit were sold in more than fifty countries, although sales focused in three major markets: Europe (58.6%), Japan (23.6%) and North America (6.2%) (Evans, 1995). Increasing competition from other fruits has led to declining prices although New Zealand has consistently earned price premiums over its competitors (Evans 1995). Development of new cultivars and kiwifruit products may hold the key to continued economic success (Jenks 1994).

1.3 NZ KIWIFRUIT INDUSTRY

1.3.1 Distribution

Almost 90% of New Zealand's commercial kiwifruit plantings are found in the Bay of Plenty (Ferguson & Bollard 1990). Orchards are also found in Northland, Auckland, Hawkes Bay, Wanganui, the Manawatu district, Nelson, and even in some areas of Marlborough and Canterbury.

1.3.2 Orchard Management And Harvesting Practices

There are many aspects to kiwifruit management, with training, shelter, pruning, spraying, pollination and thinning being particularly important operations. A calendar of typical orchard activities is presented in Figure 1-1. The plant's natural scrambling habit is contained by training it to a support structure, usually a T-bar fence or an overhead pergola. Orchard rows are usually orientated in a north-south direction to maximise use of sunlight. Natural or artificial shelter belts protect kiwifruit plants from wind, which damages vines and fruit and leads to poor pollination from reduced bee activity (Sale & Lyford 1990). Vines are pruned in winter and summer to allow air movement and light penetration around the vines; conditions which increase fruit yields and discourage

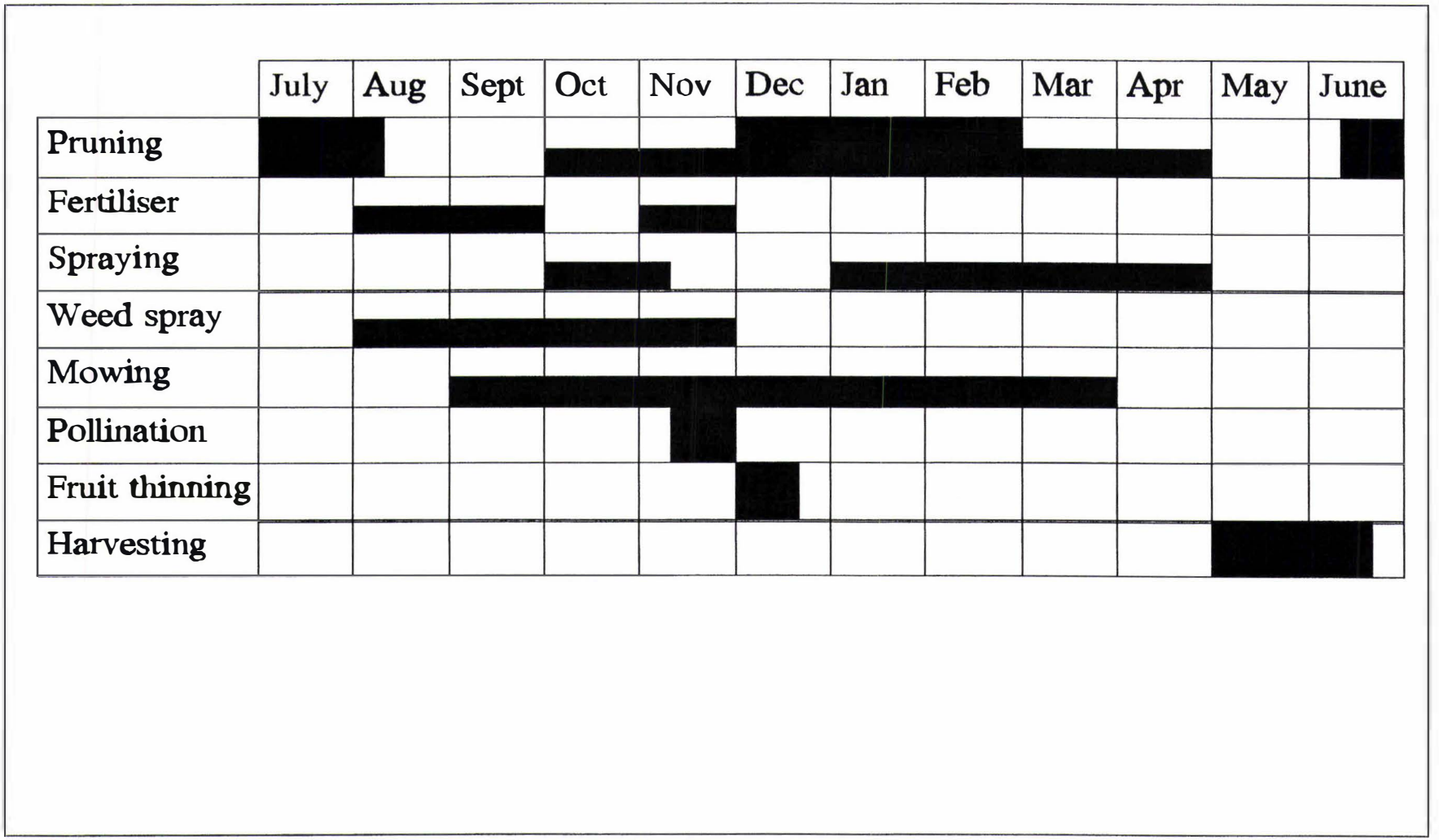


Figure 1-1. Diagrammatic representation of the annual work requirements on an established kiwifruit orchard in New Zealand. Sourced from Sale & Lyford (1990).

fungal diseases such as *Botrytis cinerea* (Sale & Lyford 1990).

Good pollination is a vital requirement for producing export-sized fruit, and this is achieved with an appropriate male:female planting ratio and by bringing honey-bees onto the orchard in November/December. Fruit thinning also reduces the number of undersized fruit.

Harvesting commences in April/May once fruit have reached an acceptable physiological maturity, determined as a total soluble solids content (TSS) of 6.2% (Harman 1981; Hopkirk 1992). The fruit is hand-harvested by snapping it off at the abscission zone at the base of the pedicel, and is placed into an apron bag which, when full, is carefully emptied into a 250 kg capacity wooden bin.

1.3.3 Postharvest Practices

Postharvest procedures include all the processes which occur after harvest until the fruit reaches its final destination, the consumer. Transportation of bins of fruit from orchard to packhouse occurs by trailer or truck. Grading takes place at the packhouse on a well-lit conveyor belt, where trained operators remove damaged and blemished fruit, followed by a machine grading for size (McDonald 1990). Fruit are then placed into packaging specified by the sole marketer, the New Zealand Kiwifruit Marketing Board (NZKMB). Common export packaging includes a single layer cardboard or wooden tray, with fruit individually contained in preformed pockets of a plastic insert (plix), cardboard tripacks containing 10-11 kg of free-flow fruit or, more recently, Europacks which contain two layers of recyclable cardboard plixes. There are eight designated fruit sizes ("fruit counts") ranging from 25 to 46 fruit per plix, with a different plix to accommodate each size. Export-sized fruit are 36 count or less. In all cases, fruit within trays are encased by a polythene liner or bag to reduce fruit dehydration (McDonald 1990).

Packaged fruit are palletised and then rapidly cooled ("precooled") prior to coolstorage at 0 ± 0.5°C and high relative humidity (at least 95%). In these optimal storage conditions fruit quality can be maintained for 4-6 months. Until recently, it was recommended that precooling and storage should take place within 24-48 hours of

harvest (Sale & Lyford 1990). Current research indicates that holding the fruit at ambient temperatures for at least two days prior to precooling (a process known as "curing") can markedly reduce storage rots (Lallu et al. 1992; Long & Bautista-Baños 1994), and many postharvest operators have adopted this technique (Evans 1992). Passive cooling, as opposed to rapid precooling, also reduces subsequent disease incidence (Lallu et al. 1992).

Fruit intended for export are checked for rots and softening following 8-10 wk storage at 0°C during the three weeks preceding shipment. On arrival at their port of destination, they often spend two or more weeks in a coolstore before distribution to a retailer or secondary coolstores (Evans 1992).

1.3.4 Industry Losses

In 1989-1991 approximately 10% of packed trays did not reach the consumer because of postharvest defects (Evans 1992). Preharvest problems account for further fruit rejects before the packaging stage. Both abiotic and biotic factors are responsible for pre and postharvest losses.

A Abiotic

Wind abrasion, sun scald, and damage from frost, hail and hormone sprays are non pathogenic causes of rejections before and at harvest, but soft fruit (whole fruit firmness below 1 kg) and those with soft patches (localised areas below 1 kg) are the major cause of postharvest abiotic losses, accounting for the greatest spoilage in the 1991 season (Table 1-1). (Fruit firmness (kg), which is measured with a penetrometer (refer Chapter 2, Section 2.1.5), provides an indication of fruit quality and maturity.) The incidence of soft patches can be reduced by minimising mechanical damage in the postharvest handling chain (Banks et al. 1992).

Table 1-1: Principal causes of kiwifruit losses in the 1991 season*.

REASON FOR LOSS	PERCENTAGE LOSS
Soft fruit and soft patches	71.2%
Botrytis cinerea rot	16.0%
Other	12.8%

*Data reproduced from Banks et al. (1992). (This was the latest data available when this research was started.)

B Biotic

Biotic losses are caused by pests and diseases. Leafroller (Lepidoptera, Tortricidae) caterpillars, whose feeding creates blemishes on the fruit, are the major pest, with up to 50% fruit damage on unsprayed vines (Sale & Steven 1984). Adherence to the spray schedule devised by the New Zealand Ministry of Agriculture and Fisheries minimises losses. Broad-spectrum organophosphate insecticides form the core of this spray programme, and in 1984 a selective biological agent, *Bacillus thuringiensis*, was introduced (Steven 1990). In some orchards, pest numbers are also monitored using pheromone traps and visual inspection, so that sprays can be timed to coincide with peak insect numbers, rather than spraying according to a calendar schedule (Steven 1994). Using this technique the number of sprays applied within a season can be reduced.

Greedy scale (*Hemiberlesia rapax*) is the second most important pest of New Zealand kiwifruit. Although this insect rarely occurs in sufficient numbers to exert a debilitating effect on vine growth, it does cause small superficial blemishes on fruit, and its presence on the fruit creates a quarantine problem (Steven 1990). Control is achieved by monitoring and applying organophosphates during the growing season and oils during the dormant season.

Other minor pests include the root knot nematode and grass grub beetles, which decrease root vigour and defoliate young vines respectively, and two spotted mite and wheat bug, which contaminate fruit at harvest. In addition, various sap sucking insects, such as passionvine hoppers and mealy bugs, excrete a sugary honeydew which stimulates the growth of sooty mould fungi, whose presence creates black stains on the fruit.

Diseases are also responsible for significant fruit losses. Among the most important are *Phytophthora* and *Armillaria* root rots, bacterial blossom blight, *Sclerotinia* field rot, *Botryosphaeria* ripe rot and *Botrytis cinerea* storage rot. Root rots can result in plant growth reduction and even death (Agrios 1988; Brook 1990b). Adequate soil drainage and removal of felled trees can help reduce incidence.

Pseudomonas viridiflava is the causal agent of blossom rot which reduces the subsequent fruit crop. This bacterium can also infect leaves from late spring onwards, producing necrotic lesions (Agrios 1988). At present there is no satisfactory method of controlling this disease (Brook 1990b).

Sclerotinia sclerotiorum is responsible for the most important preharvest disease of kiwifruit (Pennycook 1985b). The fungus produces large spreading lesions on wounded leaf tissue, and can spread into healthy leaves from tissues already infected. The fungus also rots blossoms, particularly male flowers and fruits. Fruit with deep watery lesions are shed prematurely, but smaller lesions may heal in dry weather, leaving an unsightly scar (Brook 1990b). The risk of *Sclerotinia* infection can be reduced by removal of senescent blossom tissues by an air-blast spray application, pruning male vines after pollination, and by timing the application of dicarboximides to coincide with ascospore release (Pennycook 1985b).

Unlike the diseases previously described, ripe rot caused by *Botryosphaeria dothidea* is a postharvest problem. Infection remains latent until fruits are harvested and begin to ripen. Symptoms appear on fruit held at ambient temperatures, thus may develop shortly after harvest or once the fruit is removed from coolstore. Fungicide applications to shelter belt trees and elimination of kiwifruit prunings aim to reduce the amount of inoculum present in orchards (Pennycook 1985b).

Botrytis cinerea stem-end rot is the major postharvest disease of kiwifruit (Brook 1990a; Poole & McLeod 1992). In addition to spoilage of diseased tissue, fruit infected by *B. cinerea* may contribute to further losses by producing small amounts of ethylene in coolstorage, which lead to premature softening and reduced storage life of healthy fruits

within the same tray (Pennycook 1985b; Qadir 1994). In 1989, *B. cinerea* was the most important factor associated with postharvest losses (Hopkirk & Clark 1992), and cost the industry ten million dollars (Cheah et al. 1992). Symptoms and control measures are described in detail in the following sections.

1.4 *BOTRYTIS CINEREA*

1.4.1 Taxonomy And Morphology

Taxonomically, *Botrytis cinerea* is classified in the fungal kingdom Eumycota, subdivision Deuteromycotina, class Hyphomycetes, order Moniliales and family Moniliaceae (Agrios 1988). These deuteromycete fungi are characterised by an absence of sexual reproduction and sexual structures (Agrios 1988). A discomycete sexual form of *B. cinerea*, known as *Botryotinia fuckeliana*, does exist but occurs rarely in nature (Grindle 1979). *B. cinerea* is a facultative, necrotrophic parasite which produces septate grey hyphae, hard-walled black sclerotia and clusters of one-celled ovoid hyaline/ash-coloured multi-nucleate conidia borne on the tips of long branched conidiophores (Barnett 1960).

1.4.2 Host Range

Botrytis diseases are probably the most common and widely distributed diseases of vegetables, ornamentals and field crops throughout the world (Agrios 1988). *B. cinerea* parasitizes over 200 hosts worldwide (Jarvis 1980a), and has been recorded on 108 different species in New Zealand (Pennycook 1989a). It is a serious pathogen of fruit, including grapes (Heaton & Dullahide 1993), blackberries and raspberries (Bristow 1991) and strawberries (Hutton & Persley 1993). *B. cinerea* is especially troublesome as a storage pathogen because growth, sporulation and conidial germination can occur at 0°C (Jarvis 1980b). Prior to 1973, storage rot of New Zealand kiwifruit was virtually unknown (Beever 1979). Today *B. cinerea* rot is the most important disease of kiwifruit in coolstore (Brook 1990a; Johnson & Cooke, 1993).

1.5 *BOTRYTIS CINEREA* INFECTIONS OF KIWIFRUIT

1.5.1 Etiology And Symptoms Of Infection

The life cycle of *B. cinerea* on kiwifruit is shown in Figure 1-2. *B. cinerea* becomes conspicuous in kiwifruit orchards during late blossom and petal fall (Pennycook 1985b) as the pathogen invades highly susceptible senescent floral tissue (Brook 1990a). The amount of *B. cinerea* inoculum present on wounded and/or dead vegetative tissue in the field prior to harvest is an important determinant of the subsequent number of fruit rots (Manning & Pak 1993; Elmer et al. 1994). The fungus completes its life cycle by overwintering in the field as sclerotia, which germinate in spring to produce conidia that are capable of recolonizing the host once conditions are favourable (Brook 1990a).

At harvest and during postharvest operations, picking wounds become contaminated by *B. cinerea* spores or hyphal fragments (Pennycook 1985a; Brook 1992) which subsequently penetrate the fruit via the vasculature of the stem wound (Sharrock & Hallet 1992b) to produce primary stem end rot. First symptoms appear 4-6 weeks after fruit has entered coolstorage (Brook 1992). A conspicuous external darkening commences at the stem end and advances with a sharply defined front towards the distal end of the fruit (Pennycook 1985b and Plate 1-1). Internally, affected flesh is soft and glassy with a water-soaked appearance (Plate 1-1). In advanced infections, mycelium and powdery grey conidia may be visible on the outside of the rotten fruit. External mycelium frequently spreads to adjacent fruit within the tray, causing secondary rots or "nesting" at points of contact (Pennycook 1985b; Brook 1992). Once fruit are senescent, *B. cinerea* is one of several fungi that can cause "breakdown" or ripe rots that develop anywhere on the fruit (Brook 1990a, 1992). Secondary and ripe rots are of minor importance compared to the stem end rots (Brook 1992).

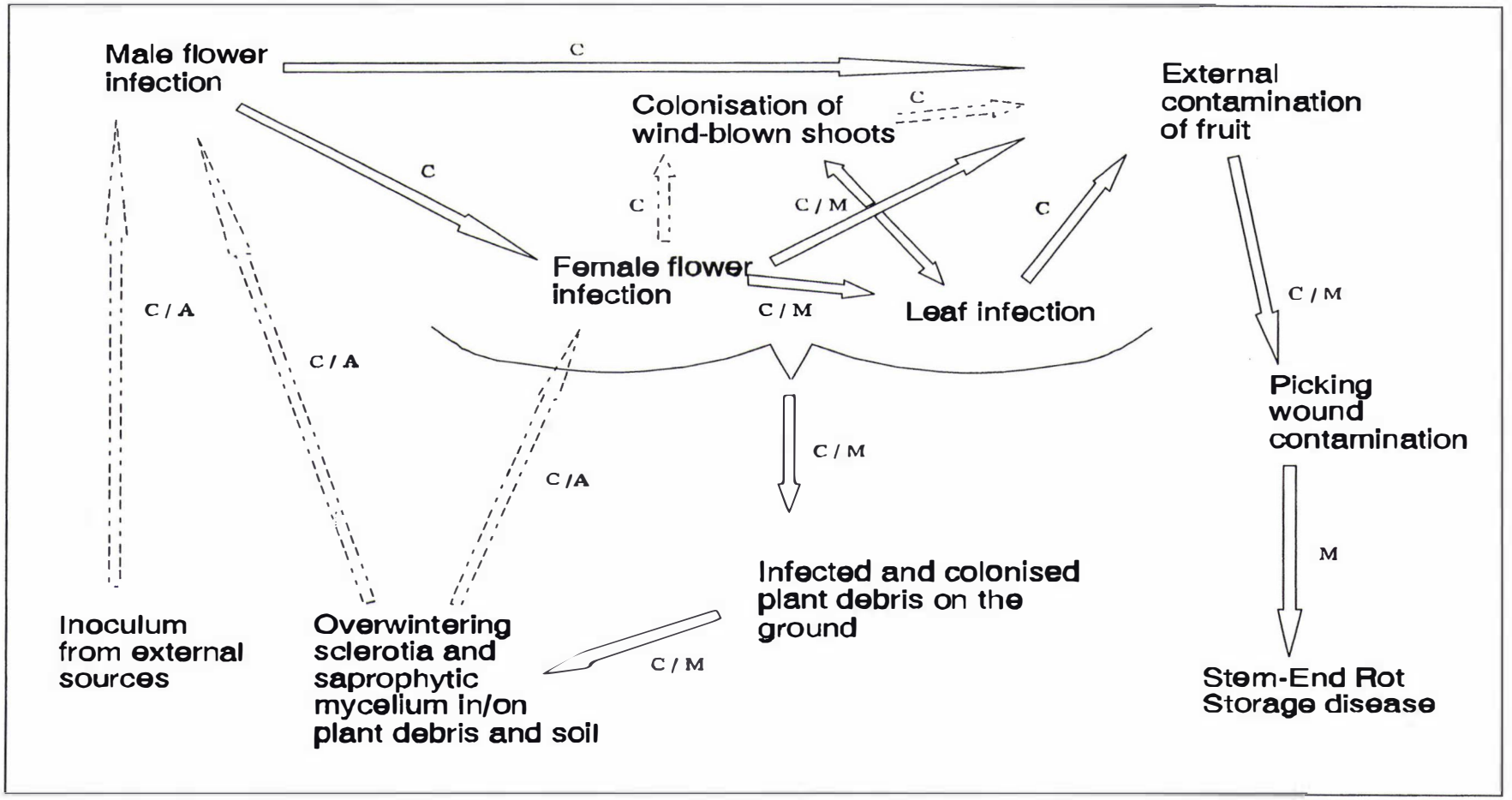


Figure 1-2. Life cycle of *B. cinerea* on kiwifruit. C = conidia, M = mycelia, A = ascospores. Arrows represent the dominant inoculum sources, based on systematic sampling of host tissues (on the ground and in the canopy) at key growth stages (flower opening, full bloom, petal fall, mid-fruit and harvest). Solid arrow = strong experimental evidence, broken arrow = no evidence to date. Information adapted from Elmer et al. (1993).

Plate 1-1. Symptoms of *Botrytis cinerea* stem end rot on kiwifruit. External symptoms (right hand fruit) comprise a darkening of infected fruit tissue that starts at the stem end and advances with a sharply defined front towards the distal end. Infection has progressed about halfway through the fruit in the picture. Internal symptoms (left hand fruit) are the glassy water soaked appearance, initially green but changing to brown, and the mushy texture of the diseased tissue.

1.6 CONTROL OF *B. CINEREA* ON KIWIFRUIT

Current industry recommendations for control of *B. cinerea* rots on kiwifruit involve application of dicarboximide fungicides during late blossom-early petal fall to prevent the build up of *B. cinerea* sporulation on senescing petals, and also at preharvest to reduce the level of inoculum present at harvest (Pennycook 1985b). Orchard sprays may reduce spore levels at harvest, but the potential infection site cannot be protected because it is not directly accessible at the time of spraying, and dicarboximides are contact fungicides with limited eradicant activity (Cheah et al. 1993; Pyke et al. 1994).

Another problem associated with fungicide application is resistance. Pommer & Lorenz (1982) reported that dicarboximide-resistant *B. cinerea* strains were readily generated in the laboratory, but exhibited low vigour and only appeared in the field after high selection pressure through prolonged intensive pesticide use. In New Zealand, most dicarboximide resistance studies have focused on *B. cinerea* populations on grape, but the same principles of limiting build up of resistant populations through restricted fungicide use apply to kiwifruit. In accord with Pommer & Lorenz (1982), Beever et al. (1989) found that high-level resistant isolates were frequently selected in the laboratory, but displayed poor fitness and were rarely observed in New Zealand vineyards. However, ultra low-level resistant isolates were relatively fit, although not as fit as sensitive strains. Moreover, Nair & Hill (1992) noted that some *B. cinerea* strains isolated from grape in Australia exhibited dual resistance to dicarboximides and benzimidazoles and possessed considerable fitness. Resistant strains have been detected in most growing areas of New Zealand and have been linked with loss of fungicide efficacy (Pak et al. 1990), but in general their build-up on kiwifruit can be restricted by applying no more than three sprays within a growing season (Pennycook 1986).

Besides preharvest spray applications, the only other industry-approved control method in current use is curing. Curing fruit at ambient temperatures for up to 48 h between picking and packing can markedly reduce infection, although it does not provide complete control and success of this treatment is sometimes variable (Beever 1992). This is possibly due to inadequate control of other factors, such as relative humidity, during

the curing process (Morris et al. 1989). The technique has been adopted by many postharvest operators in recent years (Brook 1992; Evans 1992). The mechanisms by which curing reduces infection in kiwifruit await elucidation.

At the experimental level, it has been shown that a single postharvest application of fungicide within 24 h of harvest provides more effective and reliable control than current orchard spray programmes (Pennycook 1989b). However, this control method is disallowed by importing countries (Pennycook 1985b). Moreover, use of postharvest sprays poses some serious dilemmas. Pyke et al. (1994) found that the incidence of resistant strains increased with postharvest compared to preharvest fungicide applications, and that residues were higher on dipped fruit. Disposal of used fungicide solutions and decayed dipped fruit that may act as a reservoir for fungicide-resistant inoculum could also present problems. With increased consumer opposition to fungicides, alternative control measures require urgent consideration.

Other experimental control methods include hot water dips, sulphur dioxide (SO_2) fumigation, biological control and exploitation of host resistance. Cheah et al. (1993) found that dipping fruit at 46°C for 8 min provided 64% control without heat damage. This treatment was residue-free, but there was a trade-off between adequate control of decay and fruit injury (Cheah et al. 1992).

Reports on control achieved by SO_2 fumigation are conflicting. Cheah et al. (1991) found that ten to thirty minutes exposure to 1600-3200 ppm SO_2 gave 98% control of *B. cinerea* rot without adverse side effects, but Brook (1990a) stated that SO_2 concentrations above 1000 ppm damaged fruit.

Field trials of some biocontrol agents are promising but there are no commercial products available. A postharvest application of a *Bacillus* sp. strongly inhibited *B. cinerea* (Cheah et al. 1991), and the *Trichoderma* metabolite 6-pentyl-α-pyrone (6-PAP) gave absolute control of *B. cinerea* in coolstored kiwifruit for over three months (Hill 1994). Antibiotic production is the most common selection criteria for biological control agents (BCAs), but these chemicals may present the same problems of resistance and

residue toxicity normally associated with pesticides. This situation could be remedied by use of a technique recently developed by Darryl Cook of Massey University, which selects BCAs on the basis of attachment rather than antibiosis. Despite this development, registration of BCAs is a complex, costly and time consuming process involving assessment of toxicity, pathogenicity, genetic stability and environmental impact (Woodhead et al. 1990), and it may be some years before biocontrol agents are available for commercial use.

Inadequacy of current experimental and commercially-accepted control measures necessitates further research into control of *B. cinerea* on kiwifruit. Host resistance, the ability of a plant to prevent or retard disease development, represents an exciting area for disease control because it is usually residue-free and non-toxic to the environment. Moreover, host resistance tends to be more durable than chemical control owing to its multicomponent biochemical nature, whereas fungicide efficacy is often drastically reduced by a single mutation in the pathogen. Despite the potential, techniques for enhancing host resistance have been vastly under-utilised, as past studies in the field of biocontrol tended to focus on microbes in the soil or rhizosphere rather than defence mechanisms in the plant (Sequeira 1990). In kiwifruit, the area of host resistance has been largely ignored because Hayward fruit appeared to "exhibit little or no resistance to *B. cinerea* stem end rot" (Brook 1990a). However, there are several types of resistance, all of which require consideration before accepting this statement and excluding host resistance as a means of control.

According to Heath (1995), plants may exhibit basic resistance, parasite-specific, organ-specific, age-related, or induced resistance. Basic resistance protects the plant from the thousands of potential parasites for which the particular plant species is a non host, and relies on various constitutive defences and/or defences inducible by a wide variety of organisms. Race- or parasite-specific resistance is usually effective against a single parasite, and typically involves a gene for resistance matched by a gene for avirulence in the pathogen (de Wit, 1992). It is often manifested by development of a hypersensitive response - the rapid localized death of a limited number of plant cells surrounding the inoculation site which contains the pathogen (Sequeira 1990; Cornelissen

& Melchers 1993). In contrast, induced resistance, organ-specific and age-related resistance differ from the previous two types in that they can be expressed in plants that under different circumstances would be considered as susceptible (Heath 1995). Resistance levels can vary in different plant parts, increase or decrease with organ or plant age and may be altered by environmental factors such as temperature, light, moisture and nutrient levels (Bell 1981).

Induced resistance differs from organ-specific and age-related resistance in that its expression relies on a prior treatment which sensitizes/turns on a plant's defence machinery, enabling previously susceptible tissue to resist invasion by a wide range of virulent pathogens (Sequeira 1983; Heath 1995). This enhancement of resistance in response to an extrinsic stimulus occurs through metabolic changes without a known alteration of the plant genome (Steiner & Schönbeck 1995). Resistance may be induced by an earlier localized inoculation with a non-pathogen or a virulent or attenuated pathogen (Ozeretskovskaya 1995), by treatment with a biogenic elicitor (of plant or microbial origin) or chemical agent (abiotic elicitor) that is not itself a fungicide, bactericide or viricide (Deverall 1995; Lyon et al. 1995), or by exposure to situations which stress the plant (Bowles 1990). Pathogens that cause local lesions are among the most effective inducing organisms (Deverall 1995). Induced resistance may be localized - effective within a few mm from the primary site of infection, or systemic - occurring at sites in the host spatially distant from the point of initial interaction (Sequeira 1983; de Wit 1985). Potential drawbacks of induced resistance are that it is sometimes associated with an unacceptable loss of yield or accumulation of undesirable secondary metabolites such as phytoalexins (Sequeira 1983; Smedegaard-Petersen & Tolstrup 1985). Avoidance of these negative aspects is therefore an important selection criteria when considering use of induced resistance in a commercial situation. Elicitor application represents one area of induced resistance which offers immediate prospects for use in plant protection (Deverall 1995), and evidence to date suggests that accumulation of secondary metabolites in elicitor-treated plants is unlikely to cause toxicity problems (Lyon et al. 1995). For example, elicitor treatment of intact capsicum was sufficient to protect against *B. cinerea* rots via phytoalexin production without the fruits sustaining unsightly damage (Adikaram et al. 1988).

Although Hayward kiwifruit do not appear to express basic or parasite-specific resistance to *B. cinerea*, preliminary data suggests organ-specific, age-related and inducible resistance may occur. *B. cinerea* conidia were more effective in colonizing pericarp than stem plug tissue, and stem plug extracts contained inhibitors of *B. cinerea* growth (Poole & McLeod 1992). Kiwifruit harvested later in the season were more resistant to *B. cinerea* (Brook 1990a). Poole & McLeod (1991) found that application of salicylic acid, an elicitor of plant defence mechanisms, reduced infection and produced changes in stem end tissues similar to ambient curing. The effect of kiwifruit curing may be due to reduced water permeability of the stem scar or an initiation of host defences or both. Work in other crops suggests the importance of the latter (Brown & Barmore 1983). Further research is needed to confirm the existence and explore the potential of these types of resistance in kiwifruit.

1.7 COMPONENTS OF HOST RESISTANCE AND THEIR IMPORTANCE IN KIWIFRUIT/*B. CINEREA* INTERACTIONS

Success of infection depends on the interaction between the environment, the pathogen and the host (Agrios 1988). All plants have the capacity to respond to invasion by a potential pathogen, and the rapidity and extent of this response frequently establishes the difference between a resistant and a susceptible reaction (Sequeira 1990). Host resistance is a non-static phenomenon depending on many biochemical components, the main features of which are summarized in Figure 1-3. These various types of defence mechanisms appear common to the five different forms of resistance (Heath 1995).

1.7.1 Physical Barriers

Compounds such as cutin, callose, lignin, suberin, hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) are hypothesized to play a role in the formation of structural barriers which physically impede pathogens. The cuticle, a membrane comprising cutin and waxes, forms the outermost layer of plant leaves and consequently the first line of defence. It acts as a chemical and physical barrier to fungal

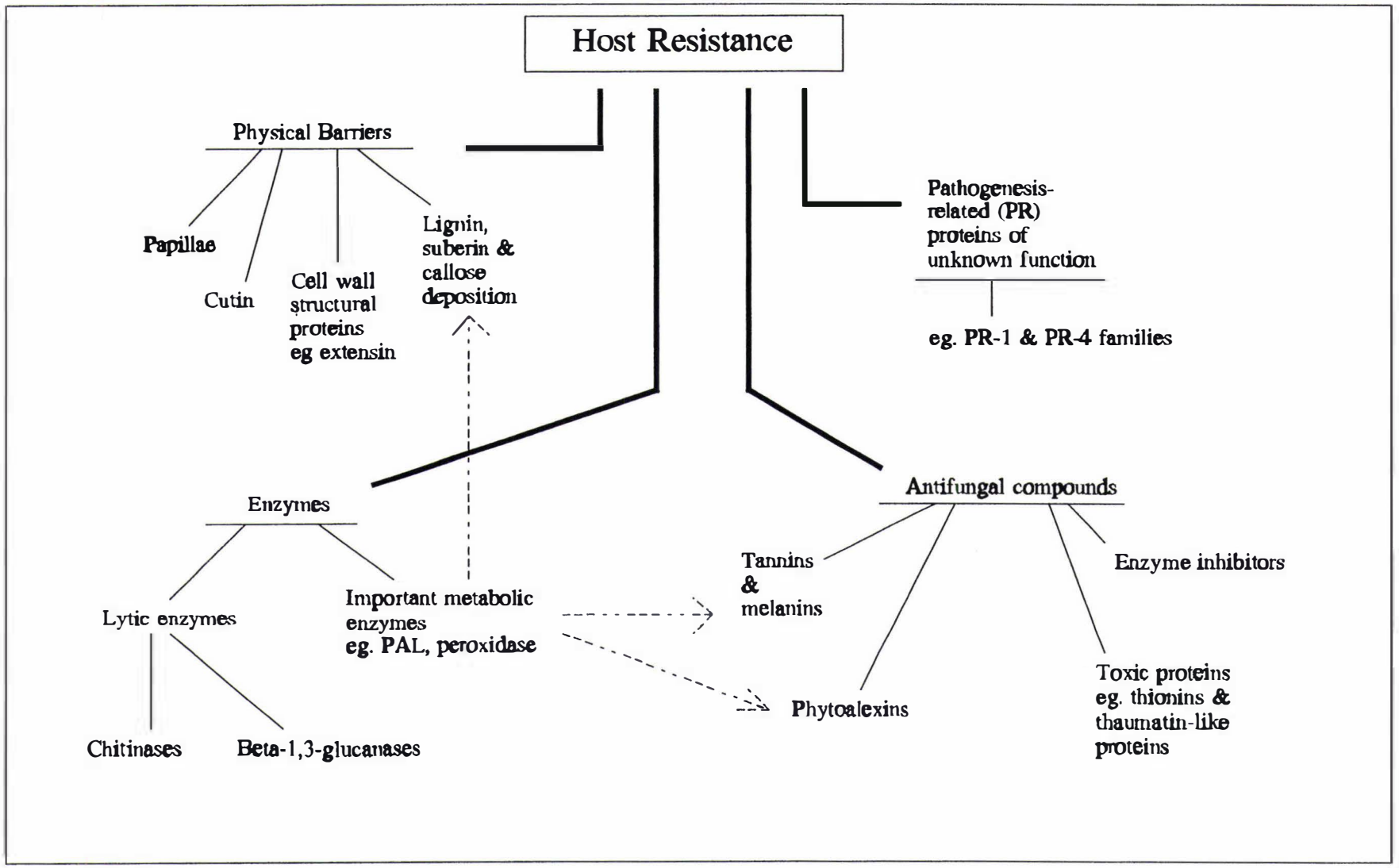


Figure 1-3. Components of host resistance.

germination and penetration. Components of wax and cutin are fungistatic to some pathogens (Wang & Pinckard 1973), but are utilised for growth by others (Kolattukudy 1980). Evidence for the role of cutin as a physical barrier comes from inhibition of *Fusarium solani* f. sp. *pisi* cutinase, which prevents infection of peas without any effect on pathogen germination or spore growth (Maiti & Kolattukudy 1979).

Deposition of cellulose and callose carbohydrates on the inside of the cell wall, just outside the plasmalemma, occurs in response to wounding and infection by fungi, viruses or nematodes (Aist 1976; Bostock & Stermer 1989). Cell wall appositions may continue until they become dome shaped or elongate and are called papillae (Bell 1981). Callose, a β-1,3-glucan, is the most commonly identified constituent in papillae but other components whose copresence may be required for efficacy (Bell 1981) include lignin, cellulose, protein, peroxidase, pectin, suberin, gums and silicon (Aist 1976). There has been some considerable debate over the role of papillae. Some consider that papillae impede the progress of penetration pegs, whilst an alternate theory suggests that papillae formation is a general wound response associated with, but not causally related to, penetration failure (Aist 1976). There is experimental data to support both hypotheses. For example, Sherwood & Vance (1980) found that cycloheximide-induced blockage of papillae formation enabled three previously avirulent pathogens to penetrate 12 species of Gramineae. Conversely, Smart et al. (1986) removed callose from barley papillae by laminarinase digestion without affecting resistance to *Erisiphe graminis*.

Lignin is an insoluble phenolic polymer formed by condensation of hydroxycinnamyl alcohols which are derived from phenylalanine via the phenylpropanoid pathway (Bell 1981). Lignin forms covalent bonds with cellulose, pectates and structural proteins in plant cell walls or related matrices and ester linkages with fatty acid polyesters to yield suberin (Brown 1989). The occurrence, speed and extent of lignification have been correlated with resistance in many host-parasite combinations. For example, rapid lignification of wounded wheat leaves, induced by a prior inoculation with a non-pathogen, appears a significant factor in resistance to *Fusarium graminearum* and *Penicillium oxalicum* (Ride & Barber 1987). Suberized cells are rarely penetrated by pathogens (Bell 1981).

Highly cross-linked polymers of hydroxyproline-rich glycoproteins (HRGPs) provide the framework for deposition of cellulose and pectins and consequently are important structural components of plant cells (Sequeira 1990). Insoluble HRGPs are known as extensin. Soluble HRGPs, presumed precursors of extensin, also present in cell walls are released in response to wounding and fungal infection (Sequeira 1990) and may protect underlying cells from pathogen attack and desiccation (Cooper et al. 1987). Kimmins & Brown (1973) considered that glycoprotein formation was a response to wounding rather than to a pathogen. However, Sequeira (1990) stated that fungal infection alone was sufficient to initiate production. HRGP insolubilization within the cell wall is thought to occur via a progressive increase in inter- and intra-molecular cross links catalysed by peroxidases (Bowles 1990).

The relative importance of physical barriers in preventing infection by *B. cinerea* varies in different hosts. There was no differential response in callose deposition between resistant and susceptible cultivars of French bean following *B. cinerea* infection (García-Arenal & Sagasta 1977). Healed wounds in apples, which were high in lignins and callose as well as phenolic substances, were significantly more resistant to *B. cinerea* spores than freshly inflicted wounds (Lakshminarayana et al. 1987). Induced resistance to *B. cinerea* in carrot root slices and tissue cultured callus was associated with increased suberin and lignin synthesis respectively (Heale & Sharman 1977).

In kiwifruit, physical barriers do not appear to play an essential role in *B. cinerea* resistance. Structural changes, such as lignification, were not apparent in fresh or aged wounds at the picking scar (Poole & McLeod 1992) or in response to curing (Sharrock & Hallet 1992a). Bautista-Baños (1995) detected some production of lignin and/or suberin, but not cutin or cellulose, in kiwifruit stem scar tissue in response to infection by *B. cinerea*. She concluded that physical barriers were not largely responsible for differences in infection between treatments. Although phenylalanine was rapidly and efficiently converted into lignin-like polymers (McLeod & Poole 1994), fruit retained their ability to transfer fluids from the stem zone for some days after harvest so mechanical barriers within vascular bundles, the main paths for infection, were unlikely to be an important factor in early resistance (Poole et al. 1993). Conversely, Hallet &

Sharrock (1993) observed a significant decrease in the ability of the stem wound to take up a coloured dye (2% v/v acid fuchsin in water) via the phloem cells three days after stem removal. However, resistance developed on a more rapid time scale (within 4 h of stem removal) than the decline in dye uptake capacity. Moreover, *B. cinerea* spores are too large to pass through pores in the phloem and physical impedance of the xylem vessels was not determined.

1.7.2 Antifungal Compounds

Plant products with antibiotic action include tannins, melanins, thionins, lectins, thaumatin-like proteins and phytoalexins. Tannins inactivate enzymes produced by some microorganisms (Hart & Hillis 1972). Melanins accumulate in necrotic cells and are the brown/black pigmented condensation products of dihydroxyphenolic quinones and tannins (Bell 1981). It has been suggested that melanins may function in a similar manner to lignin, or that they may inhibit wall degrading enzymes of pathogens (Brown 1989). However, evaluation of the importance of melanin in resistance is difficult because the transient, unstable nature of quinone and free radical intermediates, together with the resistance of melanin to enzyme or acid hydolysis, preclude quantitative measurement (Bell 1981). Unequivocal answers regarding defence roles of melanins await the development of melanin-deficient mutants.

Toxic proteins include thionins, lectins, thaumatin-like proteins and various enzyme inhibitors. Thionins are a small family of basic proteins found in leaves and seed endosperms. They exhibit toxic properties to a range of organisms and are induced in response to pathogen challenge and stress (Bowles 1990). Lectins are enzymic and non-enzymic proteins exhibiting multiple non-catalytic binding sites for carbohydrates (Bowles 1990). Virtually nothing is known of the role of lectins, although numerous speculative roles have been ascribed including storage, carbohydrate transport, and recognition in various cell-cell interactions (Callow 1977). Certain lectins, e.g. ricin and phytohemagglutin, are known to be phytotoxic (Bowles 1990), although presumed antifungal properties of another lectin, wheat germ agglutinin (WGA), were shown to be due to low level contamination of the lectin with endochitinase (Schlumbaum et al. 1986). Conversely, Broekaert et al. (1989) showed that a chitinase-free chitin-binding

lectin from stinging nettle rhizomes (*Urtica dioica* agglutinin, UDA) was a potent inhibitor of various fungi, including *B. cinerea*. However, Lerner & Raikhel (1992) determined that UDA mRNA encodes a chitinase-like catalytic domain in addition to two tandem N-terminal chitin binding domains. The chitinase domain appears to be cleaved during protein processing, and chitinase activity was observed when this catalytic domain alone was expressed in *Escherichia coli*. To add further fuel to the lectin verses chitinase debate, there appear to be some chitinases which may exhibit lectin-like functions; for example, Class I chitinases which contain a chitin binding domain with high homology to lectins like WGA (Graham & Sticklen 1994).

Thaumatin-like (TL) proteins are so named on the basis of sequence homology to the intensely sweet-tasting protein thaumatin derived from a West African rainforest shrub. TL proteins inhibited growth of various important plant pathogens, including *Fusarium oxysporum* (Huynh et al. 1992), *Phytophthora infestans* (Woloshuk et al. 1991), and *Alternaria solani* (Borgmeyer et al. 1992), and the human pathogen, *Candida albicans* (Roberts & Selitrennikoff 1990). Modes of antifungal action have not been clearly elucidated, but appear to include inhibition of pathogen enzymes (Richardson et al. 1987) and non-enzymic membrane permeabilization (Roberts & Selitrennikoff 1990; Woloshuk et al. 1991).

Phytoalexins are lipophilic, low molecular weight antimicrobial compounds comprising many diverse chemical classes, including isoflavonoids, flavonoids, diterpenes, sesquiterpenes, polyacetylenes, coumarins, isocoumarins, terpenoids, furanoacetylenes and polyenes (Bell 1981; Sequeira 1983). They are either not detectable or are present at extremely low concentrations in healthy plants, but accumulate locally in large amounts in response to microbial challenge or stress (Albersheim & Valent 1978; de Wit 1985). At higher concentrations, phytoalexins are also toxic to animal and plant cells, and peak concentrations in tissues almost always coincide with the onset of visible necrosis frequently associated with the hypersensitive response (Bell 1981). Phytoalexin induction represents one of the most rapid gene activation systems known in plants (Sequeira 1990) and therefore may be very important in the early stages of localized defence.

One convincing line of evidence for the importance of phytoalexins in resistance comes from a strong correlation between the ability of certain fungi to metabolize phytoalexins to less toxic products and high virulence (Sequeira 1990). This relationship only appears to hold true for plants that produce one major phytoalexin. For instance, most pea cultivars produce one predominant phytoalexin, pisatin, and VanEtten et al. (1980) found that virulent isolates of *Nectria haematococca* were able to demethylate this toxin whereas avirulent isolates could not. However, solanaceous plants produce multiple phytoalexins and attempts to correlate virulence with detoxification of individual phytoalexins have been unsuccessful (Ward & Stoessl 1977).

Antifungal compounds, in particular phytoalexins, appear to play an important role in many plant-pathogen interactions (Darvill & Albersheim 1984; Kokubun et al. 1995) and phytoalexins can suppress *B. cinerea* growth on capsicum (Adikaram et al. 1988), broad bean (Rossal et al. 1980), lettuce (Bennett et al. 1994) and grapes (Pezet & Pont 1990; Jeandet et al. 1995). Expression of a foreign phytoalexin in transgenic tobacco increased resistance to *B. cinerea* (Hain et al. 1993).

Proteinaceous inhibitors of the enzymes from invading pathogens or pests represent another aspect of host resistance. Alterations of plant cell wall components, tissue maceration and cell death are common features of infection by pathogens. These changes are brought about by cell wall-degrading enzymes which are produced by most bacteria and fungi (Misaghi 1982). Host inhibition of such enzymes may therefore play an important role in the prevention of infection. Abu-Goukh & Labavitch (1983) provided some of the first evidence of the role of polygalacturonase-inhibitor proteins (PGIPs) in defence of pear fruit. Cervone et al. (1990) observed that a PGIP isolated from bean inhibited the polygalacturonases of four different fungi. Further evidence for the defensive role of enzyme inhibitors comes from correlative increases in the levels of certain plant-produced proteinase inhibitors in response to mechanical wound-damage, microbial infection and insect attack (Richardson et al. 1987). Wound-inducible serine proteinases, for instance, have been identified in several plant species, including the Solanaceae and monocots (Ryan 1981). Trials where resistance has been enhanced through constitutive expression of proteinase inhibitors in transgenic plants provide more

direct evidence (Hilder et al. 1987).

Information on the roles of antifungal compounds in kiwifruit defence against *B. cinerea* is sparse. There are no comprehensive reports on the existence and effects of thionins, lectins, or TL proteins in kiwifruit. Enzyme inhibiting proteins do not appear to play a vital role in kiwifruit defence against *B. cinerea.* Various studies and reviews suggest that cutinases, pectinases, cellulases and proteases play a role in pathogenesis by *Botrytis* species (Verhoeff & Warren 1972; Verhoeff 1980; Movahedi & Heale 1990; Salinas 1990; Kamoen 1992). Multiple forms of cellulolytic and pectinolytic activities are produced by *B. cinerea* on kiwifruit, but pericarp and stem plug extracts were unable to inhibit these enzymes (Sharrock & Hallet 1992a). Moreover, the inability of externally-sourced enzyme inhibitors to reduce infection suggested that *B. cinerea* glucose/xylose oxidases and polygalacturonases were not vital for pathogenicity on kiwifruit, and the contribution of aspartate protease, an important pathogenicity factor on other plant hosts (Movahedi & Heale 1990), was also questionable (Sharrock & Hallet 1993). In the same study, browning was observed in the stem plug vasculature of healthy fruit, which occurred at a higher frequency in inoculated than uninoculated fruit, although only 30-40 fruit were examined. Melanins and phytoalexin accumulation may be associated with this browning. Phytoalexins have not been measured directly, but the high level of phenylalanine ammonia lyase (PAL) activity and the rapid rate of phenylalanine metabolism which results from inoculation and elicitor application are suggestive of increased production of phenylpropanoid phytoalexins (Poole et al. 1993). Preliminary evidence suggests that tannins do not play a central role in resistance. Poole & McLeod (1992) reported that the stem zone was markedly higher in tannin content than the main body of the fruit, but tannic acid did not inhibit *B. cinerea* growth in vitro. Further study on the roles of antifungal compounds in *B. cinerea*/kiwifruit interactions is warranted.

1.7.3 Pathogenesis Related Proteins Of Unknown Function

Pathogenesis-related (PR) proteins were first described in tobacco, following infection of the plants with tobacco mosaic virus (TMV), and are characterized by their induction by pathogen attack and stress-related situations, their relative resistance to heat and protease digestion and their solubility at low pH (Bowles 1990; Borgmeyer et al. 1992).

Basic and acidic isoforms of PR proteins are secreted into the intercellular and intracellular spaces of leaves (Bol et al. 1990). Individual PR genes have also been shown to be developmentally regulated, and currently there are five recognized groups of PR-proteins, designated PR-1 through PR-5 (Cutt & Klessig 1992). PR-2, 3 and 5 have been identified as β-1,3-glucanases, chitinases and TL proteins respectively, but biological functions or enzyme activities have not been assigned to the PR-1s and PR-4s. Their induction by pathogens suggests a role in defence, although constitutive expression of PR-1a protein in transgenic tobacco did not increase resistance to TMV, indicating that expression of this gene was insufficient to provide protection against the viral pathogen (Linthorst et al. 1989). Chitinase and β-1,3-glucanase activities have been measured in kiwifruit (McLeod & Poole 1994) and these are discussed further in Section 1.7.4, but there are no reports to confirm or deny the existence of the other PR groups in this crop.

1.7.4 Enzymes

Proteins with defence-related enzymic activities include key enzymes in phenylpropanoid metabolism and other important biochemical pathways, as well as lytic enzymes. The various branch pathways of phenylpropanoid metabolism which lead to the production of numerous defence related products, including lignin, phytoalexins and melanins, derive their building units from three core reactions catalysed by phenylalanine ammonia lyase (PAL), coumaric acid 4-hydroxylase and 4-coumarate CoA ligase (Bowles 1990). The PAL-mediated reaction is particularly important because it represents the entry point into all these pathways. PAL has been found in all green plants and levels of activity generally increase in response to wounding, infection and light exposure in etiolated tissue (Camm & Towers 1973). The authors noted that although concomitant increases in the levels of PAL and of phenolic compounds have been demonstrated in many plants, correlative increases are not always the case.

Peroxidases are involved in a number of important biochemical pathways, including the later stages of phenylpropanoid metabolism. Whilst these enzymes are not considered to have a direct antimicrobial function, and their role in plant defence is the subject of debate, they may contribute to resistance through their insolubilization of

hydroxyproline-rich glycoproteins (HRGPs) by formation of intermolecular cross-links, and via their role in lignin, suberin and melanin synthesis (Bowles 1990). Peroxidase activities generally increase in wounded and in infected tissues (Bowles 1990; Svalheim & Robertson 1990). Interpretation of this observation is complicated by the fact that peroxidases are also produced by bacteria, fungi and nematodes (Bell 1981).

The two most important classes of lytic enzymes thought to be involved in host resistance are the β-1,3-glucanases and chitinases. Pathogenic fungi also produce chitinase, which complicates evaluation of its role in plant defence (Boller, 1985). However, the widespread distribution of these β-1,3-glucanases and chitinases in higher plants, their ability to digest major chitin and β-1,3-glucan constituents in fungal cell walls, and their induction by ethylene, pathogens, elicitors and other stresses all suggest their involvement in host resistance (Sahai & Manocha 1993). The ability of chitinases to inhibit *B. cinerea* growth appears to depend on the particular pathogen-host interaction. For example, chitinases from wheat, tobacco and thorn apple were unable to inhibit the growth of *B. cinerea* in vitro (Broekaert et al. 1988), whereas chitinases from two fungal biocontrol agents *Trichoderma harzianum* (Lorito et al. 1993b) and *Gliocladium virens* (Di Pietro et al. 1993) were effective inhibitors of this pathogen.

The findings of several researchers suggest that enzymes may play a significant role in kiwifruit defence against *B. cinerea.* Increases in PAL, peroxidases and plant-produced chitinase activities were concomitant with infection by *B. cinerea*, although differences in peroxidase concentrations between inoculated and control fruit were small. There was no consistent relationship between endogenous β-1,3-glucanases and infection (McLeod & Poole 1994). Stem plug tissues exhibited greater resistance than pericarp tissue (Poole & McLeod 1992), and higher chitinase activity was found in the stem plug compared to the rest of the fruit (McLeod & Poole 1994). In addition, a high molecular weight heat-labile component, possibly a protein, which inhibited *B. cinerea* germination and germ tube extension, was present in infected stem plugs and in kiwifruit subjected to prolonged storage at 0°C (Sharrock & Hallet 1992a).

Host resistance in kiwifruit, as in other plants, appears to comprise multiple components.

In particular, antifungal compounds and host defence enzymes are two areas of kiwifruit resistance that merit further investigation.

1.8 CHITINASE

1.8.1 Classification

Chitinases are enzymes which hydrolyse β-1,4 linkages between two consecutive N-acetylglucosamine residues of chitin (Flach et al. 1992). These authors noted that chitin is one of the most ubiquitous polymers in nature and consequently these hydrolytic enzymes are found in an array of organisms, including plants, bacteria, fungi, insects, marine invertebrates and some fish which feed on invertebrates. They have been isolated from most plant organs, including seeds (Wadsworth & Zikakis 1984; Majeau et al. 1990; Yamagami & Funatsu 1993), bulbs (Van Damme et al. 1993), roots (Spanu et al. 1989; Dumas-Gaudot et al. 1992; Ludwig-Müller et al. 1994), cotyledons (Rasmussen et al. 1992), leaves (Boller et al. 1988; Fink et al. 1988; Kombrink et al. 1988; Joosten & de Wit 1989; Conrads-Strauch et al. 1990), stems (Pegg & Young 1982; Kozlowska & Krzywanski 1989; Kim & Hwang 1994), flowers (Lotan et al. 1989; Leung 1992; O'Garro & Charlemange 1994) and fruit (Sharrock & Parkes 1990; El Ghaouth et al. 1991). Despite their varied location, plant chitinases are typically monomeric, 25 to 36 kDa proteins with a predominantly endo-lytic mode of action (Boller 1988; Punja & Zhang 1993).

A Nomenclature

Enzyme nomenclature, based on the mode of cleavage of the β-1,4 bond, is marred with confusion. The international system of enzyme nomenclature proposed by Bielka et al. (1984), which assigns a unique classification number to each enzyme, describes endochitinase activity (classification number: EC 3.2.1.14) as the random cleavage of chitin polymers liberating oligomers such as chitobiose, chitotriose, chitotetraose and higher homologues. Whilst there is general agreement in the literature regarding this definition, there is considerable variation in exochitinase nomenclature. Boller (1988) used the Bielka et al. (1984) description system which lists one chitinolytic enzyme with

an exo mode of action: N-acetyl-β-glucosaminidase EC 3.2.1.30, that hydrolyses "terminal, non-reducing N-acetyl-β-D-glucosamine residues in chitobiose and higher analogues". Other researchers consider that exochitinases remove diacetylchitobiose rather than the monosaccharide from the non-reducing end of chitin in a stepwise fashion (Robbins et al. 1988; Roberts & Selitrennikoff 1988; Manocha & Balasbramanian 1994; Hodge et al. 1995). Harman et al. (1993) found that the Bielka et al. (1984) terminology was insufficient for the three distinct classes of chitinolytic enzymes produced by *Trichoderma harzianum*, and proposed two terms to describe exochitinases: N-acetyl-β-glucosaminidase, as described above, and chitin 1,4-β-chitobiosidase which releases chitobiose from chitin. It therefore appears that exochitinases can have different modes of action. The classification system proposed by Harman et al. (1993) has been adopted by other researchers (Flach et al. 1992), except that only chitobiosidase is specifically named as an exochitinase. Kunz et al. (1992) used exochitinase as a general term to describe both types of enzyme that remove monomers and dimers from the non-reducing ends of chitin. This simple terminology, which encompasses all of the above definitions, was adopted in this study because it distinguishes exo-modes of action from "random" endo-acting events without the need to characterize product sizes of exochitinase digestion.

B Genetic Classes

Plant chitinases can also be divided into classes on the basis of their structure and amino acid sequence homologies (Shinshi et al. 1990). Several of these classes may occur within the same plant species. For example, Class I, II and III chitinases are present in tobacco (Legrand et al. 1987).

i Class I

Class I chitinases contain an amino terminal cysteine-rich domain that is approximately 40 amino acids in length and has putative chitin-binding properties, a highly conserved chitinolytic catalytic domain, and a variable proline-rich hinge region between the two domains (Raikhel & Lee 1993). These chitinases are located in the plant cell vacuole. A short carboxy-terminal propeptide is responsible for vacuolar targeting (Neuhaus et al. 1991). Basic isoforms predominate, but two acidic chitinases with cysteine-rich

domains have been found in poplar (Davis et al. 1991). Flach et al. (1992) proposed that two subclasses should be defined: Class Ia for basic and Ib for acidic chitinases. Extracellular acidic PR4 chitinase from bean (Margis-Pinheiro et al. 1991) was originally considered to belong to the Ia subclass (Flach et al. 1992), but has since been redefined as a Class IV chitinase (Graham & Sticklen 1994). Class I includes the majority of chitinases described to date (Punja & Zhang 1993; Graham & Sticklen 1994), and members tend to have higher specific activities than Class II chitinases because the chitin-binding domain increases catalytic activity (Graham & Sticklen 1994). Tobacco Class I chitinases, for instance, had 10- to 15-fold higher specific activities and greater antifungal activity than their Class II counterparts (Sela-Buurlage et al. 1993). Enhanced antifungal activity against *Trichoderma viride* was also observed in Class I verses II chitinases from tomato (Joosten et al. 1995).

ii Class II

Class II chitinases comprise a monomeric catalytic domain with strong sequence homology (60-64%) to the catalytic domain of Class I chitinases, but lack the cysteine-rich chitin-binding domain (Punja & Zhang 1993). They are generally acidic, extracellular proteins (Graham & Sticklen 1994).

iii Class III

Class III chitinases differ from Classes I and II in the amino acid sequence of the catalytic domain, and they do not have a chitin-binding domain. They may be acidic or basic and are compartmentalised in the extracellular volume (Flach et al. 1992). Proteins in this class can also act bifunctionally as lysozymes, although they differ in the strength of their substrate affinity (Boller 1985), and therefore may be involved in defence against bacterial pathogens. Class III chitinases have been shown to accumulate both locally and systemically in cucumber in response to fungal and viral infection (Métraux et al. 1988).

All three types are inducible by infection, stress and elicitors but Class I and II chitinases are also expressed in an organ-specific and age-dependent manner in uninfected plants (Sahai & Manocha 1993). Ethylene treatment induces Class I chitinases

(Flach et al. 1992) and a Class III chitinase in Azuki bean (Ishige et al. 1993). Although chitinase lysozymes are predominantly in Class III, some Class I chitinases can also display lysozymal activity (Graham & Sticklen 1994).

iv Class IV

Collinge et al. (1993) proposed a new chitinase class to include a basic sugar beet isoform (Mikkelsen et al. 1992), basic rape chitinase ChB4 (Rasmussen et al. 1992) and acidic bean PR4 chitinase (Margis-Pinhiero et al. 1991). These chitinases contain a chitin-binding domain, hinge region and catalytic domain, but are distinguishable from Class I chitinases on the basis of amino acid (aa) sequence (Graham & Sticklen 1994 and serology (Mikkelsen et al. 1992). In addition, four deletions, one within the chitin-binding domain and three within the catalytic domain, shorten the final protein product (241-255 aa) relative to the 300 or so residues in Class I chitinases (Collinge et al. 1993; Graham & Sticklen 1994). These enzymes lack a C-terminal vacuolar target sequence and therefore may fulfil a similar antifungal role to Class I chitinases, but in the apoplast (Graham & Sticklen 1994).

While class designations are useful, they do not represent absolute divisions because some chitinases are not easily categorised. For example, an endochitinase from the seeds of Job's tears (*Croix lachrymosa-jobi*) has high amino acid sequence homology to Class I chitinases, but is the only known chitinase to dimerize forming a 52.5 kDa protein with α-amylase inhibitor function (Ary et al. 1989).

1.8.2 Endogenous Functions

Numerous lines of evidence suggest that chitinases contribute to host resistance. Natural substrates for the enzyme are common in fungal cell walls but are not present within the plant, although Benhamou & Asselin (1989) and Spaink et al. (1993) suggested that lipophilic chitin derivatives found in extracts of plant cell walls might act as chitinase substrates. Chitinase alone and in concert with β-1,3-glucanase has been shown in vitro to inhibit growth of fungi (Schlumbaum et al. 1986; Broekaert et al. 1988; Mauch et al. 1988b; Leah et al. 1991; Sela-Buurlage et al. 1993) and of bacteria, through lysozymal cleavage of peptidoglycan (Boller et al. 1983; Majeau et al. 1990). Moreover, chitinase

and β-1,3-glucanase appear to release elicitors from fungal cell walls, which in turn stimulate further defence responses (Keen et al. 1983; Kurosaki et al. 1987). In addition, Benhamou et al. (1990) demonstrated that chitinase accumulates around fungal hyphal material in planta.

Further testimony comes from the increases in endogenous chitinase activity in response to various factors which elicit defence mechanisms and/or stress the plant, such as exposure to ethylene (Boller et al. 1983; Ishige et al. 1993); infection by fungal, viral or bacterial pathogens (Mauch et al. 1984; Métraux & Boller 1986; Roby & Esquerre-Tugaye 1987a; Tuzun et al. 1989; Conrads-Strauch et al. 1990; Rasmussen et al. 1992; Cordero et al. 1994); application of chitin derivatives such as chitosan (Hirano et al. 1990a; Hirano et al. 1992; Wilson et al. 1994; Chang et al. 1995); treatment with chemicals (Irving & Kuc 1990) including heavy metals (Jacobsen et al. 1992) and necrotizing salt solutions (Métraux & Boller 1986); temperature shock (Van Damme et al. 1993; Margis-Pinheiro et al. 1994); exposure to UV light (Brederode et al. 1991) and ozone (Ernst et al. 1992); and by wounding through insect (Bronner et al. 1991) or nematode feeding (Roberts et al. 1992) or by mechanical techniques (Van Damme et al. 1993; Zhang & Punja 1994). Disparate stresses may either induce different isoforms, for example, *Phytophthora megasperma* f.sp. *glycinea* infection and mercuric chloride treatment lead to the accumulation of distinctly different isoforms of chitinase in soybean tissue (Yi & Hwang 1996), or they may induce the same isoforms (Zhang & Punja 1994). Although pathogen infection increases ethylene production, Mauch et al. (1984) demonstrated that chitinase induction by pathogen attack is not dependent on the production of ethylene.

Chitinase induction in response to the aforementioned stimuli does not in itself provide definitive evidence of the role of chitinases in host resistance because these agents can simultaneously enhance other defence reactions. Also, chitinase accumulation may occur to similar extents in both compatible and incompatible reactions (Vogelsang & Barz 1993; Kim & Hwang 1994) or, paradoxically, may even be higher in the susceptible response (Kragh et al. 1990). The latter situation may be explained by greater fungal biomass and stress in the diseased plant, both of which may induce chitinase activity.

In addition, measurements of total activity in diseased plant tissues may also include fungal chitinases with endo or exo activities (Sahai & Manocha 1993), which are not always easily distinguished from the plant isoforms.

In planta evidence of the role of chitinases in resistance comes from the work of Broglie & Broglie (1994) who enhanced chitinase levels in transgenic canola and tobacco, and in doing so increased resistance to *Rhizoctonia.*

Although there are many positive correlations between chitinase activity and resistance, these two factors do not always appear to be related. For example, Nakajima et al., (1992) observed that although *Fusarium oxysporum* infection induced cucumber chitinases, crude extracts and purified chitinases from the plant were unable to inhibit growth of this fungus in vitro. Similarly, Broekaert et al. (1988) and Verburg & Huynh (1991) showed that purified chitinases from various plants were not able to inhibit the growth of some fungi containing chitin in their cell walls. Mauch et al. (1984) did not find any significant differences between chitinase induction in pea pods infected with a compatible and an incompatible strain of *Fusarium solani.* Albrecht et al. (1994) noted that patterns of chitinase induction in response to symbiotic and pathogenic infection of *Eucalyptus* were indistinguishable, although the reverse was true for mycorrhizal infections of pea (Dassi et al. 1996). Also, increased chitinase expression in transgenic plants does not always elevate resistance to selected pathogens (Nielsen et al. 1993). The absence of any increase in resistance in the latter situation, however, does not eliminate the possibility that the pathogen is under some form of control by the low levels of wild-type chitinase. This could only be demonstrated unequivocally by use of mutated/transformed plants where the expression of chitinase activity had been totally curtailed. In all these examples, chitinases appear to either have no role or a secondary role in retarding pathogen development following infection or alternatively, they could require simultaneous action of other factors such as β-1,3-glucanases, which may be limiting in transgenics.

The ability of chitinases to repress disease appears to depend on several factors. Variations in the proportion of chitin verses other cell wall components such as lipids

and proteins, along with differences in accessibility of this chitin, might explain the diverse levels of pathogen susceptibility to lysis by chitinase. Chitin and β-1,3-glucans are generally found in the innermost layers of the cell wall and thus may be protected, except at the hyphal tips (Wessels 1986). Spanu et al. (1989) found that chitinases from leek readily bound to the cell walls of mycorrhizal hyphae which had been treated at 100°C to eliminate soluble polysaccharides and proteins. However, chitin in untreated hyphae appeared inaccessible to the plant chitinases.

The sites at which endogenous chitinases accumulate, relative to the locations invaded by the pathogen, can also influence the potential role of chitinases in the defence response. Obligate biotrophs and/or intercellular pathogens may never encounter the vacuolar forms of chitinase, whereas necrotrophs and intracellular hyphae would be affected by both extracellular and intracellular forms. Extracellular chitinases (Classes II, III and IV) could either play an initial role in limiting pathogen growth upon hyphal entry into the host, or release elicitors thereby inducing other defence mechanisms. Elicitor release, rather than direct inhibition, is thought to be the main role of the Class II chitinases (Graham & Sticklen 1994). Mauch & Staehelin (1989) proposed that the role of vacuolar chitinases is to form a last line of defence at a late stage of infection when they are released upon cell lysis. According to this theory, the fungicidal effect of vacuolar isoforms is maximised by the high specific activities of Class I chitinases, the delayed reaction which allows time for accumulation of potentially lethal concentrations, and the sudden release which precludes fungal adaptation. These authors also suggested that the presence of chitinases in the apoplast of infected tissue might be caused by release of vacuolar forms into the extracellular space upon host cell lysis, rather than by active secretion. However, other research indicates that extracellular forms are actively secreted. For example, Fink et al. (1988) found that chitinases in oat leaves were located extracellularly prior to fungal infection. In addition, extra- and intracellular isoforms are in different genetic classes coded by separate genes (Section 1.8.1, Part B).

There are several other factors which may influence the outcome of an interaction between host chitinases and an invading pathogen. Although chitinase accumulates in both sensitive and resistant responses, incompatibility is often linked to the rapidity and

extent of chitinase induction (Joosten & de Wit 1989; Rasmussen et al. 1992). The outcome may also depend on which specific isoforms are induced, since various isoforms from a given host may differ in substrate-binding characteristics, specific and antifungal activities (Majeau et al. 1990; Huynh et al. 1992; Sela-Buurlage et al. 1993). Reactions may also be stronger in some plant tissues than others. For example, chitinase induction was significantly higher in pepper flowers compared with leaves, following simultaneous inoculation of both parts of the plant (O'Garro & Charlemange 1994). Effectiveness of chitinases against fungi may also require simultaneous action of β-1,3-glucanase or other antifungal substances (Mauch et al. 1988b). Benhamou et al. (1990) suggested that induction of glucanases spatially ahead of chitinases may stimulate chitinase activity by release of pathogen glucan elicitors, and increase accessibility by dissolution of glucans in which the chitin polymers may be embedded. Overall, given the complexities of plant-pathogen interactions, the expression of chitinases in combination with one or more other defence mechanisms is likely to have a greater antifungal effect and presents an exciting possibility for disease control.

Although some chitinases are stress-inducible, many are also expressed constitutively, generally at low levels, and appear to be regulated at specific stages in plant growth, suggesting a possible developmental role (Punja & Zhang 1993). De Jong et al. (1992) reported that development of a temperature sensitive somatic embryo mutant from carrot required the presence of a glycosylated acidic endochitinase. Chitinases may play a role in flowering and/or reproduction. They are found in the sepals and ovaries of tobacco flowers but not in petals, styles or stigmas, although they can be induced by elicitor application to the style (Lotan et al. 1989). This variation in chitinase expression between different floral parts may indicate a developmental role or, more simply, a differential need for protection of critical or more readily infected tissues. In petunia, the activity of one chitinase expressed only in the stigma increases five-fold following anther dehiscence. Since expression is restricted to the stigma, this chitinase may have some role in pollen germination (Leung 1992). Despite the correlations between chitinase expression and plant development, specific roles of chitinases in plant morphogenesis await elucidation.

1.8.3 Tracking Chitinases

Scientists seeking to understand the functions of chitinases in plants use many techniques, including biochemical measurement of enzyme activity in crude and purified plant extracts, localization of the enzymes within plant tissue by immunocytochemistry, in vitro analysis of the substrate specificities and antifungal activities of purified chitinases, and examination of the consequences of altered genetic expression through the production of transgenic plants. Successful application of most of these techniques involves a suitable enzyme assay. For example, it is impossible to isolate chitinases from other plant components unless the progress of purification can be monitored. Similarly, performance of transgenic and control plants are typically compared on the basis of enzyme activity and resistance to pathogens. Choice of a suitable assay is therefore an important component of any study on chitinases. Chitinase assays are numerous and the advantages and disadvantages of various methods are examined at length within this thesis (Chapters 4 and 5).

1.9 OVERALL OBJECTIVES

Inadequate control of *B. cinerea*, as outlined in this literature review, remains one of the major problems that bedevils the kiwifruit industry. Host resistance offers an exciting but under-utilised alternative method of control. There is a need to further establish the importance of host resistance in kiwifruit/*B. cinerea* interactions, and in particular to examine the promising areas of antifungal compounds and defence enzymes. Preliminary work has indicated that chitinases may be an important defence mechanism, but detailed study requires optimization of measurement and purification techniques.

The overall objectives of this study were to:

1) ascertain the role of host resistance in *B. cinerea* infections,
2) optimise chitinase assays and purification techniques for use in the kiwifruit system,
3) distinguish between host and fungal chitinases,
4) examine changes in chitinase activity in response to development, infection and

elicitation, and

5) determine the importance of chitinases in kiwifruit defence against *B. cinerea*.

2 GENERAL MATERIALS AND METHODS

This chapter describes general materials and methods employed in several experiments. Methodology details unique to individual experiments are found in the materials and methods sections within the appropriate chapters.

2.1 FIELD TRIALS

2.1.1 Harvest Details

Export quality, 36 count sized fruit (102 g mean weight) were harvested between 8 and 9.30 am from one of three geographical sites, depending on availability. Fruit for the 1992 maturity experiment were harvested from vines on a pergola system in a commercial Wanganui orchard (Lat S 39° 56 Long E 175° 03). Fruit for the 1993-94 chitosan experiments were collected from pergolas in commercial orchards at Levin (Lat S 40° 37 Long 175° 17). All other fruit came from the Massey University Fruit Crops Unit (Lat S 40° 21 Long E 175 37), where a T-bar training system was used. In 1992, fruit were snapped from the pedicel, but in subsequent years they were harvested with the pedicels attached, and the pedicels were removed immediately prior to treatment, to minimise any curing effect. At each harvest, orchard relative humidity, air temperature, fruit firmness (kg) and total soluble solids (TSS) were recorded (Section 2.1.5). Ambient air temperature and relative humidity were measured outside of the vines, using a swinging hygrometer.

Fruit were collected in picking bags, then transferred to cartons for transport back to the laboratory. In the laboratory, a soft nail brush was used to remove sepals from the fruit prior to pedicel removal, in order to provide a uniform environment around the picking scar.

2.1.2 Treatment Application

The fruit were placed, stem scar uppermost, in 36 count plastic plixes in single layer

commercial cardboard trays with polyethylene liners. One 17 µl droplet of inoculum and/or chitosan solution was applied to the exposed stem scar of each fruit, using a 15 ml dropper bottle with a pipette tip attached - a technique developed by Dr. Greg Tate of Crop Health Services, Hawkes Bay. Fruit were left in ambient conditions for 1-2 h to allow the inoculum/chitosan droplets to dry. Boxes of fruit were subsequently packed and placed in stacks in a 0°C coolstore.

2.1.3 Inoculum Production

Spores were collected from a pure culture of *Botrytis cinerea* (Persoon: Fries), obtained from diseased kiwifruit in Palmerston North and named isolate K3. Cultures were grown on malt agar in vented petri plates under a 12 h:12 h light dark regime at a distance of 40 cm from lights, comprising two Philips blacklight blue tubes (TLD, 36W/08) and a Philips 34 W white deluxe tube. Subculturing was performed at 3-4 week intervals to maintain viability and pathogenicity. Malt agar was produced by adding one litre of hot reverse osmosis water (Section 2.2) to 25 g malt (Maltexo NZ Ltd) and 15 g agar (Difco), then autoclaving at 120°C for 15 minutes.

Spore suspensions were prepared in aseptic conditions. Cultures (10-14 days old) were flooded with sterile 0.01% (v/v) Tween 20 (Polyoxyethylene sorbitan monolaurate, Serva) and dislodged with a flamed glass hockey stick. The suspension was filtered through sterile muslin to remove hyphal fragments and shaken with glass beads to break up clumps of spores. Counts were made using a haemocytometer and concentrations adjusted to produce the required number of spores per 17 µl droplet.

In 1992, spore suspensions were produced on the day of the experiment. Subsequently it was found that spore suspensions could be frozen for up to seven months (Sharrock & Hallet 1992c) without loss of viability. In 1993 and 1994, the spore suspensions were prepared in advance, aliquots frozen at -20°C and defrosted on the day of use. Inoculum viability was determined for all suspensions by plating 1 ml onto malt agar plates and counting percent germination in three randomly chosen microscope fields of view after 10 h incubation in ambient conditions. Only suspensions with >95% germination were used.

2.1.4 Field Trial Controls

Each field trial included control fruit which received one 17 µl droplet of sterile 0.01% Tween 20 without spores, but otherwise were treated identically to fruit from other treatments.

2.1.5 Maturity Indicators

Between 9 and 18 fruit were harvested from within 1 m of the end of the central leader, and from above shoulder height on T-bars. A 1 cm slice was cut from each end of the fruit and the juice squeezed onto the prism of a hand-held Atago N-20 refractometer (brix range 0-20%). Juice from each end was tested separately at 20°C and the results were averaged. Prior to use, the refractometer was calibrated with water on the prism (Harman & Hopkirk 1984), and the prism was cleaned with distilled water and a tissue between each sample.

Firmness (kg) was measured by removing a 5 mm square of skin from the central area of each side of the fruit, and applying a hand-held penetrometer with an 8 mm probe (R. Bryce Mod. FT327) to these areas.

2.1.6 Assessment Of Infection

Percent infection was assessed after 4-12 weeks coolstorage at 0°C. Fruit were assessed in ambient conditions then returned to coolstorage. The number and position of infected fruit in each box were recorded. Infected fruit had soft zones with a water-soaked appearance extending downwards from the stem scar. Sporulation around the stem scar was present in more advanced infections.

2.2 LABORATORY EXPERIMENTS

Detailed recipes for all buffers used in laboratory experiments are given in Appendix I. Sample calculations of enzyme activity from the different assays are shown in Appendix II. Unless otherwise stated, all water used in laboratory experiments was town supply

water which had been passed through a reverse osmosis (RO) unit, followed by further purification via a Milli-Q 12 inch mixed bed ion exchange filter.

2.2.1 Preparation Of Crude Enzyme Extracts

Crude enzyme extracts were used as the starting point for protein purification. Exo- and endochitinase activity was measured in both crude and partially purified extracts which were also applied to SDS and native polyacrylamide gel electrophoresis (PAGE), and isoelectric focusing (IEF) gels to assess protein composition.

The extraction technique, a modification of Sharrock & Hallet (1992b) is described below:

Stem plugs, comprising the stem scar region and the underlying woody pin of tissue (Plate 2-1), were removed from kiwifruit, using a 9 mm core borer. The stem plug was separated from surrounding softer pericarp tissue by scraping with a scalpel blade. Plugs were then snap frozen in liquid nitrogen and transferred to -80°C for storage.

Extraction was performed on a small scale by crushing 1-3 frozen stem plugs in a specially constructed (Massey University Agricultural Engineering) 21 mm steel mortar and pestle (Plate 2-2). The crushed tissue was homogenised on ice in 3-5 volumes (v/w) of extraction buffer (0.1 M sodium acetate (Sigma Chemical Company), 0.2% (w/v) sodium dithionite (GPR, BDH Chemicals Ltd) and 0.02% (w/v) sodium azide (BDH Chemicals), pH 5.8) for approximately one minute, using an Ultra-Turrax tissue homogeniser (IKA-WERK, Janke and Kunkel, Model TP18/10). A 1:5 sample/buffer extraction ratio was used for tissue extracted at Massey University, Palmerston North, and 1:3 for extractions at Mt Albert Research Centre, Auckland, because the shape of test tubes available at Massey University required a larger volume for successful homogenisation. The homogenate was centrifuged at 14,600*g* for 15 minutes at 4°C. The resulting supernatant was then filtered through Whatman No. 1 filter paper and stored at -80°C until required.

Plate 2-1. Longitudinal section through a kiwifruit showing the stem plug - an area comprising the stem scar (created by pedicel removal), and the underlying pin of woody tissue.

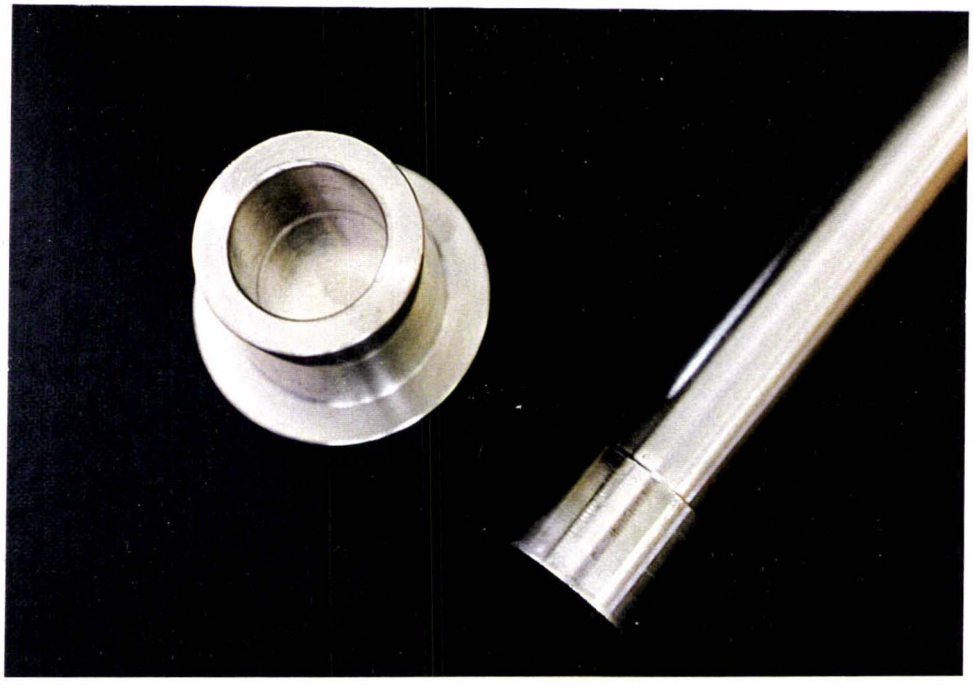


Plate 2-2. Stainless steel mortar and pestle used in preparation of enzyme extracts. One to three kiwifruit stem plugs were placed in the mortar, and the pestle was positioned on top then struck with a hammer to crush the tissue.

In 1994-95 experiments, 1 mM dithiothreitol (DTT; Sigma) replaced sodium dithionite in the extraction buffer, because dithionite may lead to reduction of proteins in the extract. Sodium tetrathionate (10 mM), gifted from Teresa Wegrzyn of HortResearch, Mt Albert, Auckland, was also included in the initial extraction buffer to inhibit actinidin (Wegrzyn & MacRae 1992).

For bulk extractions involving 10 g or more of tissue, the tissue was first cryomilled in liquid nitrogen. Three volumes of extraction buffer, and 1% (w/v) polyvinylpolypyrrolidone (PVPP, Laboratory grade reagent, BDH) to bind phenolics and tannins, were then added. The suspension was subsequently homogenised, centrifuged and filtered as for small scale extractions.

2.2.2 Classification Of Enzyme Extracts

At some stages of this research numerous different enzyme extracts were used. These extracts were differentiated with the following general classification system:

year of harvest and extract production / cured(C) or not cured(N) / inoculated(I) or uninoculated(U) / healthy(H) or diseased(D).

For example, extract 95/C/I/D would have been produced in 1995 from cured fruit that were inoculated and had visible signs of infection following 12 weeks coolstorage at 0°C. More specific details about each extract, such as the duration of the curing period and the weight:volume extraction buffer ratio, are found in the materials and methods sections within the appropriate chapters.

2.2.3 Preparation Of Substrates

A Glycol chitin (1% (w/v), pH 4)

The chitinase substrate used in overlay gels, and the viscometric and Calcofluor enzyme assays was 1% (w/v), pH 4 glycol chitin. It was prepared using the method of Trudel & Asselin (1989) with the same buffer compositions, but the gel was left for 50 minutes at room temperature after addition of acetic anhydride (Analar, BDH), and centrifugations steps were done at 27,000*g* for 30 minutes. (Appendix III details the

entire modified method.)

B Regenerated chitin (pH 6)

Regenerated chitin (reacetylated chitosan) was the substrate used in the colorimetric endochitinase assay of Boller et al. (1983). The method used to produce the substrate, a modification of Molano et al. (1977), is described below:

Crab shell chitosan (10 g, practical grade, Sigma) was ground with a mortar and pestle with slow addition of 250 ml of 10% (v/v) acetic acid (Analar, BDH). The mortar was covered with parafilm and left in the dark overnight to allow further reaction. Methanol (900 ml, analytical reagent (AR), May and Baker), was slowly added to the chitosan with mixing. The mixture was filtered through a Buchner funnel with nylon mesh (60 μm) under vacuum. Acetic anhydride (five 1.5 ml aliquots) was added slowly on a magnetic stirrer. The resultant gel was left to stand for 30 min at room temperature and then was cut into small pieces. The liquid that oozed out was decanted. Gel pieces were placed in a Waring blender and homogenised with 300 ml of methanol until a fine syrupy liquid resulted. The liquid was filtered through a 10 1 Buchner funnel using glass fibre filter paper (Whatman, GF/C) on top of Whatman No. 4 filter paper. The precipitate was washed with water (4x) until a pH of 6 was obtained. The chitin was resuspended in 600 ml of water with 0.04% (w/v) Na-azide and stored at 4°C.

C Tritiated chitin

The preparation of tritium-labelled chitin was similar to that described for regenerated chitin.

Chitosan (10 g) was ground in a Waring blender, and then pressed through a 500 μm sieve (Endecotts Ltd), and 2.5 g of the resultant material was suspended in a mortar and pestle with 50 ml of 10% (v/v) acetic acid. The mixture was covered with parafilm and left overnight in the dark at 20°C. Methanol (225 ml) was stirred into the mixture, which was then filtered through glass wool on a Buchner funnel under vacuum. Methanol was added to the filtrate to return the total volume to 225 ml. The remaining steps were performed in a fume hood using standard radioactivity precautions (gloves, glasses, lined

trays and lab coat).

A 3 ml aliquot of tritiated acetic anhydride (500 mCi/mmol, Amersham), containing 25 mCi of radioactivity, was added to the filtrate mixed vigorously on a magnetic stirrer. A further 0.75 ml of "cold" (unlabelled) acetic anhydride was added, and the resultant gel left in a stoppered flask for 1.5 h at room temperature. The gel was cut into smaller pieces and the liquid that oozed out was discarded. Gel pieces were placed in a Waring blender and homogenised with methanol. The material was placed on a Millipore sintered glass funnel and washed under vacuum with one litre of methanol followed by 5 one-litre volumes of distilled water (until radioactivity in the washings was negligible), to remove any hydrolysed [^{3}H]-labelled reagent. The gelatinous substrate was homogenised with 300 ml of Milli-Q water containing 0.02% (w/v) sodium azide (NaN_3), and stored at 4°C. Aliquots were counted and dry weights were determined to obtain specific activity of the substrate ($2.3x10^6$ dpm/mg).

2.2.4 Enzyme Activity Measurements

Different assays were used to distinguish the two types of chitinase activity (endo- vs exo-). Endochitinases randomly cleave chitin polymers, and generally attack molecules comprising three or more N-acetylglucosamine residues. (Jeuniaux 1966; Molano et al. 1979; Harman et al. 1993). There is some confusion about exochitinase nomenclature (refer Chapter 1, Section 1.8.1, Part A for a detailed discussion), but the definition adopted in this study is that exochitinases cleave dimers (β-D-N,N′-diacetylchitobiose) and/or monomers of N-acetylglucosamine from the non-reducing end of chitin chains (Kunz et al. 1992).

A Colorimetric assay of Roberts & Selitrennikoff (1988)

Exochitinase activity was quantitatively determined using the chromogenic assay of Roberts & Selitrennikoff (1988), with modifications described by Sharrock & Hallet (1992b). Exochitinase activity cleaves a dimer or two individual monomers of N-acetylglucosamine from *p*-nitrophenyl-β-D-N,N′-diacetylchitobiose releasing *p*-nitrophenol, a coloured product.

The chromogenic substrate, *p*-nitrophenyl-β-D-N,N′-diacetylchitobiose (Sigma) was dissolved (0.1 mg/ml) in substrate buffer (50 mM sodium acetate, pH 5.6 with 0.02% (w/v) NaN_3), and stored in a foil-covered container at 4°C. For each treatment, four 10 µl aliquots of test extract were placed into the wells of a flat-bottomed microtitre plate (Nunc 96F). Fifty microlitres of 0.1 M sodium tetraborate (May and Baker Ltd) were added to one or two of the four wells per treatment to preinhibit the reaction. Substrate (90 µl) was then added to all wells, and well contents were mixed. The plate was wrapped in parafilm (American National Can), sealed in an airtight polythene bag and incubated for 3 h at 37°C in a water bath. Tetraborate stopper (50 µl) was added to the previously uninhibited wells, and absorbance was read at 405 nm (Labsystems Multiskan Biochromatic plate reader). The difference between the average absorbances of preinhibited and uninhibited wells was attributed to exochitinase activity.

Change in absorbance over time was converted into nmol of *p*-nitrophenol produced per minute per ml of extract using the molar absorption coefficient ε of a 1 M *p*-nitrophenol solution and Beer's law.

B Viscometric assay

This assay was used to determine the presence/absence of endochitinase activity in the early stages of experimental work. The assay measures the decrease in substrate viscosity caused by chain length reduction through enzymatic cleavage. Endo- rather than exo-activity is measured principally, because exochitinase activity creates minimal reduction in chain length. Commonly, two replicates were tested for each sample, the time consuming nature of this assay making further replication impractical. It was superseded by the Calcofluor and radioactive assays.

An aliquot (10µl) of test extract or water (control) was vortexed with 400 µl of substrate (described below), and drawn into a 3.5 mm diameter barrel Haake syringe containing a 3.175 mm gold coated ball-bearing. The syringe was inverted and small air bubbles, which would impede bearing movement, were removed by agitating the bearing, using a magnet held against the outside of the syringe. (This caused bubbles to rise to the mouth of the syringe where they could be expelled.) The inverted syringe was placed

inside the viscometer (Haake 001-1926) at 37°C. The time between mixing of extract and substrate, and obtaining the first reading on the viscometer, was noted. Generally this loading process took about one minute.

The bearing was pulled to the top (mouth) of the inverted syringe by a cycling magnet within the viscometer, and released to descend through the liquid by gravity. The rate of descent was affected by the viscosity of the liquid, and was measured as the time taken in milliseconds (ms) for the bearing to fall between two light barriers 2 cm apart in the viscometer (Plate 2-3). This was recorded on a 286 computer attached via its RS232C port to the viscometer (Plate 2-4). Every 30 seconds the magnet returned the bearing to the mouth of the syringe, and the whole process was repeated. The data was used to generate a curve of changes in viscosity (ms) over time for each sample. Curves were used to make qualitative comparisons between samples (Fig. 2-1, A).

The viscometric substrate was prepared by diluting 1% (w/v) pH 4 glycol chitin in pH 5 citrate phosphate (McIlvaine) buffer. The actual dilution factor (2.2x = 450 µl glycol chitin, 550 µl citrate phosphate buffer) was chosen to produce an initial fall-time of 9,000-10,000 ms (i.e. time taken for the ball bearing to fall between the light barriers.) If the fall time was much above 10,000 ms, the ball was picked up by the rotating magnet before completing its 2 cm fall. If the initial viscosity was too low, the ball descended too quickly for reliable estimation of reduction in fall time due to enzyme activity. The diluted substrate was then microwaved for 1 minute (medium power) to ensure complete solubilisation of all solid particles. It was filtered through a 70 µm cell strainer (Falcon) to remove any remaining extraneous particles, degassed under vacuum in a syringe, and 400 µl was placed in an Eppendorf tube ready for addition of 10 µl of test extract.

Quantification of the assay was achieved by converting sample fall time after 15 minutes in the viscometer into dynamic viscosity (mPa/s), using the equation supplied by the Haake Microviscometer instruction manual (refer Appendix II). It was assumed that all samples tested at any one time had approximately the same starting viscosity. Fifteen

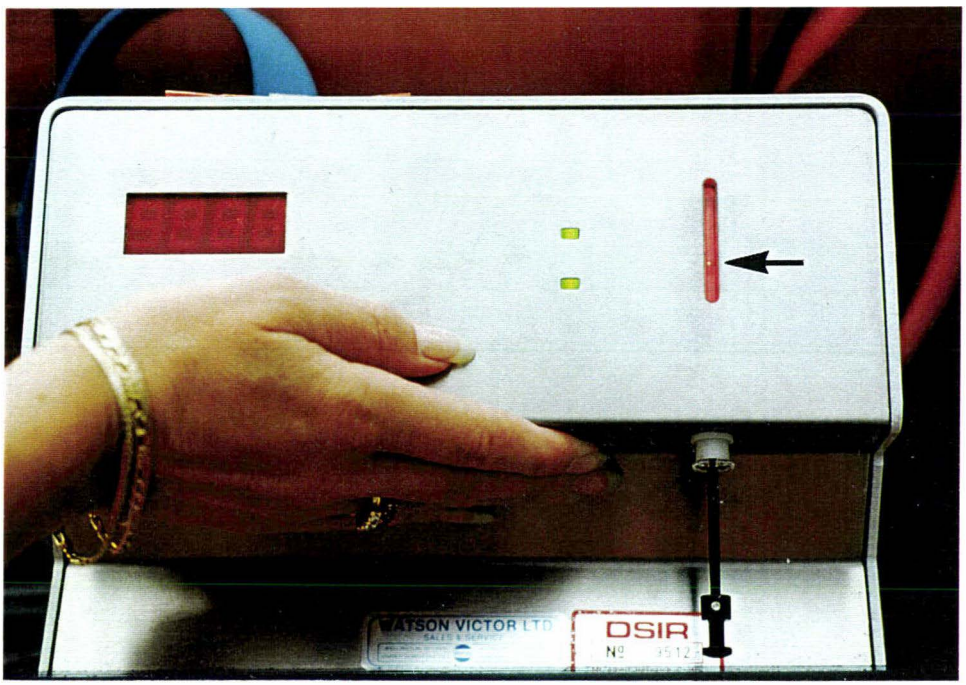


Plate 2-3. Close-up of the Haake 001-1926 viscometer showing the position of a gold ball bearing (arrowed) moving through liquid in an inverted syringe. The time taken for the bearing to descend 2 cm between the two green lights on the viscometer was recorded in milliseconds.

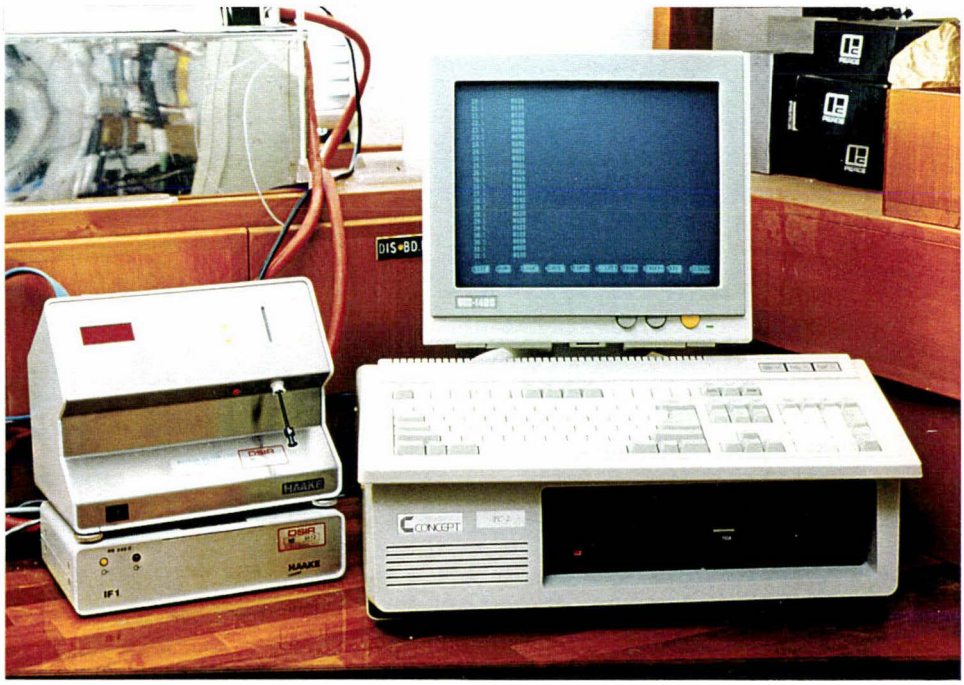


Plate 2-4. The complete viscometer system. Temperature was maintained at a constant equilibrium by circulating water from a temperature-controlled water bath through the viscometer via rubber hosing. Readings from the viscometer were transferred directly to a computer at 30 second intervals.

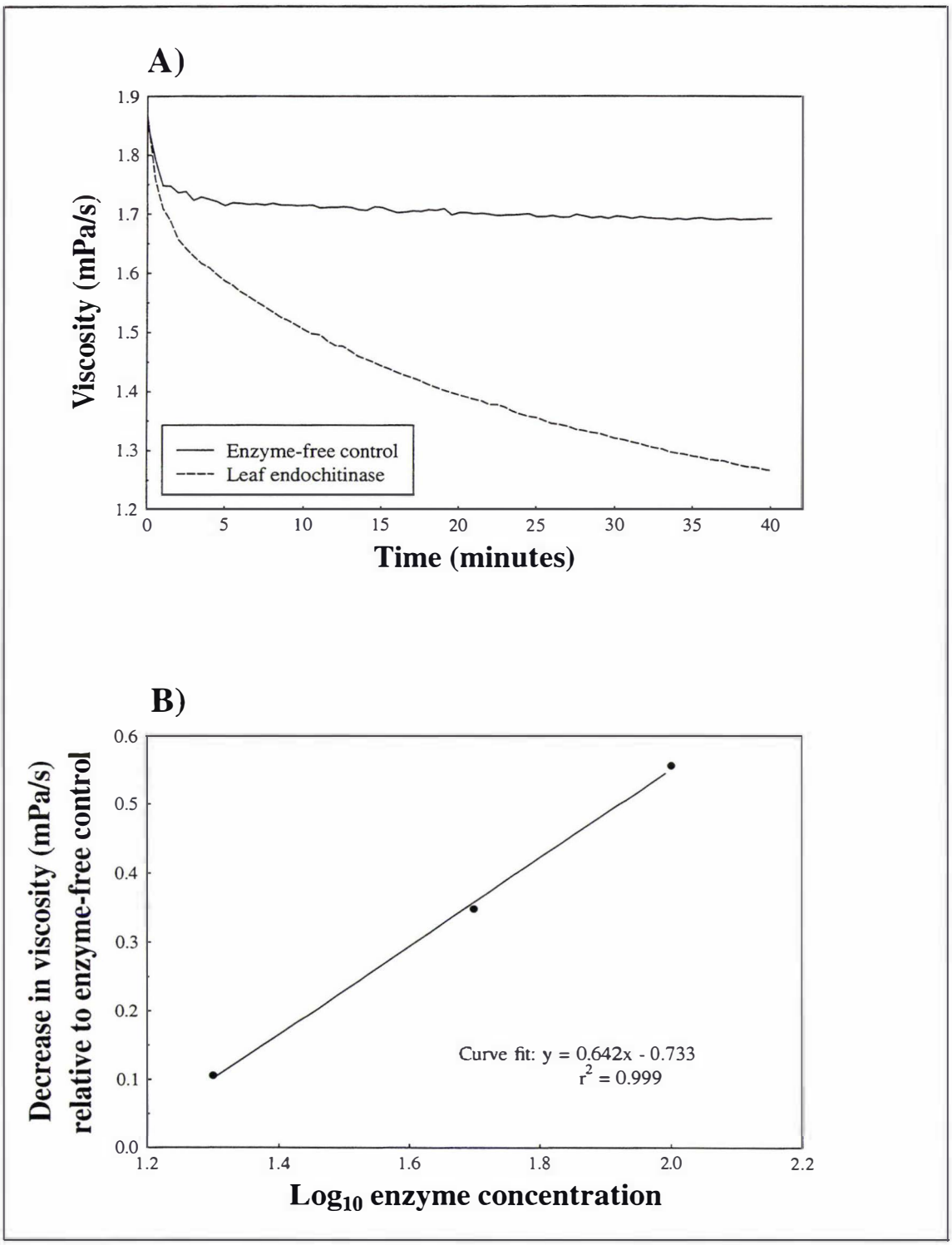


Figure 2-1. A) Changes in the viscosity of glycol chitin solution over 40 minutes at 37°C in the presence or absence of kiwifruit chitinase. The reaction mixture in the viscometric assay comprised 400 µl of 1% (w/v) glycol chitin diluted 2.2x in pH 5 McIlvaine buffer, and 10 µl of crude chitinase extract or water. B) The relationship between enzyme concentration and decrease in viscosity, relative to the enzyme-free control.

minutes allowed adequate time for control viscosity to stabilize. (Initially control viscosity decreased as the sample heated up to the temperature of the water jacket in the machine. Once the control had reached 37°C, its viscosity stabilized, whereas in samples containing chitinase, it continued to decrease because of substrate degradation.) Decreases in dynamic viscosity after 15 minutes, in samples containing chitinase, were found to be linearly related to $\log_{10}$ of enzyme concentration (Fig. 2-1, B), allowing endochitinase activity to be expressed as a percentage loss of viscosity compared with the enzyme-free control.

C Calcofluor petri dish assay

Sharrock & Gill (1991 unpubl. data) modified Trudel & Asselin's (1989) method of staining undegraded glycol chitin with Calcofluor to enable detection of endochitinase activity in agar plates. The assay allowed rapid assessment of the presence or absence of endochitinase activity in large numbers of fractions generated by protein purification. It was also used to qualitatively rank samples with large differences in chitinase activity. The assay was the most sensitive of all the assays used, detecting levels of chitinase activity as low as 0.07 nmol N-acetylglucosamine/min/ml, but had poor resolution (see Chapter 5), so at least ten-fold concentration differences were required before a significant change in the radius of the reaction zone was observed. For quantitative measurement of chitinase activity, where greater resolution was required, the Molano et al. (1977) radioactive assay was employed - this assay resolved 2-3 fold concentration differences. Further modifications to the Calcofluor method were made in the course of this study (refer Chapter 5), and the final protocol adopted is described here.

Six grams of agar powder (Oxoid) were added to 390 ml of pH 5 citrate phosphate (McIlvaine) buffer containing 0.02% (w/v) azide. The suspension was microwaved until boiling to dissolve the agar. After 10 minutes cooling time, 4 mls of 1% (w/v) pH 4 glycol chitin was added and mixed thoroughly. (Glycol chitin is damaged by extreme heat.) The mix was poured into 8.5 cm diameter petri plates (20 ml/plate). Once the agar had set, seven evenly spaced 5 mm diameter wells were punched into the agar, using a cork borer attached to a vacuum pump. The plates were stored in sealed plastic bags at 4°C until required.

Thirty microlitres of test extract or control (0.1 M sodium acetate extraction buffer or reverse osmosis (RO) water), was placed in each well. The plate was sealed with parafilm and placed in a 37°C incubator for 5 h. The plate was then covered with 10 ml of 0.01% (w/v) Calcofluor white M2R (Fluorescent brightener 28, Sigma) in 500 mM tris(hydroxymethyl)aminomethane hydrochloric acid (Tris-HCl) buffer, pH 8.9, containing 0.02% (w/v) sodium azide. The Calcofluor solution stains the glycol chitin. After ten minutes, the plate was rinsed in RO water several times and left covered with water for 15 minutes.

The water was removed, and the plate was viewed under long wavelength UV light (350 nm) in a Chromato-vue lightbox (Ultra-violet Products, Inc.). Zones in which the glycol chitin had been degraded appeared as dark circular areas around the wells containing test extract, while the remainder of the plate fluoresced blue/white due to the stain attached to the glycol chitin (Plate 2-5). The plate was inverted and the perimeters of the reaction zones marked with an indelible pen. Diameter measurements (mm) were taken at two perpendicular points and the results averaged. Reaction zone radial extension (mm) was then calculated by subtracting the radius of the well (2.5 mm) from the total radius (diameter/2).

Commonly three replicate measurements were made for each sample tested, each replicate comprising a well on a different plate.

D Colorimetric assay of Boller et al. (1983)

A variation of the colorimetric assay of Boller et al. (1983) was used to quantify exo- and endochitinase activities in the same samples. Regenerated chitin was used as a substrate instead of colloidal chitin. Modifications were made to this assay in the course of this study (refer Chapter 5) in an attempt to improve its sensitivity. The radioactive assay was eventually used in preference because it was considerably more sensitive.

The principle of this assay is that the first incubation step releases N-acetylglucosamine dimers and monomers as a result of exochitinase activity (as defined by Kunz et al., 1992), as well as soluble intermediate chain length chitin oligomers from endochitinase

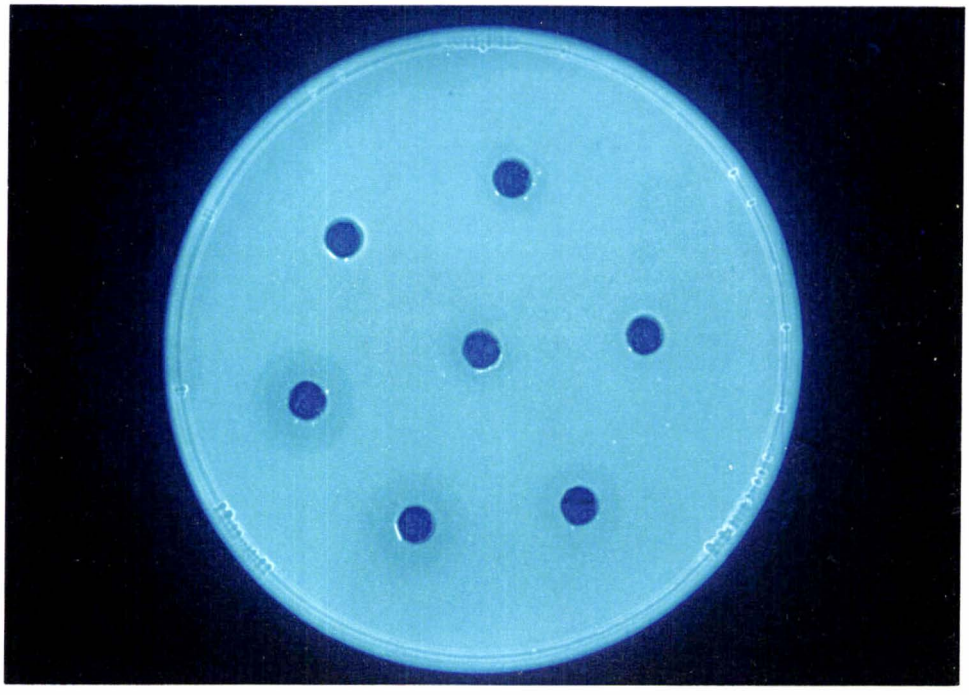


Plate 2-5. Glycol chitin petri dish assay for endochitinase activity. Test samples were placed in 30 µl wells punched into agar containing the glycol chitin substrate. After incubation to allow for enzymic digestion of the substrate (typically 5 h at 37°C), the plates were stained with Calcofluor white. Glycol chitin degraded areas were visible as dark circles against a fluorescent blue background. Dark rings surrounding the two wells in the bottom left hand corner of the plate indicate that the samples in these wells have chitinase activity.

digestion of the suspended polymeric chitin substrate. Activity of exo-acting N-acetylglucosaminidase and chitobiase (which splits chitobiose into two N-acetylglucosamine molecules) can be quantified at this point by going to the third step directly. The soluble oligomeric products of endochitinase digestion are further degraded to monomers by snail gut enzyme during a second incubation step. In the third incubation, dimethylaminobenzaldehyde (DMAB) reagent reacts with the monomers to produce a coloured product. Boiling between each incubation stops each reaction from proceeding any further.

To distinguish exo- and endochitinase activity, tubes with and without snail gut enzyme were prepared. Water was substituted for snail gut enzyme in the tubes without snail gut enzyme, but in all other respects the two sets of tubes were treated identically. The difference in results between tubes incubated with and without snail gut enzyme is normally attributed to endochitinase activity.

Specific details of the final assay protocol adopted in this study are described below. (A flow diagram of all these steps is also found in Appendix II.) Test extract (0.1 ml) was placed in each 1.5 ml Eppendorf tube. Water (0.1 ml) replaced the test extract for an enzyme-free control. A chitinase enzyme standard was produced by substituting the test extract with 0.1 ml of *Streptomyces griseus* chitinase 1 mg/ml (Sigma) in 10 mM sodium acetate (NaOAc) buffer, pH 5.6.

Substrate/buffer mix (0.1 ml of reacetylated chitosan (see Section 2.2.2, Part B) and 0.1 ml of 0.1 M NaOAc buffer, pH 5.6) was added to each of the tubes, which were vortexed and incubated at 25°C for 2 h with continual shaking on a orbital shaker (IKA-Vibrax, Janke and Kunkel, Model VXR), with a dial setting of 1400.

The tubes were boiled for 3 min to stop chitinase action and centrifuged for 10 min at 11,000*g* to remove unreacted substrate. A 150 µl sample of each supernatant was transferred to a second set of Eppendorf tubes, and 20 µl of snail gut enzyme/buffer mix (crude extract of β-glucuronidase (*Helix pomatia*, Sigma) mixed in a 1:3 ratio with 0.5 M potassium phosphate buffer, pH 7.1) was added. Tubes were vortexed and incubated at

37°C for 1 h without shaking.

A 35 µl aliquot of 1 M sodium borate buffer, pH 9.8 was added, and the tubes were boiled for 3 min to stop the snail enzyme reaction. The tubes were cooled rapidly in an ice bath, and 1 ml of DMAB (Analar, BDH) freshly diluted 1:4 in glacial acetic acid, was added. The tubes were incubated at 37°C for 20 min, and the absorbance of 1 ml aliquots of their contents was measured at 585 nm on a spectrophotometer (Pye Unicam, PU 8600 UV/VIS, Philips).

At least two tubes with reacetylated chitin, and one containing water instead of substrate, were set up for each sample. The substrate-free tube was used to measure background absorbance.

A series of standards was prepared by adding 35 µl of 1 M sodium borate buffer, pH 9.8 to 170 µl of 0.1, 0.25, 0.5 or 1 mM N-acetylglucosamine (Sigma). Steps following borate buffer addition were identical to those described above. Standard curves relating absorbance to N-acetylglucosamine concentration were constructed.

E Radioactive assay of Molano et al. (1977)

The radioactive assay of Molano et al. (1977), redescribed by Cabib (1988), was used with some modifications. It was the most reliable and sensitive of the quantitative assays used, but was relatively time consuming, expensive and did not differentiate between exo- and endochitinase activity.

A mixture of 5 µl 1 M NaOAc, pH 5.5, 30 µl tritiated chitin suspension (see Section 2.2.2, Part C), 30 µl test extract and 35 µl 0.1 M NaOAc extraction buffer, pH 5.8 was incubated in screw cap Eppendorf tubes at 25°C on an orbital shaker (IKA-Vibrax, Janke and Kunkel, Model VXR), with a dial setting of 1400, for 18 h (or 3 h for very active extracts). The 1 M NaOAc buffer, pH 5.5 was added to bring the pH closer to the pKa of acetate, thereby increasing its buffering capacity. The reaction was stopped by adding 0.2 ml of 10% (w/v) trichloroacetic acid (AR, May and Baker) to each tube. The tubes were centrifuged at 11,000*g* for 15 min, and 250 µl of each supernatant was spun at

11,000*g* for 15 min through a 0.45 μm microfuge filter (Alltech). Filtrate (200 μl) was transferred to mini scintillation vials and 3 ml of ASCII (Amersham) scintillant was added. The tubes were vortexed and radioactivity measured using a scintillation counter (Beckman LS2800) for 1 minute intervals, preset using the tritium window setting. Repeat counts were made after 12 h. Water replaced test extract in the no enzyme control. Tubes containing substrate + scintillant, and scintillant only, were included. Each sample was assayed in triplicate.

The microfuge filtration step was used in all the radiochemical experiments described in Chapter 5, as well as Experiment 6 in Chapter 6. This step was omitted in all other later experiments because of concerns that repeated stringent washing conditions were damaging the filters, and that the cumbersome filtration step had not significantly reduced inherent variation in the assay.

2.2.5 Protein Estimation

Total protein content was determined using the Bradford (1976) assay, adapted for use in microplates, with reagents from the Bio-Rad protein assay II kit (No. 500-0002). Bovine serum albumin (1.4 mg/ml) was dissolved in water to produce a standard, and 2 ml aliquots were frozen until required. Water and diluted protein standard were mixed in various ratios to produce concentrations ranging from 8-80 μg/ml of protein.

Test extract or standard (160 μl) was pipetted into each microplate well, and 40 μl of Bio-Rad dye reagent concentrate was added. Well contents were mixed for 5 min on an orbital shaker (IKA-Vibrax, Janke and Kunkel, Model VXR), with a dial setting of 600. Following 10 min incubation at 20°C, the plate was read at 620 nm on an ELISA plate reader (Multiskan Biochromatic 348, Labsystems). The standards were used to construct a curve relating absorbance to amount of protein (μg) present.

For more concentrated protein samples, standards of 0.05-0.5 mg/ml protein were produced. Standard or test extract (10 μl) was added to 200 μl of dye reagent previously diluted in four volumes of water and filtered through Whatman No. 1 paper. Incubation and measurement were identical to that described previously.

2.2.6 Electrophoresis

Unless otherwise stated, recipes for buffers described in this section are given in Appendix I, and gel recipes in Appendix IV.

A Electrophoresis equipment

Isoelectric focusing (IEF) was performed using a Pharmacia LKB 2117 Multiphor II Horizontal Electrophoresis Unit, with a LKB 2303 Multidrive XL 3.5 kV Power Supply. The temperature was maintained at 4°C by a LWG MacDonald Refrigeration unit, with 20% (v/v) polyethylene glycol as the circulating refrigerant.

Sodium dodecyl sulphate (SDS) denaturing gels and non-denaturing native gels were cast and run in a Mini-Protean II Slab Cell (Bio-Rad). The LKB Multidrive or a Solsat Electrophoresis power system was used.

Glycol chitin overlay gels (0.01% (w/v), 200 µm thick, 110 x 245 mm) were produced using a LKB 2217-200 Ultramould gel casting kit.

B Gels, buffers, and protein markers

Precast 150 µm thick, 125 x 125 mm pH 3-10 Servalyt Precotes (Serva) were used for IEF. These were cut in half when a small number of protein samples was applied. Anode fluid 3 (No. 42984) and cathode fluid 10 (No. 42986, Serva) were applied to paper electrode wicks (Serva). Serva pI 3-10 protein test mixture (No. 39211) was used as a protein standard.

The Laemmli (1970) system, adapted for use with slab gels, was used for denaturing electrophoresis. Moulds were prepared as described in the Mini Protean manual, except that plastic Gel-Fix (Serva) was attached to the longer glass plates to facilitate gel handling during staining. Polyacrylamide resolving gels (T=12%, C=2.7%) and stacking gels (T=4%, C=2.7%) were cast using electrophoresis grade acrylamide and methylene-bis-acrylamide (Bio-Rad). β-mercaptoethanol was omitted from the sample buffer. Low molecular weight range SDS-PAGE standards (No. 161-0304, Bio-Rad) were used.

Low pH discontinuous native slab gels and buffers were prepared using the method described by Blackshear (1984). Riboflavin (BDH) was used as the stacking gel catalyst. Carbonic anhydrase (Sigma) and trypsinogen (Promega) markers, and low range protein molecular weight markers (V524A, Promega) were used.

Glycol chitin overlays were used in conjunction with IEF, native and SDS gels to detect bands with chitinolytic activity. The mould was prepared by attaching a special plastic film (GEL-FIX for PAGE, Serva) to the longer glass plate of the LKB Ultramould gel casting unit with a thin water layer. A 0.2 mm thick spacer was placed on top and secured in a flat position with plastic side clips. One side of the shorter glass plate was covered in a liquid called Plot (Serva), diluted 60-fold in water, which prevents gel adherence to this upper plate. Once dry, the glass was placed treated side downwards on the spacer to the right of the spacer window.

Substrate (20 ml, sufficient for 2 overlays) was prepared by mixing 0.25 ml of 1% (w/v) pH 4 glycol chitin (see Section 2.2.2, Part A) with 19.75 ml of pH 5 McIlvaine buffer. Two millilitres of fresh acrylamide stock (T=40%, C=2.7%) and 8 ml of substrate were mixed and degassed for ten minutes in a sealed Buchner flask under vacuum, then 80 µl of fresh 10% (w/v) ammonium persulphate (Bio-Rad) and 20 µl of N,N,N′,N′-tetramethyl-ethylenediamine (TEMED, research grade, Serva) were added. The gel was then poured into the spacer window, whilst simultaneously sliding the short glass plate across the window. The gel was left to set overnight, with a weight on top of the mould. Glass plates were subsequently removed and the gel surface was covered with non-stick plastic (GEL-FIX for covers, Serva). The gel sandwich was stored in a sealed bag at 4°C until required.

C Sample/standard preparation and application

IEF samples were applied using a clean dry silicone rubber applicator strip (Serva) in the centre of the gel. Sample volumes of up to 10 µl were applied to wells in the applicator strip. Best results were obtained when defrosted samples were first vortexed to resuspend and mix the contents, then centrifuged at 11,000*g* for 5 min to remove any undissolved material that would otherwise cause streaking. One microlitre of protein test

mixture 3-10 (No. 39211, Serva), diluted three-fold in water, was applied.

For SDS gels, concentrated extracts were diluted 1:4 with Laemmli (1970) sample buffer. Extracts in sample buffer were then heated to 95°C for five minutes to denature the proteins. For native gels the heat step was omitted, water was substituted for SDS and β-mercaptoethanol, and methylene blue was used instead of bromophenol blue in the sample buffer. Samples were centrifuged (11,000*g*, 5 min) to remove any precipitate that would cause streaking, and 20 µl of supernatant was applied to each well in the stacking gel. Less concentrated samples, used in early SDS gels, were freeze dried (5PS Speedivac centrifugal freeze drier, Edwards High Vacuum Ltd) and directly resuspended in 20 µl of SDS sample buffer. SDS and native molecular weight standards were also diluted 1:4 in sample buffer, but only about 5 µl of this mix was applied to the gel.

D Running conditions

Current passing through IEF gels was adjusted to produce an initial voltage of 200 V. As resistance increased, the voltage required to maintain this current rose, until reaching the preset power value of 4 W for full sized gels, and 2 W for mini gels. Running conditions were then maintained at this constant wattage. Voltage continued to rise as the current fell, and the run proceeded until both were constant (usually 2.5-3.5 h). The final plateau voltage was typically 1600-1800, and the final accumulated V.h value at least 2500.

SDS gels were run at 200 V constant voltage for 45 min or until the bromophenol blue dye front had reached the bottom of the gel. Native gels received 30 mA constant current per gel, and runs continued until the methylene blue dye front had reached the bottom of the gel (usually 2-3 h).

Once electrophoresis had finished, glycol chitin overlays were applied like a blotter and were left in contact for 45 minutes-1 hour at 4°C. Since the gels received no current or voltage, proteins moved into the overlay from the underlying gel by diffusion.

E Gel staining

Proteins in slab gels were stained using the method of Merril et al. (1981), using reagents from the Bio-Rad Silver stain kit (No. 161-0443) and the Bio-Rad recommended protocol (refer Appendix V). During each step, the gel and bathing solution were shaken gently in a glass container on an orbital shaker. IEF gels received an extra preliminary 1 hour wash step in 30% (v/v) methanol, 10% (w/v) trichloroacetic acid (TCA), 3.5% (w/v) sulfosalicylic acid (SA, Analytical reagent, May and Baker) in order to remove ampholytes before silver staining.

Coomassie blue protein staining was also used for samples with higher protein content. The gel was stained for 1 h with 0.1% (w/v) Coomassie blue R-250 (Bio-Rad) in fixative (30% (v/v) methanol, 10% (v/v) acetic acid). It was destained by gentle agitation for 3 h in 30% (v/v) methanol and 10% (v/v) acetic acid, followed by overnight incubation in 7% (v/v) acetic and 5% (v/v) methanol plus Zerolit DM-F (BDH) granules, which adsorb the leached stain.

Glycol chitin overlays were stained for 10 minutes in a fresh 0.01% (w/v) solution of Calcofluor white M2R (Fluorescent brightener 28, Sigma) in 500 mM Tris-HCl buffer, pH 8.9 + 0.02% (w/v) azide. The gel was then rinsed and left in water for 15 minutes, and viewed in a Chromato-vue light box under long wave UV light at 350 nm.

F Rotofor

Electrophoretic separation was also carried out in solution, using a Rotofor cell (Bio-Rad) and the LKB 2303 Multidrive XL 3.5 kV power supply. Temperature was maintained at 5°C by a LWG MacDonald refrigeration unit. The central chamber in the cathode was covered with 0.1 M sodium hydroxide (Univar, Ajax Chemicals), and 0.1 M phosphoric acid (Univar, Ajax Chemicals) was placed in the anode compartment. The central focusing chamber was initially filled with Milli-Q water, which was removed at the end of the wash cycle, signalled by a constant current of 2 mA.

Crude extract was dialysed against Milli-Q water overnight to remove excess buffer salts. (The conductivity of the sample applied to the Rotofor should be 50 μS or less).

It was sometimes necessary to centrifuge dialysed samples to remove precipitates. Ampholyte solution - Biolyte pH 3-10 (Bio-Rad), or Servalyt pH 5-8 (Serva) - was added to the extract to give a final ampholyte concentration of 1-2%. The central focusing chamber was filled with 55 ml of the extract/ampholyte mix.

Electrofocusing proceeded at 12 W constant wattage until the voltage had stabilised (4-6 h). At the end of the run, 20 samples were harvested from the separate compartments in the focusing chamber. These were applied to IEF gels to view the effects of separation, using protein staining and activity gel overlays.

2.2.7 Lyophilisation

Samples used on gels and in protein purification were concentrated by freeze drying extracts and resuspension in reduced solvent volumes. Lyophilisation was also used for long term sample storage.

Small samples (0.2-1 ml) were frozen in Eppendorf tubes. Open tubes were placed in a 5PS Speedivac centrifugal freeze drier, and brought down to dryness over a period of 2-12 h.

Larger volumes were lyophilised in a Multi-Dry TM freeze drier (Model FD-5-84, FTS Systems). Ten millilitres of extract were placed in a 50 ml Falcon tube, and the tube was capped with aluminium foil to prevent sample loss if bumping occurred in the freeze drying process. Four to five holes were punched in the cap with a pin to allow vapour loss, and the tube was snap frozen in liquid nitrogen before placement in the freeze drier. Samples were dried for > 24 h until completely dehydrated.

2.2.8 Protein Purification

A General information

At each step of the purification process, fractions containing chitinases were identified using the Calcofluor petri dish assay. These fractions were pooled, concentrated (refer Section 2.2.7), buffer exchanged when necessary (see (B) below), and passed on to the next step in purification. Fraction composition was assessed using SDS and IEF gels and

overlays. Quantitative measures of chitinase activity and total proteins were provided by the Molano et al. (1977) radioactive and Bradford (1976) assays.

B Buffer exchange

Buffer exchange of small sample volumes (2-3 ml) was achieved by placing the sample in a 10,000 molecular weight cut off (MWCO) "Slide-a-Lyzer" cassette (Labsupply Pierce) and dialysing overnight at 4°C against the desired buffer. Alternatively, an Econo-Pac P6 cartridge (6,000 MWCO, Bio-Rad) was used. The P6 cartridge was equilibrated, according to the Bio-Rad Econo-Pac P6 instruction manual, using degassed, filtered (0.45 μm) buffers. Extract (3 ml) which had been filtered (0.45 μm), was slowly injected onto the column, and immediately the eluate was collected. The first 1.5 ml collected was discarded as this was the void volume. The next 3 ml contained the buffer exchanged sample.

Buffer exchange of large sample volumes (> 3 ml) was achieved using 10 mm diameter dialysis tubing (10 kDa MWCO, Union Carbide), tied at both ends to form a bag, and suspended in a measuring cylinder containing the exchange buffer and a magnetic stir bar. Dialysis took place overnight at 4°C.

C Chromatography

Ion exchange was performed using Bio-Rad Econo-Pac ion exchange cartridges. The Q cartridge was used for anion exchange with degassed, filtered (0.45 μm) 20 mM Tris-HCl buffer, pH 8, whereas 20-50 mM NaOAc buffer, pH 5 was used in conjunction with the S (cation exchange) column. Proteins bound to the columns were eluted with a linear gradient (0-1 M) of sodium chloride (Sigma). A Phenomenex BioSep SEC-S3000 250 x 10 mm HPLC column, with guard column, was used for gel filtration. Chromatography was controlled either by a Hewlett Packard Ti-series 1050 HPLC or by an automated Bio-Rad Econosystem. The HPLC was fitted with a titanium Rheodyne sample injector (Model 7125, Alltech). HPLC Buffers were filtered (0.45 μm) and degassed with helium. The Hewlett Packard Multiple Wavelength detector monitored protein content at 218 and 280 nm in the fractions being collected.

2.2.9 Antibody Studies

A Enzyme linked immunosorbent assay (ELISA)

An indirect ELISA system was used (Voller et al. 1979). Crude or purified kiwifruit extract was diluted in 0.1 M sodium carbonate buffer, pH 9.6 (Appendix I), and 100 µl of serially diluted antigen was applied to wells of a flat bottomed microtitre plate (Nunc Maxisorp). The plate was capped and left overnight at 4°C to allow for antigen adsorption. The plate received three 5-10 min washes in phosphate buffered saline-Tween 20 (buffer composition described in Appendix I), before 200 µl of 0.1% (w/v) polyvinylpyrrolidone (PVP, Laboratory grade reagent, BDH) was added to every well to block the plate and prevent nonspecific binding. After 90 minutes incubation at 37°C, the plate was washed three times in phosphate buffered saline-Tween 20 (PBS-Tween). Chitinase antibodies, serially diluted in PBS-Tween, were applied to the wells and incubated at 37°C for 1 h. Following three PBS-Tween washes, 100 µl of biotinylated goat-antirabbit antibody (Amersham), diluted 1000x in PBS-Tween, was added to each well. After 1 h at 37°C, the plate was washed in PBS-Tween and 100 µl of Streptavidin biotinylated horse radish peroxidase complex (Amersham), diluted 1000x in PBS-Tween, was added to each well. The plate was incubated 30 min at 37°C, then washed exhaustively (4x 10 min washes) in PBS-Tween. Freshly prepared substrate solution (0.1 ml/well) ^ containing 5 mg *o*-phenylenediamine dihydrochloride (GibcoBRL, Life Technologies), and 10 µl 30% (w/v) hydrogen peroxide (H_2O_2, Analar, BDH) in 20 ml citrate-phosphate buffer, pH 5 was added and incubated at room temperature. The reaction was stopped after 7-15 minutes by addition of 50 µl 0.5 M sulphuric acid, and the absorption of sample and control wells was recorded at 492 nm.

"No antibody" control wells were treated identically to other wells (i.e. received antigen, biotinylated goat-antirabbit antibody and horse radish peroxidase complex, substrate, etc), except that PBS-Tween buffer was substituted for the antibody raised to chitinase. "No antibody, no antigen" controls contained buffer only.

Five polyclonal chitinase antibodies were tested. Three antibodies raised to sugar beet chitinases - two basic (CH2 and CH4) and one acidic (SP), were generous gifts from Dr. Jorn Mikkelsen, Danisco Biotechnology, Copenhagen. A basic cucumber chitinase

antibody was kindly gifted by Dr. Koko Nakajima, Research Institute of Life Science, Japan. An antibody raised to the chitinase of *Janthinobacterium lividum*, a bacterium isolated from the gut of New Zealand grass grub by Dr. Dave Greenwood, Mt Albert Research Centre, Auckland, was also tested. In addition to ELISAs on kiwifruit antigens, the chitinase antibodies were also applied to bean chitinase, purified according to the method of Boller et al. (1988), and a bacterial endochitinase from the gut of *Janthinobacterium lividum* (Greenwood et al. 1993).

B Western blotting

Following SDS electrophoresis, gels were blotted onto nitrocellulose membranes (Hybond-ECL, Amersham) in a Bio-Rad Mini Trans-blot Electrophoretic Transfer Cell, as per the instructions in the Bio-Rad Transblot instruction manual. Blotting took place at 100 V over 60 min in 25 mM Tris, 192 mM glycine (Electrophoresis purity, Bio-Rad), 20 % (v/v) methanol, pH 8.3 buffer. The membranes were then transferred into 0.1 % (w/v) PVP in PBS-Tween and incubated with gentle stirring at room temperature overnight. Following the blocking step, ELISA proceeded as described above, except that reagent was applied to membranes in a container rather than to wells of a microtitre plate, all incubations were performed at room temperature, and a different substrate solution was used. This solution comprised 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (GibcoBRL, Life Technologies) in 25 ml of pH 7.4 PBS (without Tween) plus 25 μl of 30 % (w/v) H_2O_2.

Substrate incubation was carried out in the dark for 5-15 min. The reaction was stopped by dilution in PBS-Tween and the membrane was washed numerous times in PBS-Tween until all traces of substrate were removed.

2.3 STATISTICAL ANALYSIS

The purpose of this section is to familiarise the reader with basic statistical techniques used in this study, and to provide justification for procedures such as data

transformation. Frequently used statistical terms are highlighted. All statistical data analyses were carried out using the SAS system (1990). Sample SAS analyses are given in Appendix VI.

2.3.1 ANOVA And The Need To Transform Data

Experiments were carried out according to standard experimental designs (for example, randomized block design, split plot and nested). An Analysis of Variance (**ANOVA**) was then performed, followed by **multiple comparison tests** to ascertain treatment differences. A full ANOVA table (also associated with the results presented in Chapter 5, Section 5.2.3., Part A, (i)) is shown below:

Table 2-1: ANOVA of the effect of first incubation time on absorbance (raw data) in the Boller et al. (1983) assay.

Source	DF*	SS†	MS‡	F-Ratio	P-Value
TIME	5	0.0276	0.0055	7.70	0.0214
ERROR	5	0.0036	0.0007		
TOTAL	10	0.0312			

* Degrees of freedom,
† sum of squares,
‡ mean squares

However, within this thesis only the information from ANOVA tables regarding treatment significance, i.e. only the P-values are presented, where $P<0.05$ is considered to be significant.

Basic requirements for an ANOVA are that:
1) variability is homogeneous among treatments, and
2) responses are normally distributed for each treatment.

When these conditions are violated, the usual procedure is to transform the data so that the transformed values satisfy the requirements, at least approximately (Mead & Curnow 1983). In this study, data from some experiments needed such transformation before

analysis, while ANOVA could be carried out on raw data in other cases where the required conditions of ANOVA were already satisfied. When transformed data were used, results (multiple comparison tests, graphs and tables of means, etc) are presented in terms of these transformed values. Note that in such cases presenting the corresponding means etc. of the raw data may provide misleading interpretation. For example, the magnitude of differences between "raw" means in Fig. 2-2 appears large, but the corresponding differences between the "transformed" means (Fig. 2-2) may not be statistically significant. Note that transformations are chosen such that ranking of treatment effects remains the same (i.e. if "raw" mean of treatment A is larger than that of treatment B, then this order is conserved in the "transformed" data, eg. Fig. 2-2).

2.3.2 Multiple Comparison Tests

When the treatment effect is found to be significant (in ANOVA), multiple comparison tests are carried out to look at pairwise differences between treatments, using either Fisher's Least Significant Difference (**LSD**) for experiments with equal sample sizes (**balanced data**), or Duncan's Multiple Range test for **unbalanced data** (unequal sample sizes). However, "least squares means" (**LSMeans**) test and linear contrasts were also used in some cases. All tests were carried out at the 5% level of significance, i.e. $P<0.05$ indicates a significant difference. Duncan's Multiple Range test was only applicable to **main effect** (refer Section 2.3.4) treatment comparisons and not **interactions** (described in Section 2.3.4).

2.3.3 Standard Error Bars

A measure of experimental variability on graphs in scientific papers is often provided by treatment standard error bars. This method was not adopted in this thesis because these standard error bars are incorrectly used to determine significant differences between treatments. Standard error bars overestimate treatment differences, in that non-overlapping error bars might indicate that two treatments are significantly different, when in actual fact they are not, as a proper statistical comparison test such as Duncan's might suggest. The main statistical purpose of standard error bars is to indicate individual treatment variability. However, if the assumptions of ANOVA have been fulfilled and the data is "balanced", all variances and hence standard error bars should

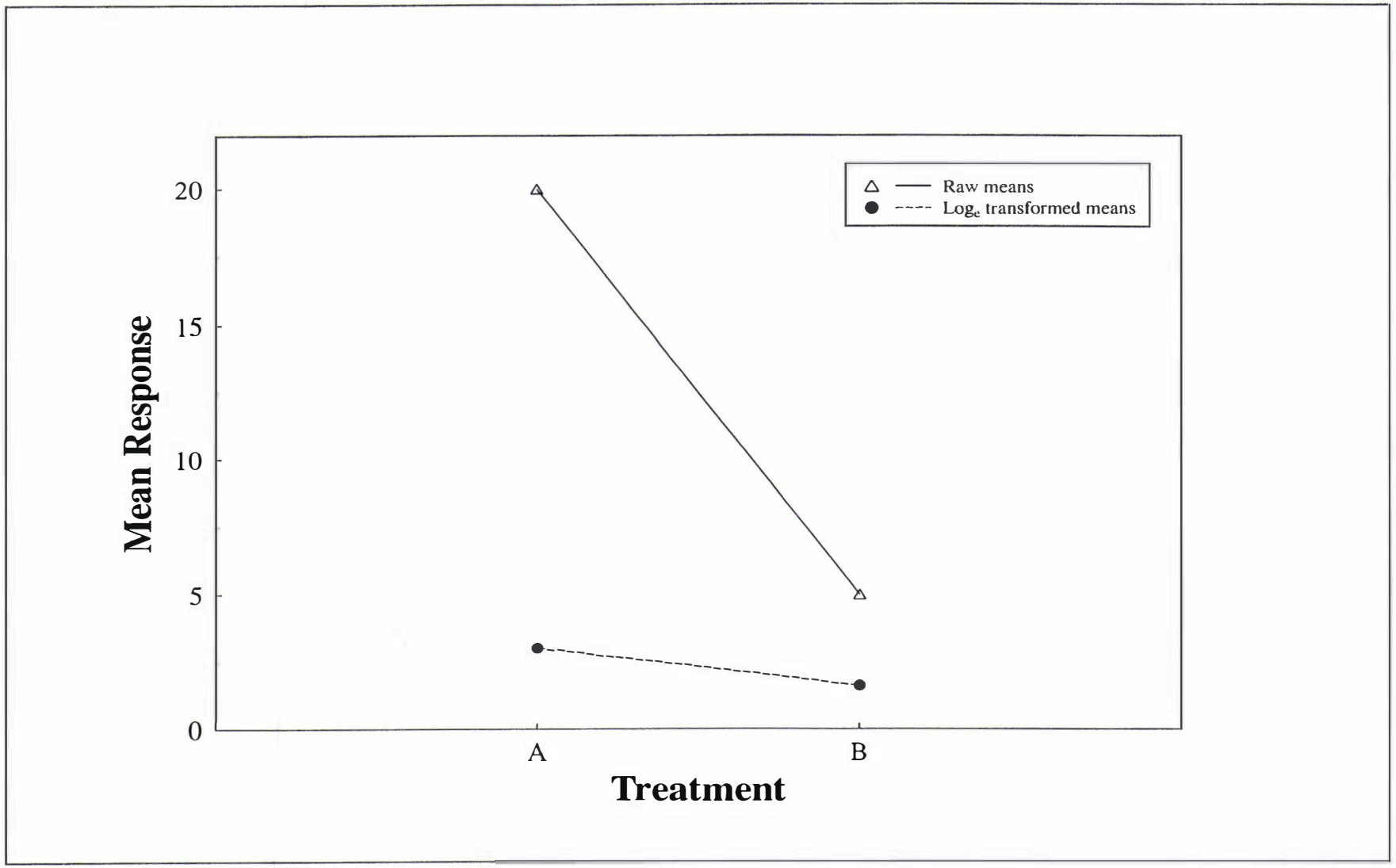


Figure 2-2. Comparison of treatment means before (solid line) and after (dashed line) a $\log_e$ transformation.

be approximately the same size. (Note that standard error bars are inversely proportional to sample size, and therefore error bars from treatments of different sample sizes are not directly comparable.) Provided the data is "balanced", the appearance of vastly different sized standard error bars on a graph merely indicates that variances are heterogenous among the treatments and the data require a transformation. Treatment differences on graphs in this thesis are indicated by a LSD bar in the case of balanced data, and Duncan's Multiple Range test for "unbalanced" data. In the latter case, different letters on graphs represent significant differences.

2.3.4 Experimental Designs

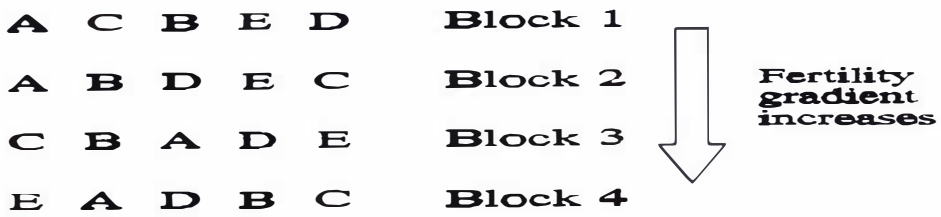
Experimental designs used in this study were completely randomized, randomized block, factorial, split plot, nested and repeated measures designs. A brief description of each design follows. The format of ANOVA tables varies for each type of design, and example formats along with more detailed information on each design are found in Ott (1993).

A Completely randomized design (CRD)

CRD represents the simplest experimental set-up, where t treatments are randomly applied to homogeneous experimental units and no restrictions are placed on the allocation of these treatments.

B Randomized block design (RBD)

In a **RBD** design, experimental units are first grouped in such a way as to block out some external source of variation that may otherwise confound treatment effects. Each treatment is then randomly applied to one experimental unit within each group or block. For example, a soil fertility gradient within a field may influence the results in an experiment measuring yields from five different crop varieties. To overcome this problem, the five varieties are randomized within b blocks such that each block represents a different soil fertility and each treatment appears once within each block i.e.



C Factorial

In a **factorial** design, the effects of two or more experimental factors on a response y are assessed. This is in contrast to the previous designs where different levels of just one experimental factor are considered. In a factorial experiment there are two types of treatment effects:

1) **main effects** due to individual experimental factors, and
2) **interaction** between the experimental factors.

These are best explained using an example. In an experiment measuring yield of two wheat cultivars (A and B) at two different fertiliser concentrations (low and high), several different scenarios are possible. The first possibility is that the cultivar and fertiliser concentration factors do not interact (Fig. 2-3, A), i.e. the effect of wheat cultivar on yield is the same regardless of fertiliser concentration. In this case, it is possible to interpret "main effects", i.e. overall, Variety A produces higher yield than Variety B (Fig. 2-3, B), and yield increases with fertiliser concentration irrespective of cultivar (Fig. 2-3, C). A second possibility is shown in Fig. 2-4, A, i.e. an "interaction" between factors exists, namely yield of cultivar A decreases with increased fertiliser concentration, and vice versa for cultivar B. In this case some "main effects" comparisons are still sensible, i.e. A is always the higher yielding cultivar irrespective of fertilser (Fig. 2-4, B). Fig. 2-5 represents a third situation where there is a significant "interaction" between factors, but "main effects" interpretation is likely to be misleading. For example, the yield of cultivar A is not always higher than cultivar B, and must be related to the level of fertiliser used.

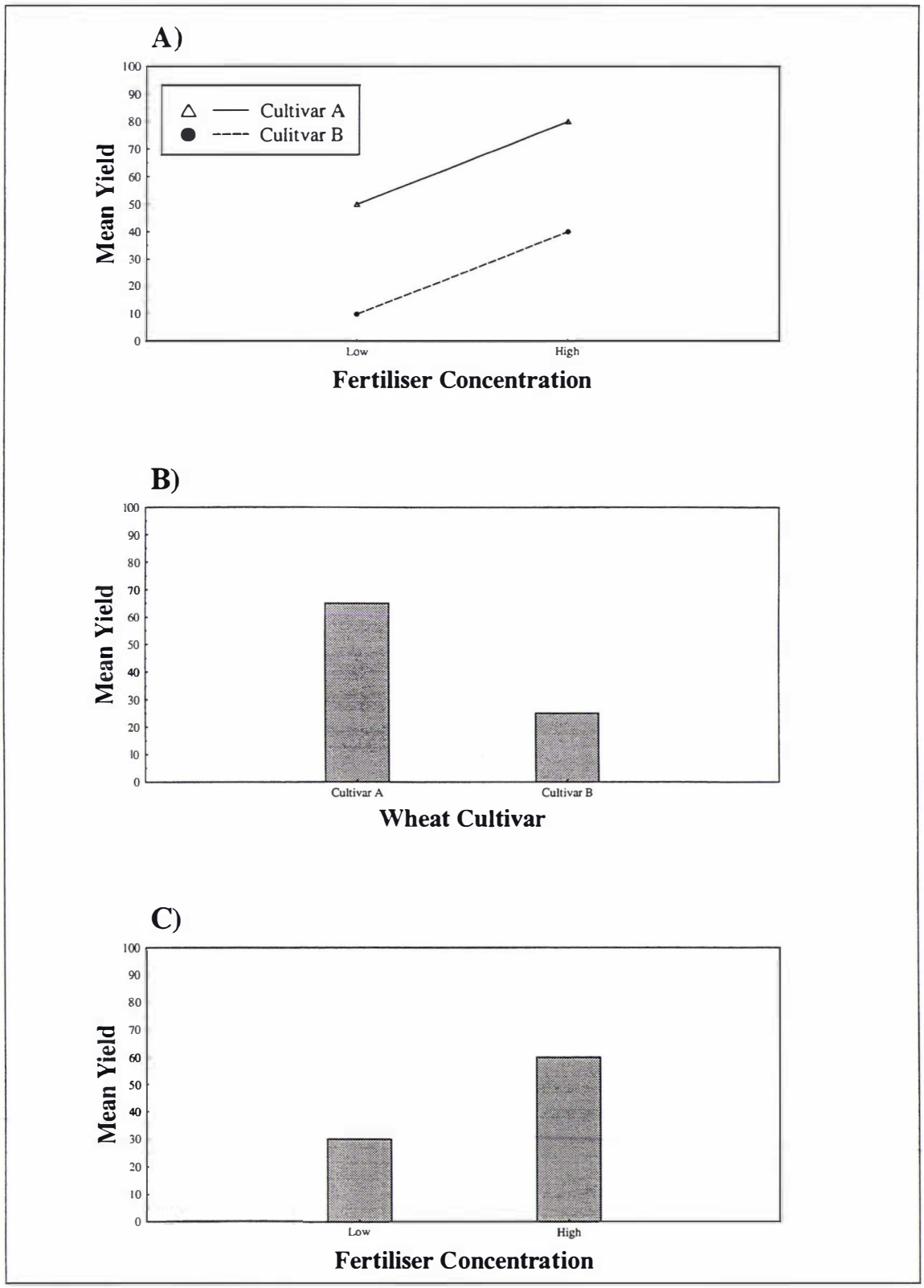


Figure 2-3. Mean yield as influenced by A) wheat cultivar and fertiliser concentration, B) wheat cultivar averaged over fertiliser concentration and C) fertiliser concentration averaged over wheat cultivar.

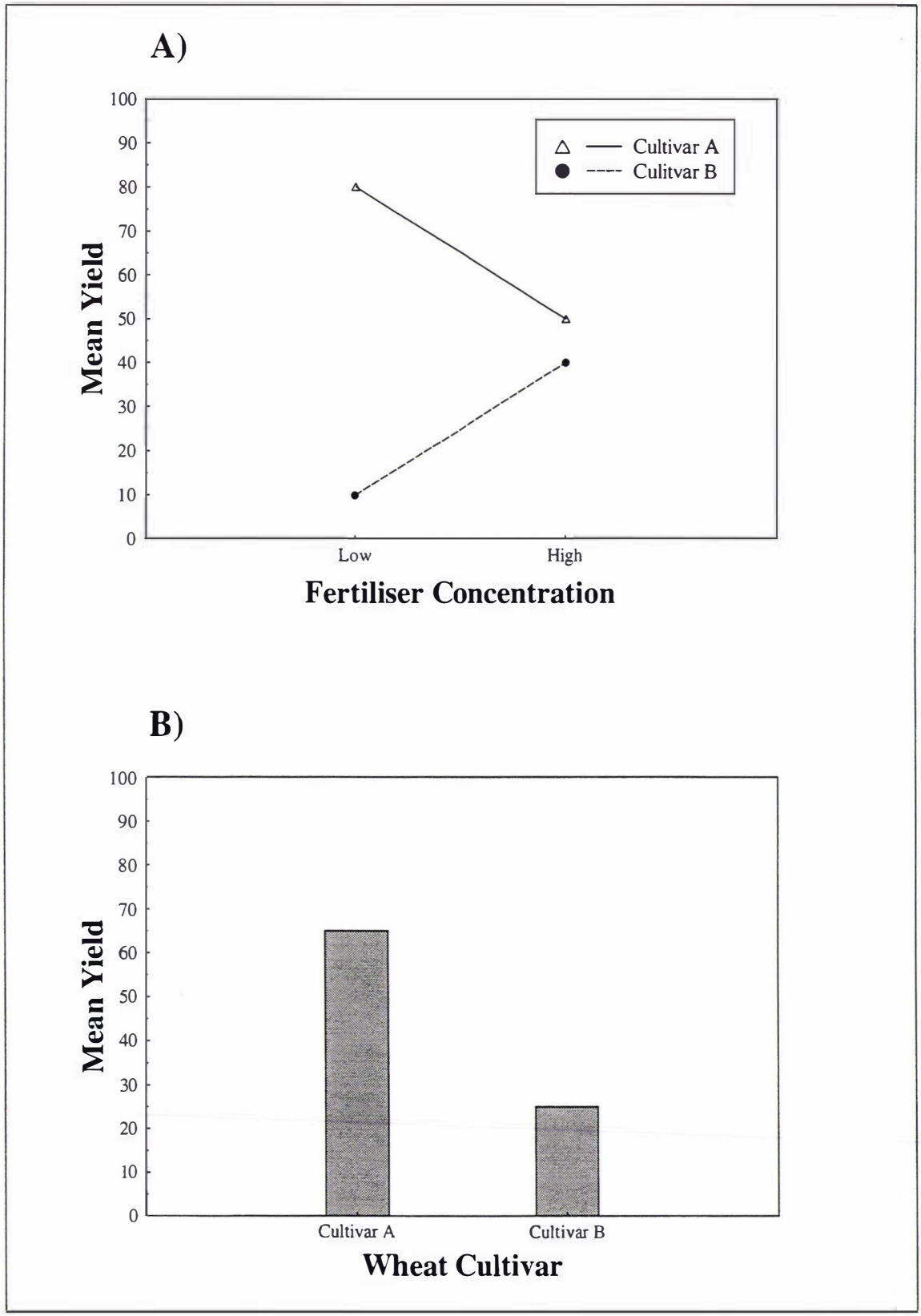


Figure 2-4. Mean yield as influenced by A) interaction between wheat cultivar and fertiliser concentration, and B) wheat cultivar averaged over fertiliser concentration.

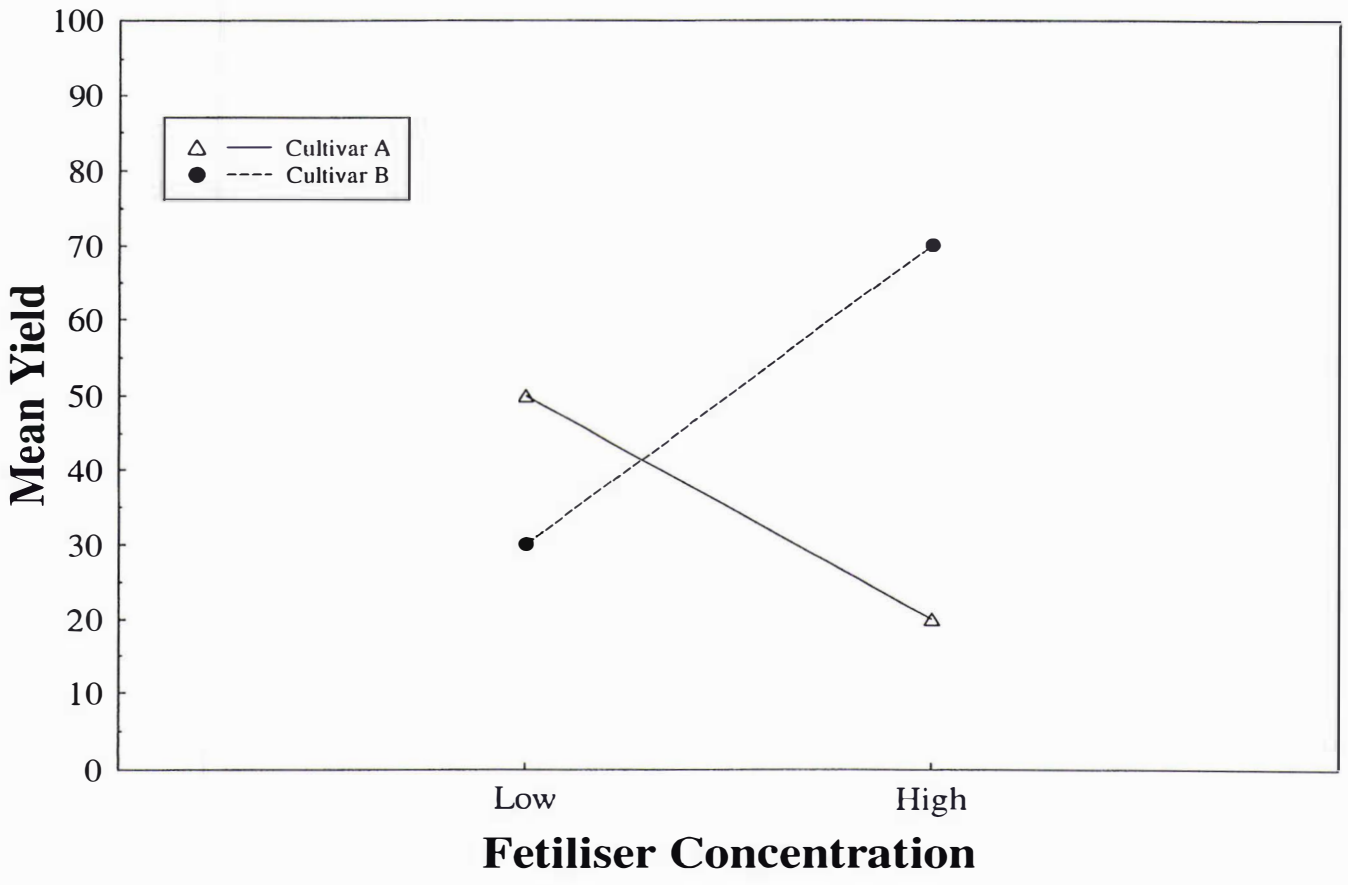


Figure 2-5. Mean yield as influenced by interaction between wheat cultivar and fertiliser concentration.

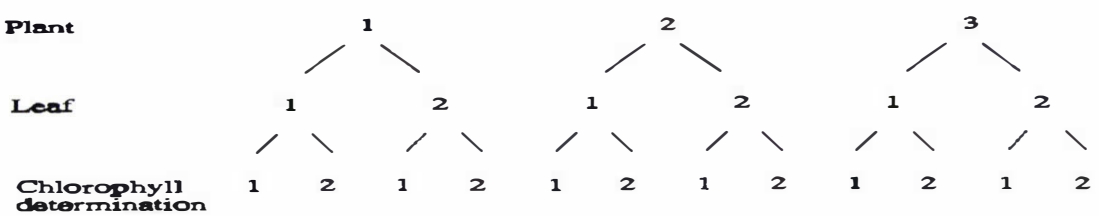
If the "interaction" effect is significant ($P<0.05$ in ANOVA), then the nature of the interaction should be explained first. Providing that "main effects" are significant ($P<0.05$), and the corresponding F-ratios in the ANOVA table are larger than the "interaction" F-ratio, interpretation of "main effects" is also meaningful (Mead and Curnow, 1983). Thus the interpretation of the "main effects" should follow that of the "interaction". This order of presentation was followed throughout this thesis for all experimental designs involving interaction and main effects (factorial, split plot and repeated measures).

D Split plot

Split plot experiments involve two or more experimental factors like a factorial design, but the size of experimental units appropriate for one factor is not practical or suitable for the other factor. As a consequence, treatment allocation takes place in two stages. First, one factor (the **main plot** factor) is randomly applied to large units, then the other factor (the **split plot** factor) is randomly applied to smaller units within each of the large units. As in the factorial case, this design also consists of main effects of "main" and "split plot" factors and the interactions between "main" and "split plot" factors.

E Nested

A **nested** experiment is one where each factor is nested within another factor. In other words, experimental factors are allocated in a hierarchical manner. For example, consider an experiment where two measurements (determinations) of leaf chlorophyll are made on each of two leaves from three different plants. The measurements are unique to each leaf, which in turn is unique to each plant i.e.



In this experiment, leaves are said to be nested within plants and determinations nested within leaves. The hierarchical nature of this design prevents interaction comparisons. For example, the interaction between plants and leaves cannot be tested because variation between leaves within a plant is not consistent with the variation between leaves within another plant. Therefore, leaves 1 and 2 from plant 1 can be compared with each other, but not with leaves 1 and 2 from plant 2.

F Repeated measures

Repeated measures is an experimental design where a response is measured on the same experimental unit at several different times. Interaction and main effects comparisons are possible, but analysis is considerably more complicated than that of a factorial design and the reader is referred to Ott (1993) for more details.

3 HOST AND INOCULUM FACTORS AFFECTING DISEASE INCIDENCE RESULTING FROM ARTIFICIAL INOCULATION

3.1 INTRODUCTION

3.1.1 Why Artificial Inoculation?

Botrytis cinerea is a major postharvest pathogen of kiwifruit (Poole & McLeod 1992), but natural infections are intermittent and variable (Beever 1992). This unpredictability presents problems for researchers trying to draw valid conclusions from experiments. In order to gain an understanding of the interaction between the pathogen, its host and the environment, and to identify factors which influence pathogen infection and survival, it is important to have reasonable levels of infection. Artificial inoculation is an important tool in pathological research because it can be used to overcome the problems of low, inconsistent infection.

In the past, artificial inoculation of kiwifruit with *B. cinerea* has been beset by the same problems associated with natural infection (Pyke 1991; Beever 1992). There is consequently a need to identify those factors required for successful infection so that techniques for artificial inoculation of kiwifruit with *B. cinerea* can be standardised, thereby reducing the variability previously associated with these types of experiments. Identification of these factors will also make it possible to reduce experimental costs, because less fruit will be required to detect treatment differences, and will highlight those factors which need to be avoided in a commercial situation in order to achieve control of the pathogen.

Factors which influence the success of infection can be divided into 3 major areas (the effect of the pathogen, the environment and host resistance), as described by the "Disease Triangle" (Agrios 1988). The artificial inoculation trials reported in this chapter examine host and inoculum factors which influence infection. Host factors were the

focus of the present study because of the potential to enhance fruit resistance, thereby minimising the use of agrochemicals, and because of a personal interest in plant defence mechanisms. Early work indicated that fruit maturity, fruit xylem pressure as measured by water potential (Ψ), and the age/condition of the picking wound were host factors that warranted further study (Pyke 1991).

3.1.2 The Effect Of Maturity

Physiological changes associated with maturation may be accompanied by changes in a plant's defence response to infection. Susceptibility to pathogen attack is influenced by maturity in many plants, including apples (Sitterlay & Shay 1960), avocado (Prusky et al. 1985), squash (Sharrock & Parkes 1990), and tomato (Lavy-Meir et al. 1989). Work by New Zealand scientists has indicated that maturity may also be important in kiwifruit. Brook's (1990a) review described experiments done by Beever in 1981, and by Pennycook and Manning in 1989, where uninoculated kiwifruit became less susceptible to infection as they matured. However, similar work using inoculated fruit (Pennycook & Manning 1982 unpubl. data) showed no increase in resistance as fruit matured (Brook 1990a). Further work is required to determine whether artificial inoculation had overcome any inherent resistance.

Many factors such as environmental conditions at the time of harvest, and presence or absence of sepals can influence infection (Blakeman 1980; Pyke 1991). Since it is not known how such factors varied between the experiments described previously, it is difficult to draw overall conclusions about the effect of maturity. In this work, a standardised artificial inoculation design was used. In addition, harvest dates with similar environmental conditions were selected to reduce variability associated with external factors, and to thereby produce more consistent results.

3.1.3 The Effect Of Plant Water Status

Over three years of harvests (1985-88), Pennycook and Manning found a close correlation between vine xylem pressure, measured by water potential in detached leaves, and the amount of stem end rot in uninoculated fruit. The lowest levels of infection coincided with the highest leaf water potentials experienced in the early afternoon

(Brook 1990a). On the basis of these observations, it is worth investigating whether harvest time is an important determinant of the success of artificial inoculation experiments.

3.1.4 The Condition Of The Picking Wound

Curing, a process in which picking wounds are left for a period of time to allow wound repair and initiation of plant defence mechanisms, increases fruit resistance. It is well documented that curing kiwifruit for periods of twenty-four hours or more allows sufficient time for fruit defence mechanisms to swing into action to produce a significant reduction in infection (Pennycook & Manning 1992). However, other work indicates that changes can occur more quickly (Poole & McLeod 1994). This has serious implications for artificial inoculation experiments because there are often delays of several hours between harvest and coolstorage, during which time physiological changes that may affect results can occur. Further investigation is warranted to determine if high levels of infection can be achieved by inoculating fresh wounds.

3.1.5 Nutrients At The Stem Scar

The ability of *Botrytis cinerea* isolates to germinate in water varies greatly (Blakeman 1975; Clark & Lorbeer 1977), and the presence of exogenous nutrients on the plant surface can stimulate both the germination and growth of germ tubes markedly, provided that the epiphytic microflora do not actively compete for nutrients (Blakeman 1975). Availability of nutrients on the fruit surface may be one factor responsible for variable infection levels. The importance of nutrition for successful artificial inoculation needs to be examined.

3.1.6 Silicon Application

The application of silicon to cucumber roots reduced the severity of fungal attack through stimulation of plant defences (Cherif et. al. 1994). However, induction of resistance by means of elicitors prior to fungal infection can divert energy from normal processes and result in yield losses (Sequeira 1990). Silicon elicitation offers the advantage of inducing plant defence only in response to pathogen infection (Cherif et al. 1994). The effect of silicon on resistance of kiwifruit to *B. cinerea* is not known.

3.2 OBJECTIVES

The purpose of these artificial inoculation experiments was to examine the effect of:

1) fruit maturity at harvest on host resistance,
2) host water status and condition of the picking wound on susceptibility to infection,
3) the role of inoculum level on disease incidence,
4) the effect of nutrient addition to inoculum on disease incidence, and
5) silicon application on host resistance.

3.3 MATERIALS AND METHODS

In all artificial inoculation experiments, percent infection was assessed as described in Chapter 2, Section 2.1.6). Fruit temperature was measured using a temperature probe attached to a Grant Squirrel 1200 data logger. A box of fruit comprised a single-layer cardboard export tray with a polyethylene liner and plix insert holding 36 fruit.

3.3.1 Experiment No.1

Title : Kiwifruit maturity and susceptibility to Botrytis cinerea infection - 1992 harvest.

Fruit were harvested from a pergola system on a commercial orchard near Wanganui. Three harvests dates were chosen (15 April, 18 May, 25 May), to produce fruit of three distinct maturities within the commercial harvest range (6-12% total soluble solids concentration). Total soluble solids (TSS) was used as an indication of fruit maturity and was measured on a sample of 9-10 fruit from within the orchard at each harvest (see Chapter 2, Section 2.1.5 for details). Mean ± SEM total soluble solids (percent concentration) for each of the successive harvest dates were 5.1 ± 0.1, 8.9 ± 0.3 and 10.3 ± 0.4. Fruit and air temperature and air humidity were also measured at each harvest (refer Appendix VII).

Prior to the first harvest, six vines in each of two adjacent rows in the orchard were

selected and marked with spray paint. Two vine positions were marked on each vine using hanging tags. These positions were defined as:

Vine position 1 (VP1) < 1m from the central leader
Vine position 2 (VP2) > 1m from the central leader

On the first harvest date, each picker started at vine 1 and randomly harvested approximately thirty fruit from within the VP1 area by snapping the fruit off from its stalk into a picking bag. The picker then moved to the next vine and randomly harvested fruit from the VP1 area of the second vine into the same picking bag. Fruit from the VP1 area of twelve vines were thus pooled. The same procedure was repeated for VP2, but using a different picking bag. Fruit were transported back to Massey University Palmerston North. After removing sepals with a soft-bristled nail brush, fruit from VP1 were randomly allocated into five groups of two single-layer export trays (containing 36 fruit each), and the process was repeated for the VP2 fruit. One of five *B. cinerea* spore loadings (0, 1000, 5000, 25000 or 125000 spores/stem scar) was applied to fruit in each group of two replicate boxes at 4.5 h after harvest. Details of inoculum preparation and application are given in Chapter 2, Sections 2.1.2 and 2.1.3. Inoculum droplets were left to dry for approximately 1-2 h before fruit were packed and placed in the coolstore (0 ± 0.3°C). The process was repeated for the second and third harvests. On 25 May there was a longer delay (2.5 h) between finishing the harvest and commencing inoculation, as fewer helpers were available to pick and pack the fruit (refer Appendix VII).

Cumulative percent infection was recorded after 4, 8 and 12 weeks coolstorage.

Graphs of infection (raw data) after different time intervals in coolstorage were used to determine whether treatments affected the time required for disease initiation.

The effect of treatments on the total amount of disease present was assessed using the twelve week percent infection data. The experiment was carried out according to a completely nested fixed effects design (refer Chapter 2, Section 2.3.4), since all the fruit originated from the same unique source, namely the twelve marked vines in the orchard.

Maturity was said to be nested within VP, inoculum within maturity and replicate boxes within all of the above. A square root transformation of data was required to satisfy the assumptions of ANOVA. (Chapter 2, Section 2.3.1 explains in greater detail the rationale behind data transformation.)

3.3.2 Experiment No.2

Title : Kiwifruit maturity and susceptibility to B. cinerea infection - 1993 harvest.

The 1993 maturity experiment was carried out at the Massey University Fruit Crops Orchard, Palmerston North. Vines in this orchard were trained on a winged T-bar system. Harvest dates were chosen to obtain fruit with four distinct maturities. Mean ± SEM total soluble solids (percent concentration) for each of the successive harvest dates were 6.2 ± 0.1, 7.8 ± 0.3, 10.9 ± 0.4 and 12.0 ± 0.3. Fruit and air temperatures and air humidities during each harvest and inoculation are given in Appendix VII. Unlike the 1992 experiment, fruit were randomly harvested from vines throughout the orchard rather than from specific numbered vines.

Fruit with attached pedicels were harvested on four occasions (30 April, 17 May, 6 June, 16 June), and inoculated with 0, 25000 or 125000 spores/fruit immediately following pedicel removal approximately 1 h after harvest. Percent infection was assessed after 4, 8 and 12 weeks coolstorage at 0 ± 0.3°C. Curing was minimised by harvesting fruit with the pedicels attached, and removing the pedicel immediately prior to inoculation. The total time between harvest and coolstorage was 2-3 h, and this curing period was kept approximately constant across all four harvests (Appendix VII).

The effect of treatments on time taken for infection to develop was examined using plots of infection (raw data) after different periods in coolstorage.

Treatment effect on total disease incidence was assessed using the twelve week percent infection data. The experiment was carried out according to a split plot design (refer Chapter 2, Section 2.3.4), where the four maturities were considered as main plot treatments and the three inoculum levels as the split plot treatments. Data were square

root transformed.

3.3.3 Experiment No.3

Title : Kiwifruit water potential at harvest and susceptibility to B. cinerea infection - 1992 harvest.

Fruit without pedicels were collected from a winged T-bar system at Massey University Fruit Crops Orchard on two separate days (13 May, 19 May). Mean ± SEM total soluble solids (percent concentration) were 8.8 ± 0.4 and 10.1 ± 0.4 respectively. On each day seven harvests of four boxes (replicates) of 36 fruit each were made at 2 h intervals. Air temperatures and relative humidities at each harvest interval are given in Appendix VII. Fruit were randomly harvested from the same group of vines at each time interval, but a different set of vines was used on the second day because insufficient fruit remained on the first set of vines. At each time interval, sepals were removed and the fruit immediately inoculated with 5,000 *B. cinerea* spores per stem scar 20-75 minutes after harvest (Appendix VII). All fruit were then placed in coolstore (0 ± 0.3°C) once the inoculum droplets were dry (about 1 h later). Time lapses of approximately 3 h between harvest and coolstorage were kept constant for all treatments in the experiment.

Four boxes of uninoculated controls without pedicels were also collected at 8 am on each harvest day.

Water potential (Ψ) was measured at each time interval on two additional fruit with pedicels attached. Water potential measurements (Boyer 1995) in megapascals (MPa) were made using a pressure bomb (Soil Moisture Equipment Corp., USA). Each fruit was suspended in the apparatus by its pedicel, which was trimmed to 2 cm immediately before measurement.

Graphs of infection (raw data) after various times in coolstorage were used to determine whether the time taken for infection to develop was affected by the day of harvest and/or the harvest time.

The twelve week percent infection data set was analysed as a nested fixed effects design, with time intervals nested within days, to determine whether the harvest time affected the total disease incidence. There was no need to transform the data set because it was normally distributed and variances were homogeneous, i.e. the basic requirements of ANOVA were already satisfied (Chapter 2, Section 2.3.1).

3.3.4 Experiment No.4

Title : Condition of the picking wound and susceptibility to B. cinerea infection - 1992 harvest.

The two parts of this experiment were analysed separately.

A Fresh picking wounds

Fruit with attached pedicels were randomly harvested in fine weather from vines in the Massey University Fruit Crops Orchard on 5/5/92, and were left for 0, 6, 24 h or 6 weeks before inoculation. For the 6 and 24 h time lapses, the fruit remained in ambient conditions but for the six week time lapse, fruit were placed in coolstorage (0 ± 0.3°C) until 24 h before treatment application, when they were moved into ambient conditions to allow adequate time to equilibrate. The average total soluble solids (percent concentration) of the fruit was 6.9 ± 0.2.

There were three treatments:

i Pedicel removal

- the pedicel was completely removed immediately prior to inoculation to create a fresh picking wound, which was inoculated with one drop of spore suspension containing 5000 *B. cinerea* spores.

ii Cut pedicel

- the pedicel was trimmed to 5-10 mm immediately prior to inoculation, and 5000 spores were applied to the freshly cut surface.

iii Wounded scar

- the pedicel was snapped off, and the top 3-4 mm of the stem scar removed with a 5 mm diam drill bit (Plate 3-1). Inoculum was applied immediately after wounding.

Once inoculum droplets had dried (1 h), the fruit were placed in coolstorage. Cumulative percent infection was assessed 4, 8 and 12 weeks after inoculation and plots of this data were used to determine if the time taken for disease to appear was affected by the type of treatment.

Treatment effect on total disease incidence was assessed using the twelve week percent infection data. The experiment was carried out according to a fixed effects nested design, with treatments nested within time lapses. There were four replicate boxes of 36 fruit for each time/treatment combination, and an additional four boxes of controls - uninoculated fruit without pedicels which were placed in the 0°C coolstore immediately after harvest. A square root transformation was necessary to satisfy the assumptions of ANOVA.

B Old picking wounds

Fruit were harvested without pedicels, and were left for 0, 6, 24 h or 6 weeks before inoculation with 5000 spores/fruit and coolstorage as described in Part A. The control fruit used in Part A were also used for this experiment. Percent infection assessments were made 4, 8 and 12 weeks after inoculation. Plots of this raw data were constructed to determine whether time taken for infections to appear was influenced by wound age.

The effect of wound age on disease incidence was assessed using twelve week percent infection data analysed as a randomised block design. Data required a square root transformation.

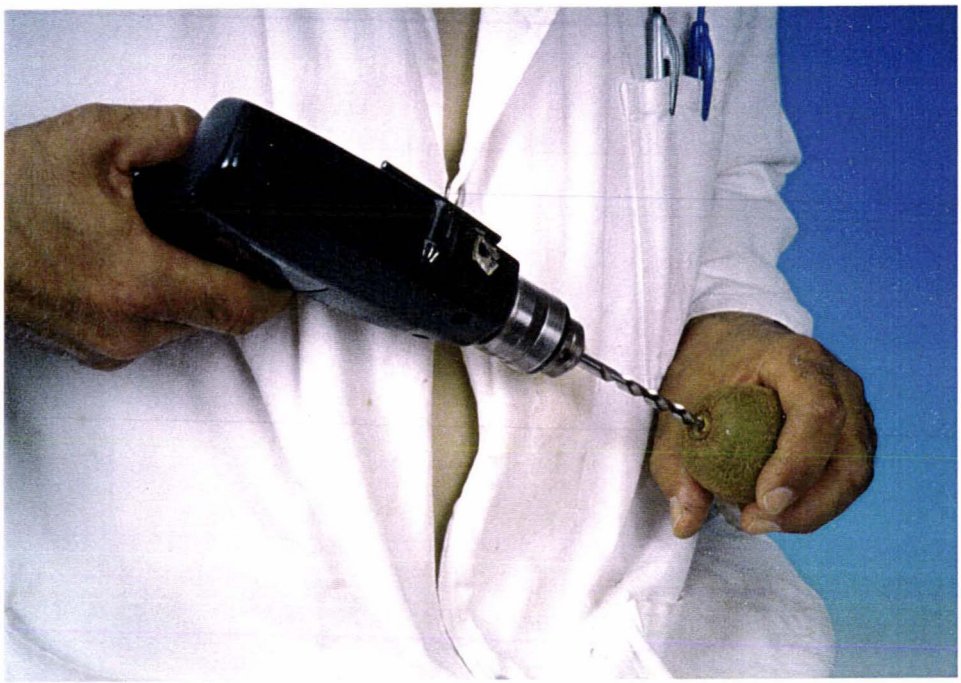


Plate 3-1. Removal of the top 3-4 mm of the stem plug with a 5 mm diam. drill bit.

3.3.5 Experiment No. 5

Title : Inoculum nutrient status and the success of artificial inoculation - 1992 harvest.

A *Botrytis cinerea* spore suspension ($2.9x10^5$ spores/ml), prepared in the usual manner (Chapter 2, Section 2.1.3), was placed in a centrifuge tube and weighed. The suspension was centrifuged for ten minutes at 3000*g*. The supernatant containing nutrients from the agar plate was discarded, and the spores were resuspended in fresh 0.01% Tween 20. The sample was recentrifuged and the precipitate resuspended in 0.01% Tween 20 to half the original weight. The double strength, washed spore suspension was divided into three batches. One batch was mixed 1:1 with a 9% solution of yeast extract (Difco), the second was mixed 1:1 with 9% sucrose (Chelsea), and the third 1:1 with 0.01% Tween 20.

Fruit were harvested without pedicels from pergolas in a commercial Wanganui orchard on 25 May 1992 in fine weather. The average total soluble solids (percent concentration) of the fruit was 10.3 ± 0.4. They were randomly divided into three groups of 108 fruit and inoculated at 7.5 h after harvest with one of the three batches of spore suspension at a rate of 5,000 *B. cinerea* spores per picking scar. The fruit were left in ambient conditions until the inoculum droplets dried (1-2 h), then were packed and stored at 0 ± 0.3°C. Cumulative percent infection was assessed after 4, 6, 10 and 12 weeks coolstorage.

The twelve week data set was analysed as a completely randomised design, with three replicates of 36 fruit per treatment. Data transformation was not required.

3.3.6 Experiment No. 6

Title : Soluble silicon (Si+) application and the success of artificial inoculation - 1994 harvest.

Fruit with attached pedicels were collected from pergola-trained vines in Levin on 23 May 1994. The average TSS of the fruit was 7.0 ± 0.3. Pedicels were snapped off approximately 3 h after harvest, immediately before inoculation with 25,000 or 0 spores

per fruit. Once the inoculum droplet had dried (about 1 h), a single droplet containing 1.7 mM or 0 mM silicon in the form of sodium silicate solution (BDH, 30% SiO_2), diluted in Tween 20, was applied to each stem scar. After the silicon-amended droplet had dried (1 h), the fruit were packed and cured for three days at 20°C before coolstorage. Percent infection was assessed after twelve weeks at 0 ± 0.3°C.

The experiment was carried out according to a split plot design, where the two inoculum loads were considered as main plot treatments and the two silicon levels as the split plot treatments. Data were square root transformed.

3.4 RESULTS

Unless otherwise stated, P-values come from ANOVA.

3.4.1 Experiment No. 1

Some infections appeared within four weeks on inoculated fruit, but not on uninoculated fruit (Fig. 3-1). Disease onset was also accelerated in fruit harvested later in the season (Fig. 3-1, B and Fig. 3-2), although this trend was not evident in uninoculated fruit where there were very low levels of infection (Fig. 3-1, A). There were no consistent patterns between the time taken for infections to occur and the vine position from which fruit was harvested (Fig. 3-1).

Differences in disease incidence associated with different inoculum loads, nested within each vine position and maturity, were significant (P-value from ANOVA=0.0001), but overall effects of vine position and maturity on percent infection were not significant (P>0.05).

Total percent infection increased with spore loading, and infection in the control fruit (no inoculum) was always lower than that in fruit inoculated with ≥ 5000 spores (Fig. 3-3). Disease incidence due to natural infection decreased as fruit matured, regardless of vine position in the control fruit (Fig. 3-3). This trend was not apparent in artificially

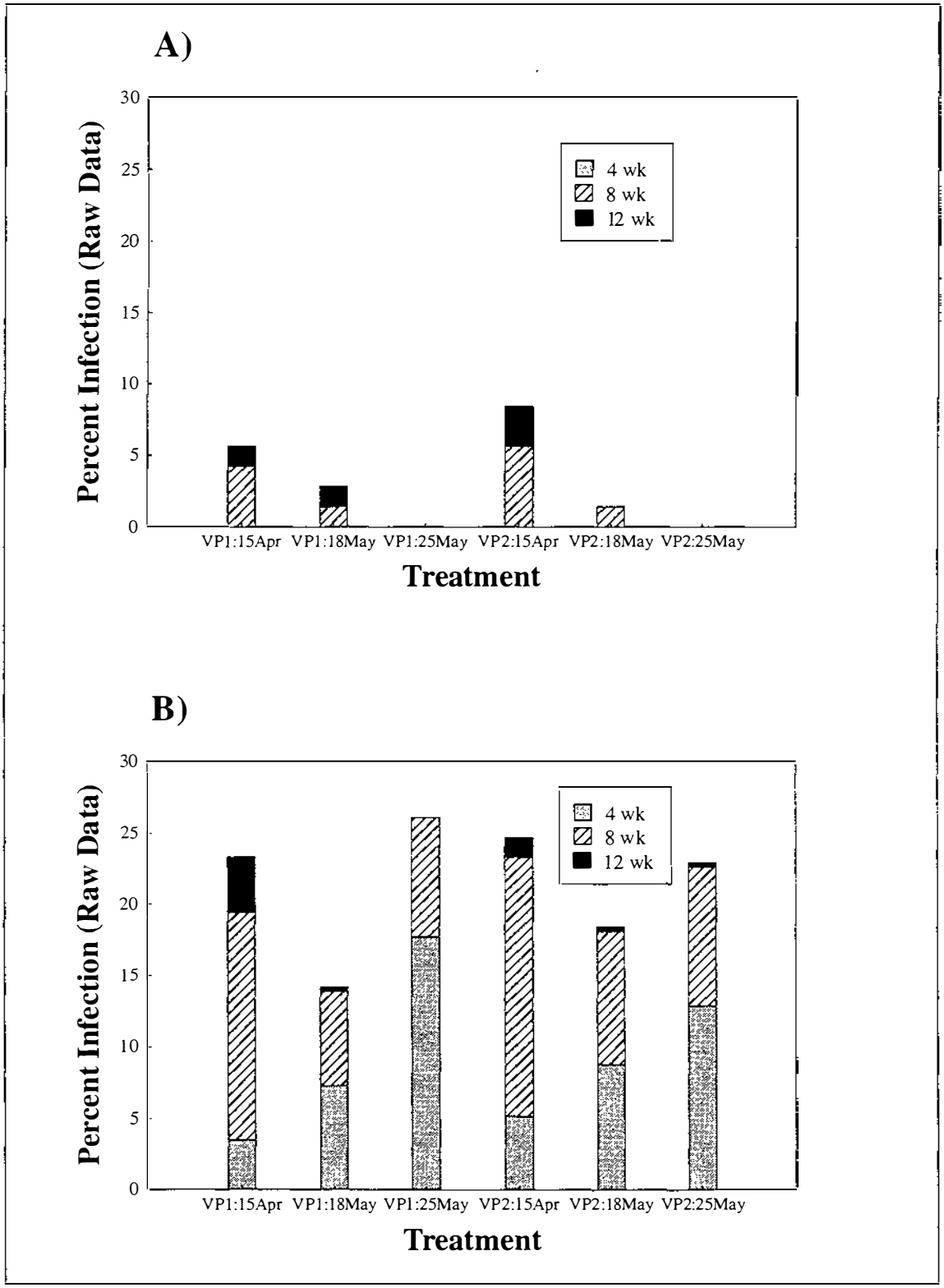


Figure 3-1. Cumulative percent infection (raw data) in fruit collected from two vine positions on three harvest dates and A) left uninoculated, or B) inoculated with 1,000-125,000 *B. cinerea* spores per stem scar. Assessments were done after 4, 8 and 12 weeks storage at 0 ± 0.3°C in 1992.

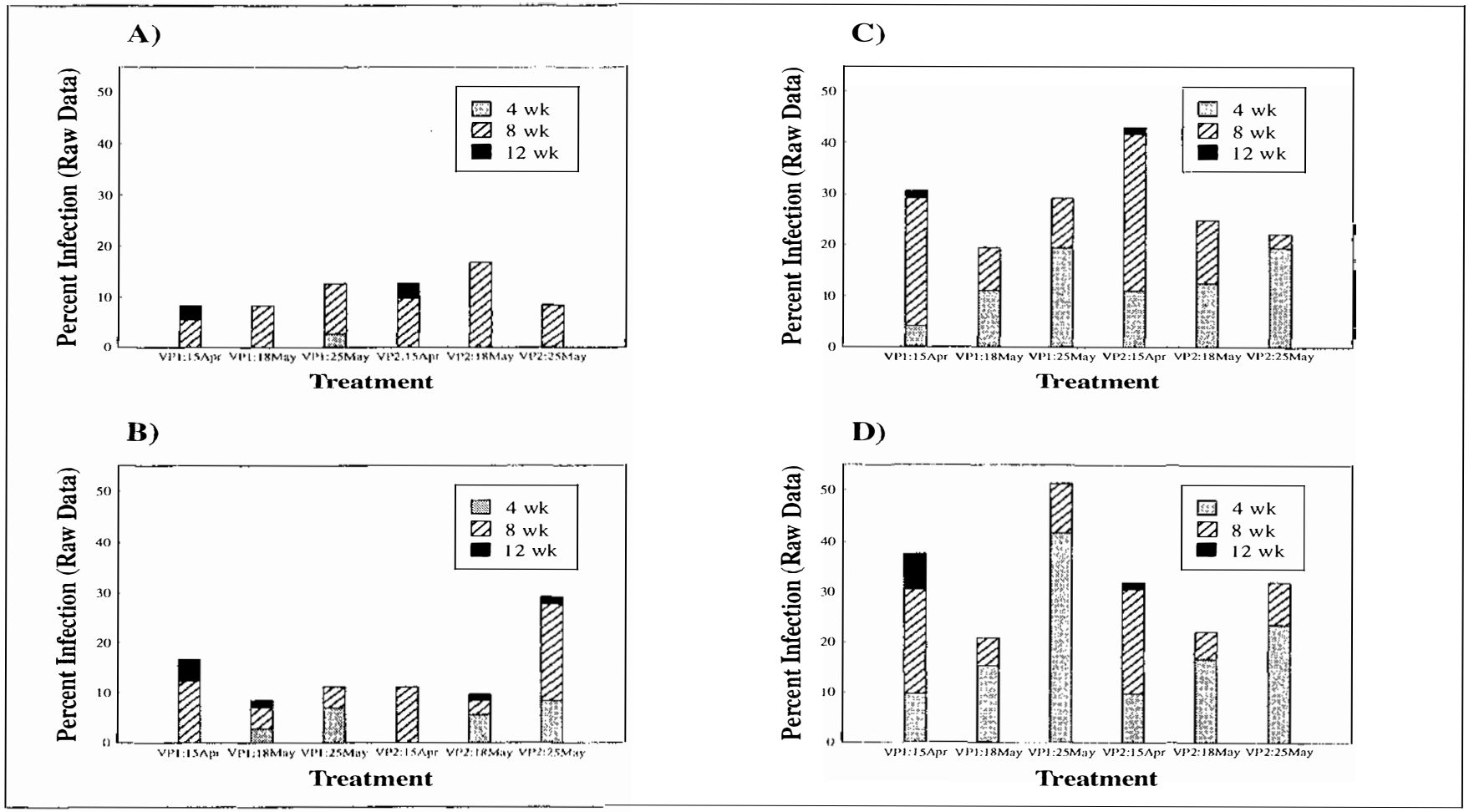


Figure 3-2. Cumulative percent infection (raw data) in fruit collected from two vine positions on three harvest dates, and inoculated with A) 1,000, B) 5,000, C) 25,000 or D) 125,000 *B. cinerea* spores per stem scar, assessed after 4, 8 and 12 weeks storage at 0 ± 0.3°C in 1992.

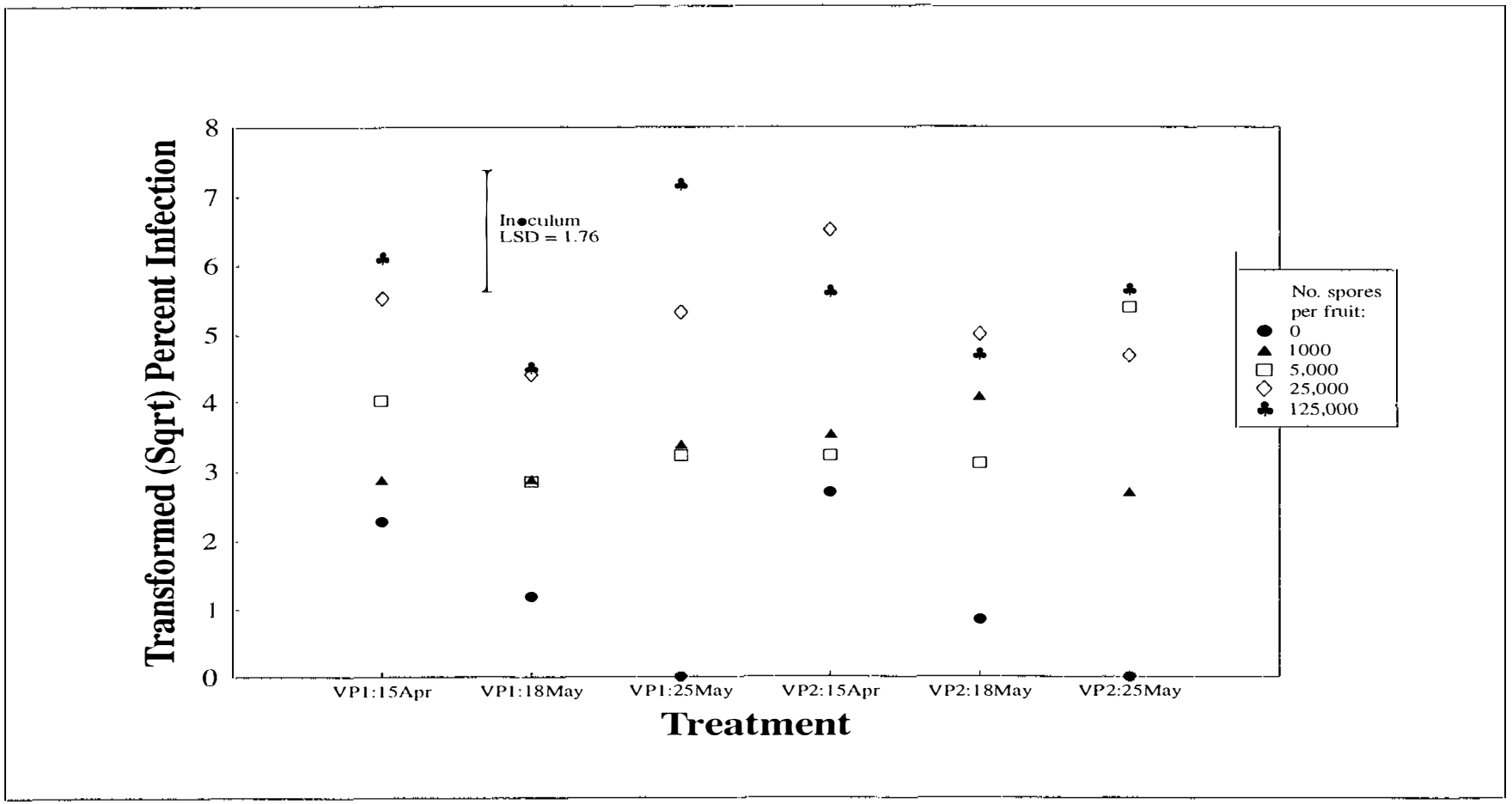


Figure 3-3. Percent infection (square root transformed) of fruit as influenced by inoculum loading nested within maturity and vine position, assessed after 12 weeks storage at 0 ± 0.3°C in 1992. Fruit were taken from VP1, vine position 1 < 1m from central leader, and from VP2, vine position 2 > 1m from central leader. LSD = least significant difference. The LSD bar applies to within-column comparisons only, owing to the hierarchical nature of the nested design.

inoculated fruit, in which infection was lowest at Harvest 2 for both vine positions (Fig. 3-3).

3.4.2 Experiment 2

The onset of infection occurred sooner in inoculated than in uninoculated fruit (Fig. 3-4). Low levels of infection in the uninoculated fruit made it difficult to determine if the harvest date influenced the time taken for infections to develop. At 25,000 spores per fruit, the appearance of infection was delayed with successive harvest dates, but this trend was not evident at the 125,000 spores per fruit inoculum loading (Fig. 3-4).

There was a significant "interaction" (refer Chapter 2, Section 2.3.4, Part C) between maturity and inoculum, (P=0.0001), which shows that differences in disease incidence associated with different inoculum loadings vary with fruit maturity (Fig. 3-5, A). (There is no LSD bar on Fig. 3-5, A because data were unbalanced, and also Duncan's Multiple Range test was not applicable to the interaction.) Infection of control fruit (no spores/fruit) was significantly higher on May 17 than at all other harvests (Table 3-1). For the 25,000 spore loading per stem scar, infection consistently decreased with subsequent harvests i.e. as the fruit matured (Table 3-1 and Fig. 3-5, A). The relationship between infection and harvest date was not as clear for fruit inoculated with 125,000 spores (Fig. 3-5, A).

Interpretation of the "main effects" (refer Chapter 2, Section 2.3.4, Part C) was also meaningful. Irrespective of inoculum loading, fruit from the later harvests had significantly lower levels of infection than the fruit from earlier harvests (Fig. 3-5, B). Infection increased significantly with inoculum loading irrespective of maturity, and control fruit always had significantly fewer infections than fruit inoculated with 25-125,000 spores (Table 3-1 and Fig. 3-5, C).

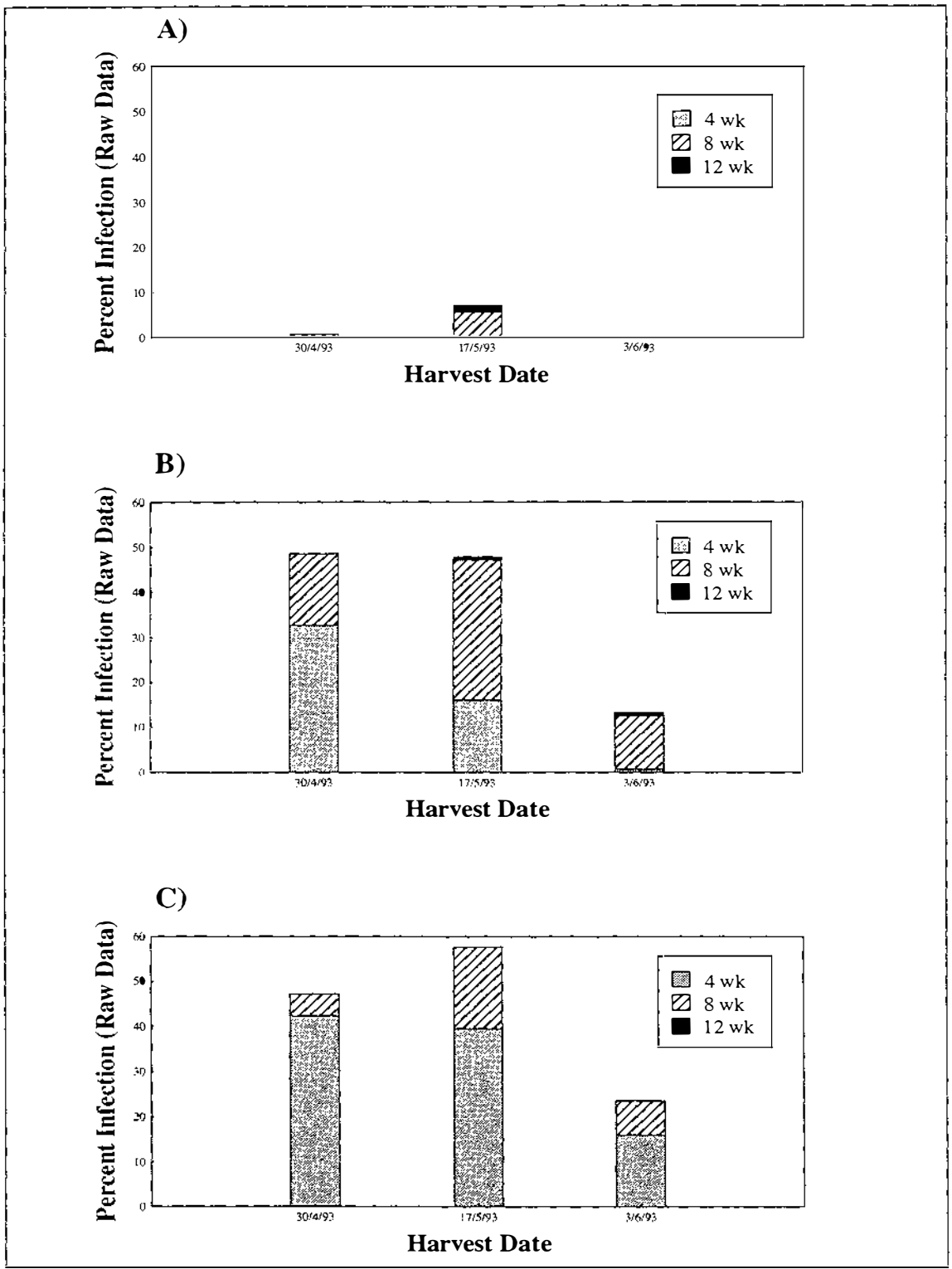


Figure 3-4. Cumulative percent infection (raw data) in fruit collected on three harvest dates and inoculated with A) no spores ("uninoculated"), B) 25,000 spores or C) 125,000 *B. cinerea* spores per stem scar, assessed after 4, 8 and 12 weeks storage at 0 ± 0.3°C in 1993. 16/6/93 data were not included in Fig. 3-4 because percent infection for this data was assessed after 12 weeks coolstorage only.

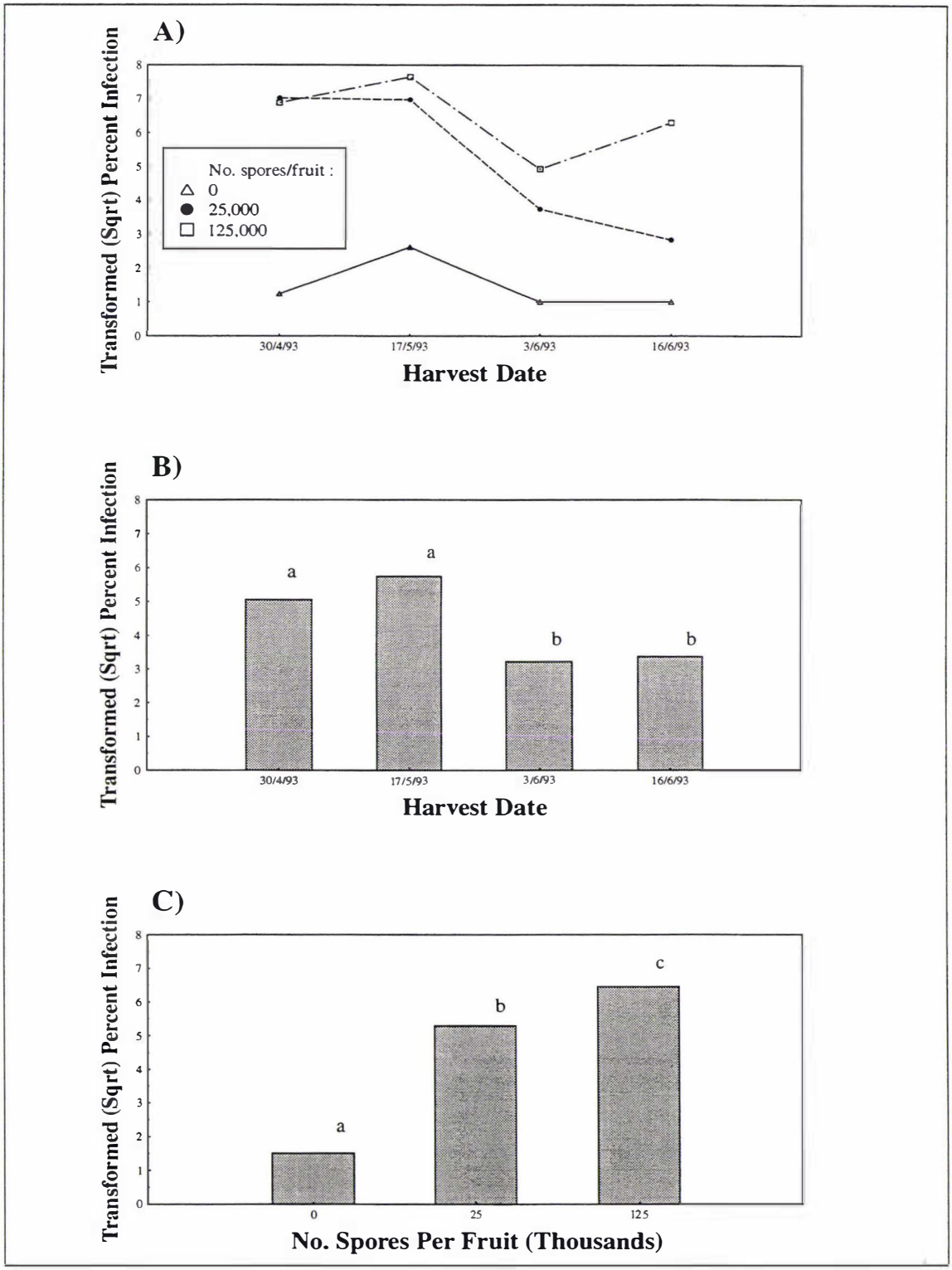


Figure 3-5. Percent infection (square root transformed) of fruit as influenced by A) the interaction between fruit maturity and inoculum loading, B) maturity averaged over inoculum loading, and C) inoculum loading averaged over maturity, assessed after 12 weeks storage at 0 ± 0.3°C in 1993. a,b,c represent significant differences in Duncan's Multiple Range test, (α=0.05).

Table 3-1: Percentage infection (square root transformed means ± SEM) of fruit at four different maturities with three inoculum loadings, assessed after 12 weeks storage at 0 ± 0.3°C in 1993.

HARVEST DATE	INOCULUM LOAD PER FRUIT (THOUSANDS) 0	25	125	**Average**
Harvest 1, 30/4/93	1.24 ± 0.24	7.02 ± 0.31	6.88 ± 0.54	5.05 ± 0.84
Harvest 2, 17/5/93	2.62 ± 0.60	6.97 ± 0.35	7.65 ± 0.25	5.74 ± 0.71
Harvest 3, 3/6/93	1.00 ± 0.00	3.73 ± 0.31	4.92 ± 0.36	3.22 ± 0.52
Harvest 4, 16/6/93	1.00 ± 0.00	2.83 ± 0.46	6.30 ± 0.34	3.37 ± 0.79
Average	1.50 ± 0.24	5.29 ± 0.52	6.45 ± 0.33	4.41 ± 0.38

3.4.3 Experiment 3

The time of day at which fruit were harvested had no effect on the time taken for subsequent infections to develop (Fig. 3-6).

Mean disease incidence was significantly lower at the later harvest (P=0.0097 and Table 3-2).

Although decreased water potential appeared to correlate with increased infection (Fig. 3-7, A and C, compared with Fig. 3-7, B and D respectively), differences in disease incidence observed at various harvest times within any one day were not significant (P>0.05 and Fig. 3-7, A and C).

Water potential also appeared to fluctuate with air temperature (see Appendix VII), and was highest at the coolest periods at the start and at the end of the day. The relationship, however, was not proportional to the temperature differential, because on 13/5/95 the temperature changed by 12.5°C over the course of the day (c.v. 3.5°C on 19/5/95), but the magnitude of water potential changes were approximately the same on both days (Fig. 3-7, B and D, and Appendix VII).

In a separate analysis (data not shown), control and treated (inoculated) fruit were compared, and the control fruit had significantly (P=0.0001) fewer infections.

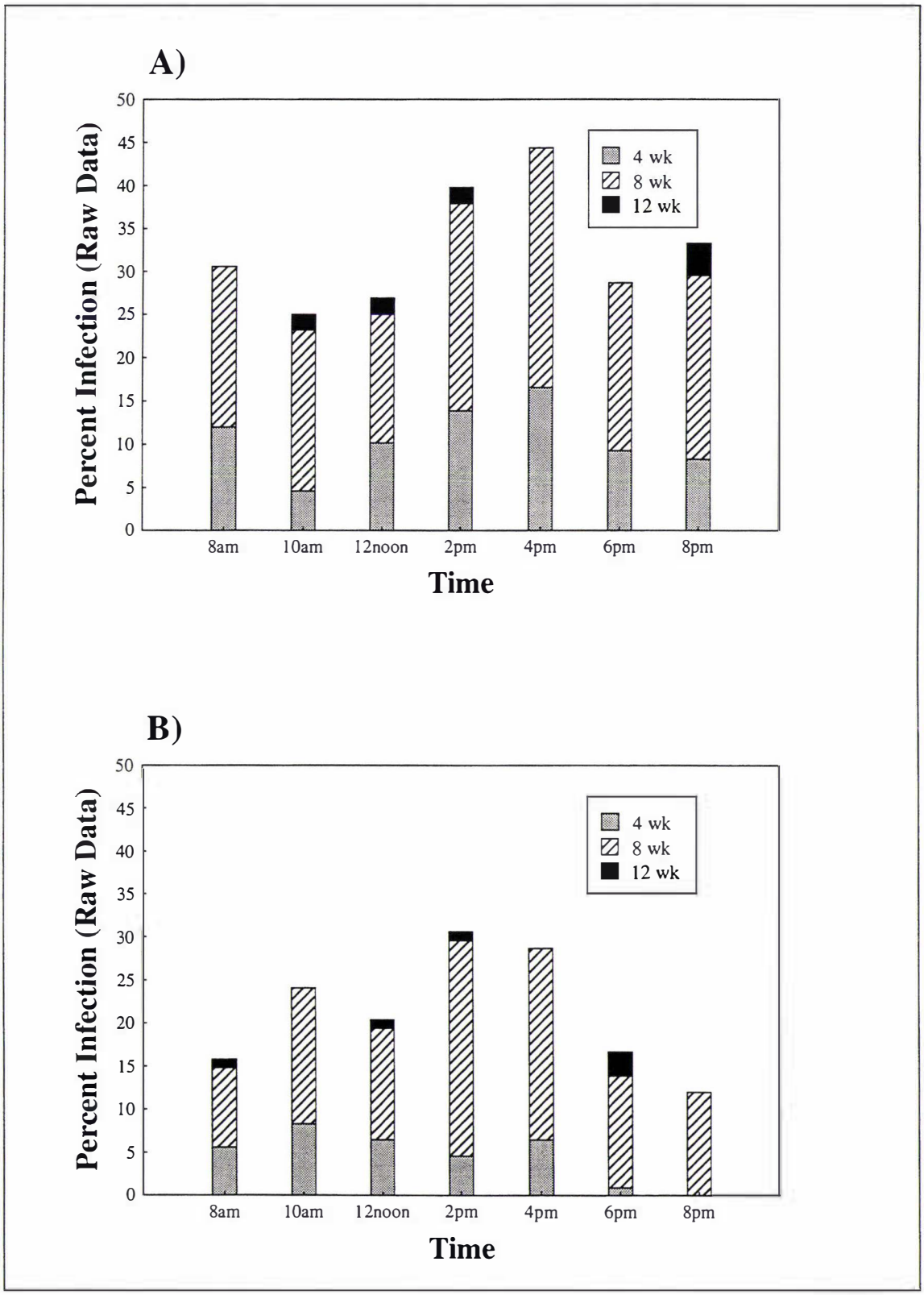


Figure 3-6. Cumulative percent infection (raw data) in fruit harvested at two hourly intervals on A) 13 May, and B) 19 May, assessed after 4, 8 and 12 weeks storage at 0 ± 0.3°C in 1992.

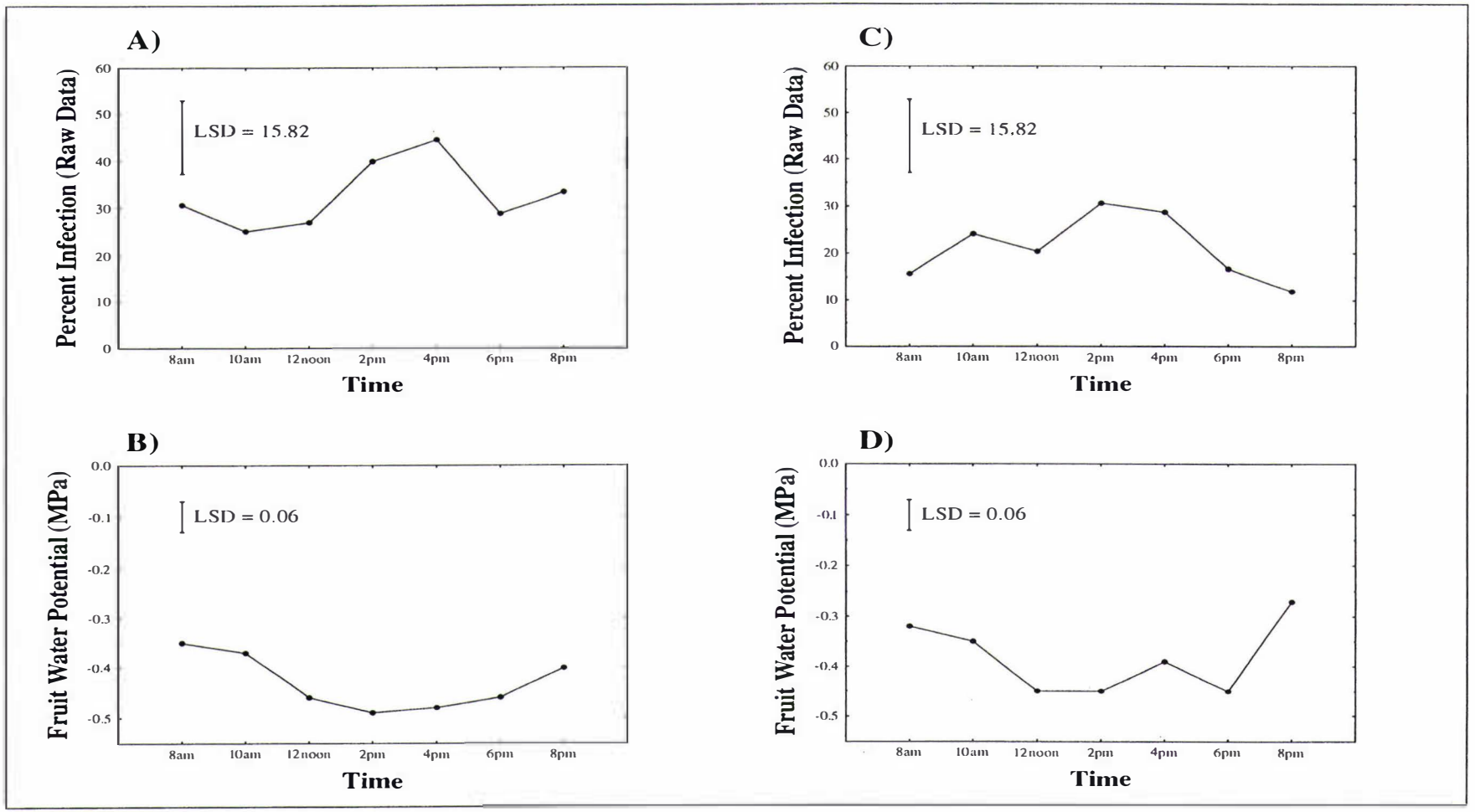


Figure 3-7. Water potential (raw data) measured in megapascals, and percent infection (raw data) in fruit harvested on two separate days, assessed after 12 weeks storage at 0 ± 0.3°C in 1992. A) 13 May, percent infection, B) 13 May, water potential, C) 19 May, percent infection, and D) 19 May, water potential. LSD = least significant difference.

Table 3-2: Percent infection (raw means ± SEM) of fruit harvested on 13 May and 19 May, averaged over harvest interval, assessed after 12 weeks coolstorage at 0 ± 0.3°C in 1992.

HARVEST DATE	PERCENT INFECTION
13/5/92	32.7 ± 2.5
19/5/92	21.2 ± 2.0
LSD* = 8.16, n† = 21	

* least significant difference
† sample number

3.4.4 Experiment 4

A Fresh picking wounds

The appearance of infection symptoms was delayed when the pedicel was trimmed as opposed to removing it entirely, or to drilling the stem scar (Fig. 3-8, B, compared with Fig. 3-8, A and C). For all three types of fresh wound, delays of up to 24 h before wounding and inoculation had no effect on the time taken for subsequent infections to develop. However, when the fruit was left for six weeks before it was wounded and inoculated, appearance of symptoms was delayed (Fig. 3-8, A, B and C). This trend was most apparent in drilled fruit (Fig. 3-8, C).

Both time lapse before inoculation, and type of wound treatment nested within time, had significant effects on disease incidence (P=0.0097 and P=0.0001, respectively).

Percent infection was high when inoculum was applied to fresh wounds made within 24 h of harvest, but was significantly reduced when applied to freshly created wounds on fruit kept in coolstore for six weeks. (Fig. 3-9, A).

Infections resulting from inoculation of fresh wounds made within 24 h of harvest were ranked consistently according to wound type. The standard practice of removing the pedicel resulted in fewer infections than leaving the pedicel attached and inoculating the trimmed end. Removing the surface layers of the stem scar with a drill was intermediate between the two (Fig. 3-9, B). Although ranking of the treatments was the same in each case over the first 24 h, differences in infection between all three treatments were not

large (except for pedicel removal at 6 h, which had significantly lower infection than drilling or pedicel trimming).

When wounds were created and inoculated after a six week delay, the relative ranking of treatments had changed and there were large differences in the amount of infection resulting from each treatment. The trend towards decreased infection at six weeks was less evident in fruit with drilled stem scars than in fruit with other types of stem wound (Fig. 3-9, B).

B Old picking wounds

As wound age at inoculation increased, a greater proportion of infections took longer to appear. This trend was evident for wounds that were less than 24 h old (Fig. 3-8, D).

Delaying inoculation following removal of the stalk at harvest significantly (P=0.0001) reduced disease incidence (Fig. 3-10). Percent infection resulting from inoculation at 24 h after pedicel removal was significantly lower than for fruit inoculated immediately after stalk removal (0 h on Fig. 3-10). After six weeks curing of the picking wound at 0 ± 0.3°C, inoculation resulted in fewer infections than in the uninoculated control.

3.4.5 Experiment 5

Infections appeared more quickly when spore suspensions were amended with yeast extract (Fig. 3-11). The infection level was significantly higher (P=0.004) in fruit inoculated with spore suspensions amended with yeast extract nutrients than in those with sucrose or with no amendment.

3.4.6 Experiment 6

There was no interaction between inoculum loading and soluble silicon (Si+) application (P>0.05). Irrespective of inoculum loading, addition of soluble silicon had no significant effect on disease incidence (P>0.05). Fruit inoculated with 25,000 spores had significantly higher infection levels (raw mean = 12.5%) than uninoculated fruit (raw mean = 0.7%), regardless of the silicon treatment (P=0.002).

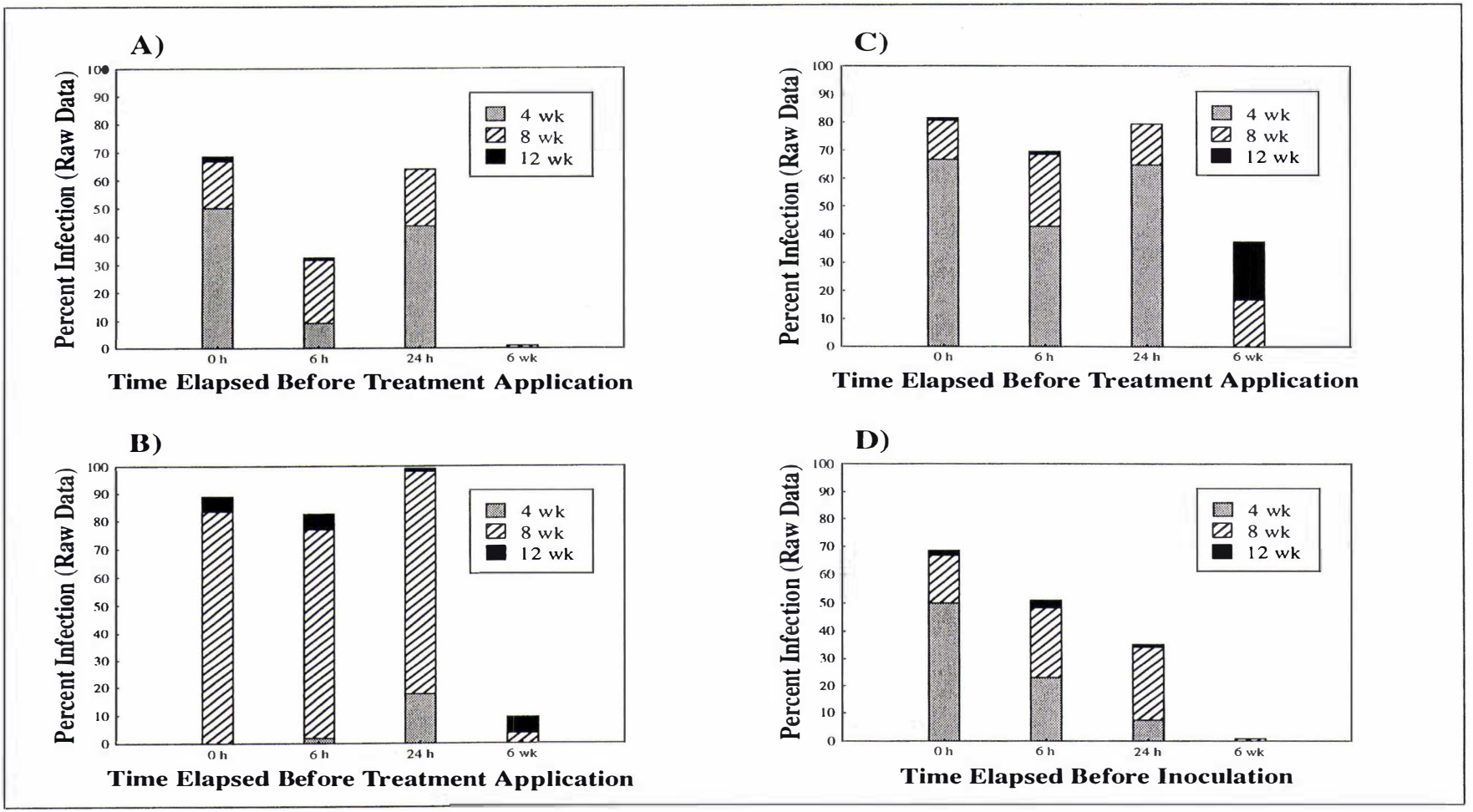


Figure 3-8. Cumulative percent infection (raw data) in fruit with fresh and old picking scar wounds, and four different time lapses before treatment application, assessed after 4, 8 and 12 weeks storage at 0 ± 0.3°C in 1992. A) fresh wound, pedicel removed, B) fresh wound, pedicel trimmed, C) fresh wound, drilled scar, and D) old wound, aged scar.

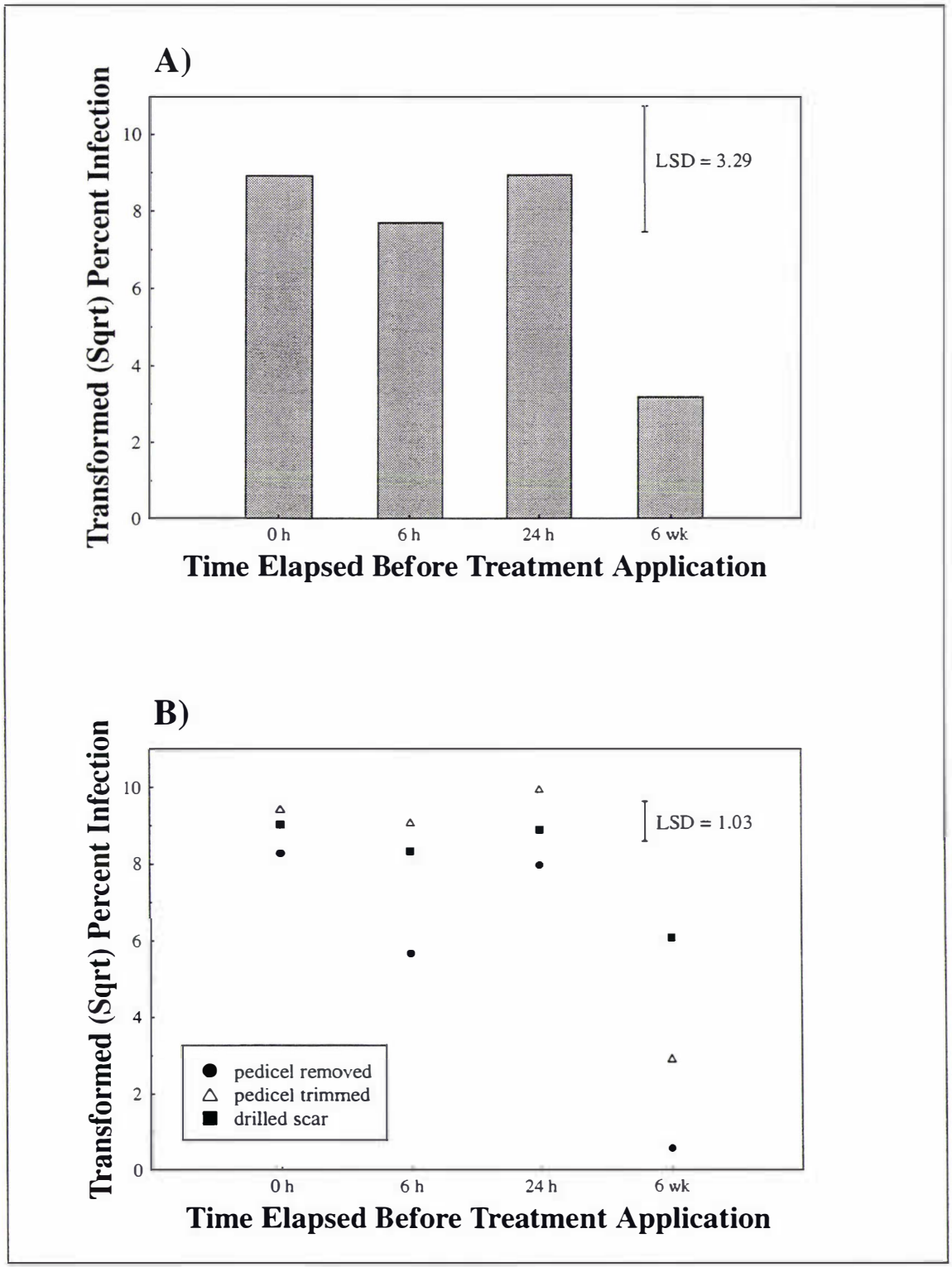


Figure 3-9. Experiment 4, part A. Percent infection (square root transformed) of fruit as influenced by A) time lapse before treatment application, averaged over type of wound treatment, and B) wound treatment, nested within time lapse before treatment application, assessed after 12 wk storage at 0 ± 0.3°C in 1992. LSD = least significant difference. The LSD bar in B) applies to within-column comparisons only.

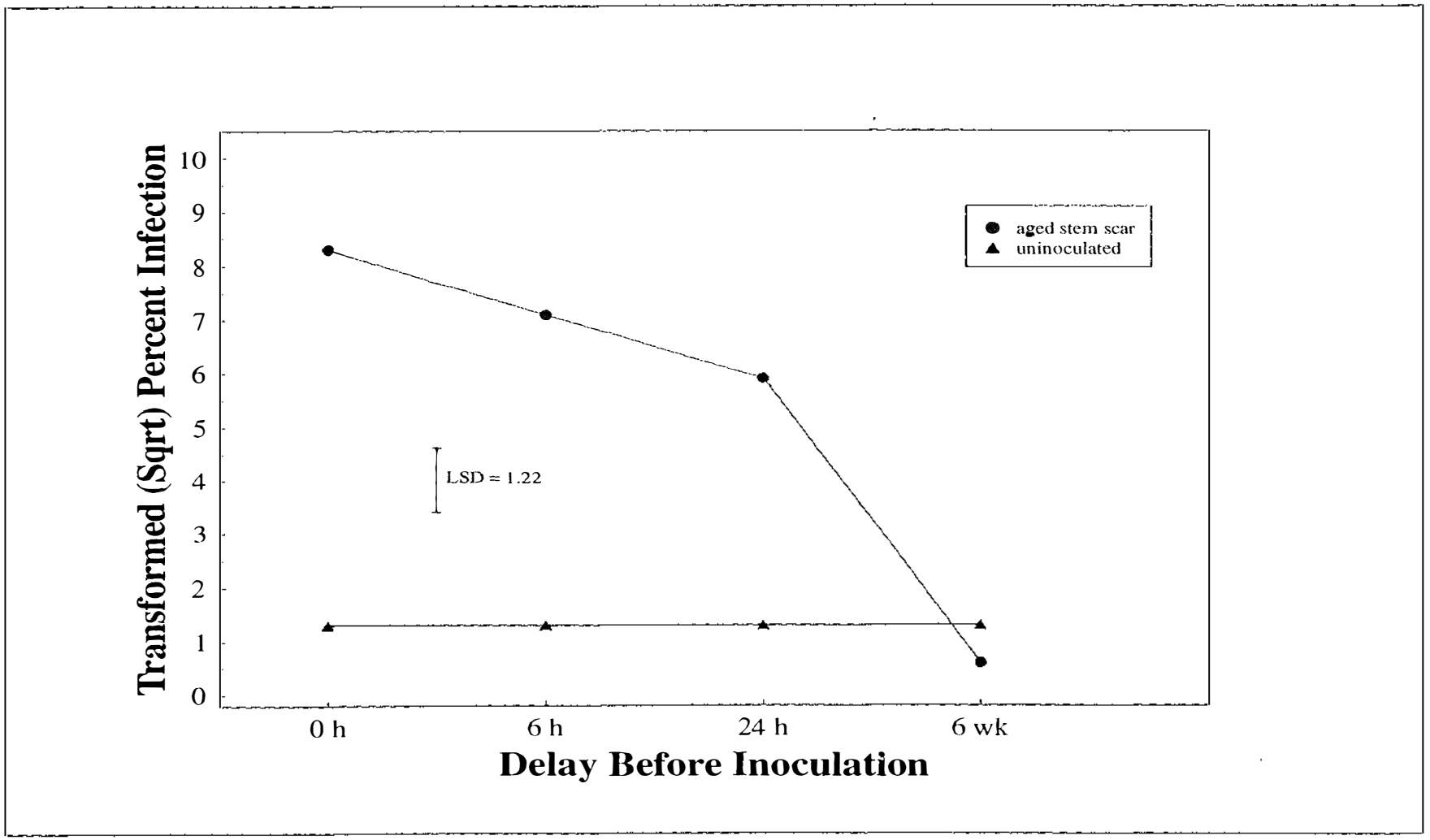


Figure 3-10. Experiment 4, part B. Percent infection (square root transformed) of fruit as influenced by wound age, assessed after 12 weeks storage at 0 ± 0.3°C in 1992. LSD = least significant difference.

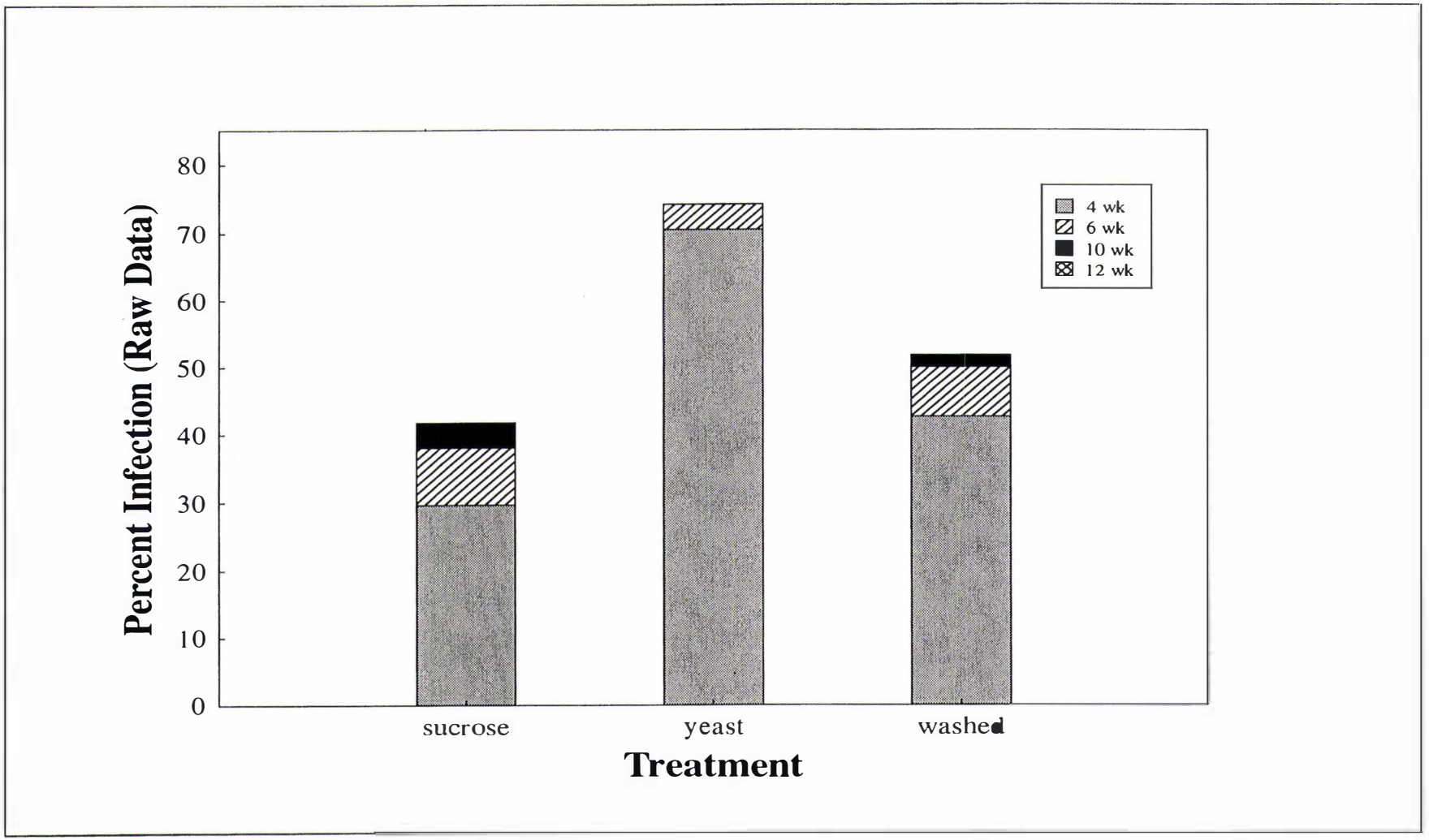


Figure 3-11. Cumulative percent infection (raw data) of fruit inoculated with three different nutrient-amended spore suspensions, and assessed after 4, 6, 10 and 12 weeks storage at 0 ± 0.3°C in 1992.

3.5 DISCUSSION

This research demonstrates that infection of kiwifruit by *B. cinerea* is affected by pathogen factors, such as nutrient status and inoculum load, and by host factors, including fruit maturation and wound repair.

Intense disease pressure caused by high inoculum loads of *B. cinerea* increased total disease incidence and accelerated symptom expression. In Experiments 1 and 2, a strong positive correlation between inoculum load and total disease incidence was observed. This is consistent with the findings of Hallet & Sharrock (1993) and Bautista-Baños (1995), although the latter author found that the level of disease varied with the pathogenic isolate. There is a tendency for fewer *Botrytis* spores to germinate when crowded together (Blakeman 1980; Bautista-Baños 1995), but this appears to be compensated by increased penetration due to a greater likelihood of some spores being well positioned to grow directly into the vascular elements of the stem scar, as well as a greater concentration of plant cell wall degrading enzymes produced by germinating spores and hyphae (Hallet & Sharrock 1993). Increased infection with high inoculum load has also been reported for other *Botrytis* species such as *B. tulipae* (Price 1970), and *B. allii* (Segall & Newhall 1960). In this study, higher spore numbers also stimulated earlier appearance of stem-end rot disease symptoms. This is consistent with the findings of other researchers, who observed a positive correlation between high disease pressure and accelerated infection on kiwifruit (Hallet & Sharrock 1993), and on banana (Stover 1972).

Nutrient availability at the kiwifruit stem plug can influence the success of *B. cinerea* infection. Yeast amendment of the inoculum significantly increased disease incidence, and all infections had appeared by six weeks of coolstorage (Experiment 5). Other research has shown that viability and pathogenicity of *Botrytis* spores have been enhanced by nutrient addition. Last (1960) found that the infectivity of aged *B. fabae* spores could be restored by addition of 0.2-5% solutions of yeast extract and simple sugars. Changes in nutrition can stimulate morphological changes in *Botrytis* species that alter pathogenicity (Lorbeer 1980), and nutrients can also stimulate superficial mycelium

development over the plant surface, and appressorial formation (Blakeman 1980). Harper et al. (1981) found that virulence of *B. cinerea* on broad bean leaves was increased when spore suspensions were amended with pollen and fruit extracts containing simple sugars. Experiment 5 shows that *B. cinerea* exhibits preference for certain nutrients, since sucrose addition resulted in reduced disease incidence, whereas yeast extract containing a complex mixture of nutrients had a stimulatory effect. Harper et al. (1981) noted that *B. cinerea* conidia suspended in sucrose alone did not cause disease of broad bean, but a combination of sucrose and Vogel's mineral salts allowed aggressive lesions to develop. Poole & McLeod (1992) found that sucrose alone could not sustain *B. cinerea* hyphal growth on agar. Blakeman (1975) concluded that both carbohydrates and nitrogen were required for germination. Normally fruit surfaces are rich in nutrients (Blakeman 1980), but there may be active competition from other epiphytic microflora which limit the success of *B. cinerea* establishment (Blakeman 1972; Blakeman 1975). Sharrock & Hallet (1992b) found that *B. cinerea* can be chemically inhibited and/or out-competed by other microorganisms present on kiwifruit picking scars. Variation in the types of nutrients and microflora present on the stem scars of different kiwifruit may contribute to the inconsistent results encountered in both natural and artificial inoculation experiments. However, addition of a suitable nutrient, such as yeast extract, to inoculum is a useful technique for improving the efficacy of artificial inoculation.

In some cases the interaction between host and pathogen factors may be important in determining the outcome of infection. In general, kiwifruit resistance to *B. cinerea* infection increases with fruit maturity at harvest (Brook 1990a; Hopkirk et al. 1990; Pennycook & Manning 1992; Pyke et al. 1993b; Poole & McLeod 1994). However, the presence or absence of this effect appears to depend on inoculum load. For example, increased resistance with fruit maturation was only evident at lower inoculum levels in this study, i.e. in the uninoculated fruit from Experiment 1 and those which received the lower inoculum dose (25,000 spores per stem scar) in Experiment 2. Similarly, Brook (1990a) quoted unpublished work by Pennycook and Manning, who observed reduced infection in later-harvested uninoculated fruit, whilst inoculated fruit from the same experiment did not exhibit a maturity effect. It appears that high inoculum loadings overcome any inherent fruit resistance, but the inoculum level cut-off point for a

maturity effect can be affected by regional differences, such as environment at harvest, etc. For example, Experiment 1 was replicated in Hawkes Bay and Riwaka by Dr. G. Tate and Mr N. Pyke respectively, and in both cases disease incidence decreased as fruit matured, irrespective of inoculum load (Pyke et al. 1993a). Experimental variability at Wanganui, Hawkes Bay and Riwaka was minimised by using the same experimental protocol and batch of inoculum. However, regional differences in orchard practices, the weather preceding harvest, stem scar microflora and spore numbers in the environment are all factors which might have accounted for differences between the sites.

In contrast to pathogen factors which increased disease incidence and expedited symptom expression, host factors examined in this study, such as fruit maturity and curing, appeared to increase resistance and slow the onset of disease. Most crops become less resistant to infection as they mature (Sommer 1982) and this is often related to decreases in antifungal compounds. For instance, green tomato fruit possess factors that suppress conidial germination of *B. cinerea*, and infections increase in parallel with the changes in colour upon ripening (Lavey-Meir et al. 1989). The reduction of antifungal compounds concomitant with ripening causes latent infections of *Colletotrichum gloeosporiodes* on avocado (Prusky et al. 1985) and *C. musae* on banana (Brown & Swinburne 1980) to reactivate. In contrast, increased resistance with ageing has been reported in tobacco (Wyatt et al. 1991), and kiwifruit in this study. Total soluble solids (TSS) gives an estimate of sugar content (Harman 1981) and is used to monitor kiwifruit development because it gives the best simple indication of when to harvest the crop to ensure adequate storage life and acceptable eating quality once the fruit is ripe (Harman 1981; Hopkirk 1992). However, the kiwifruit maturity effect on disease incidence does not appear to be attributable to TSS itself. In apples, an increased susceptibility to several fungal pathogens with ripening is thought to correlate with the accumulation of simple sugars (Sitterly & Shay 1960), whereas in kiwifruit, sugars also increase with ripening, but the stem scar of fruit becomes less susceptible to pathogens. TSS is just one of numerous components that change as kiwifruit mature (MacRae et al. 1989a, 1989b, 1990; Wegrzyn & MacRae 1991). It has been suggested (Pyke et al. 1993b) that differences in kiwifruit susceptibility as they mature could be related to physiological changes, eg. in fruit water status (investigated in Experiment 3) or biochemical changes

in the fruit. Wyatt et al. (1991) found that the biochemical changes occurring in tobacco, which conferred resistance to *Peronospora tabacina*, were attributable to increases in peroxidases, chitinases and β-1,3-glucanases as the tobacco matured.

The influence of fruit position on resistance was also considered in Experiment 1, where fruit were harvested from a pergola system at Wanganui. Concurrent experiments were carried out in 1992 by Pyke et al. (1993a) on a T-bar system at Riwaka and a pergola at Hawkes Bay. In Experiment 1 there was no correlation between disease incidence and vine position (< 1 m from the trunk or > 1 m from the trunk). At Riwaka and Hawkes Bay, the greater crop loading and distribution allowed the use of three vine positions (A - within 1m of the trunk; B - within 1m of the end of the main leader and C within 1m of the tips of laterals arising from the "B" position). Under these conditions there were significant differences in fruit infection from the different vine positions, and these differences were associated with variation in TSS throughout the plant. Fruit position on the vine is an important factor accounting for variation in fruit size, shape, soluble solids concentration (a measure of fruit maturity), flesh firmness and water content (Hopkirk et al. 1986; Smith et al. 1994). However, differences in fruit soluble solids concentrations at various vine positions on pergolas are not as great as those for T-bar trained vines (Hopkirk 1986). This factor may explain why differences in disease incidence were observed in fruit collected from several vine positions at Riwaka (T-bar) but not at Wanganui (pergola). Vine positional differences in infection were observed for the Hawkes Bay pergola system, possibly because more vine positions were sampled than at Wanganui, and overall the Hawkes Bay fruit appeared more susceptible to infection than fruit from the other two sites (Pyke et al. 1993b).

Experiment 3 results showed that normal diurnal variations in water potential do not significantly influence disease incidence resulting from artificial inoculation of kiwifruit with *B. cinerea*. Although low (more negative) fruit water potentials which occurred in the early afternoon were concomitant with higher infection, the differences in the disease incidence observed at different times of the day were not significant. Experiment 3 was duplicated at two other geographical sites, and in both cases there was no strong correlation between percent stem end rot and leaf or fruit water potential (Pyke et al.

1993a). In contrast, Brook (1990a) recorded that in 1985 Pennycook (unpubl. data) detected a strong correlation between low leaf water potential and decreased rots in uninoculated fruit, the opposite trend to that found in Experiment 3. He proposed that infection was lower in fruit with a water deficit (i.e. more negative water potential) because *B. cinerea* spores would have had less chance of establishing an infection on a dry wound than on one where xylem sap was present. Whilst much of the literature supports this theory, with some evidence that free water and/or high humidity is necessary for infection (Blakeman 1980), current research indicates that free water is not essential for *B. cinerea* spore germination and penetration (Brian Williamson, pers. comm.), and in some cases more rots occur in kiwifruit cured at low humidity (Bautista-Baños 1995).

The strong influence of water potential on disease incidence in the one-day uninoculated experiment previously described (Brook 1990a) was not observed in Experiment 3, possibly because the high disease pressure associated with artificial inoculation could have overcome any resistance related to water potential. Although changes in water potential followed a diurnal pattern, rising at night and falling during the day, large differences in water potential require changes in climatic conditions occurring over an extended period of time rather than on a day-to-day basis. Diurnal water potential fluctuations do not appear to have a significant effect on infection, but effects of long term changes in water potential still need to be investigated.

Differing rates of wound response in fruit harvested with and without pedicels in Experiment 4 indicate that the defence response is concentrated at the kiwifruit stem scar, the site of natural abscission. Fruit in Experiment 4, Part A were subjected to two wounds - an initial wound, created at harvest when the fruit was harvested with pedicels attached, and a second fresh wound (pedicel trimming, removal or stem plug drilling) produced immediately prior to inoculation. Since there was no time delay between fresh wounding and inoculation, in all cases fruit had no time to respond to fresh wounds. In fruit picked with pedicels attached, delays of up to 24 h between initial wounding at the end of the pedicel during harvest. and subsequent re-wounding and inoculation at sites closer to the fruit, were insufficient to build up plant defences to the point where

infection decreased. However, a six week delay did significantly reduce infection (Fig. 3-9). Changes in the kiwifruit as a whole accompanying maturation during the six week delay before fresh wounding and inoculation may have also contributed to reduced infection. In contrast, fruit in Experiment 4, Part B were wounded only once by pedicel removal at harvest, and this wound site at the stem scar was directly inoculated after a 0 h-6 wk delay. This resulted in significantly decreased susceptibility to infection by 6-24 h of harvest, and an increase in the average time taken for infections to appear. It appears that the accumulation of resistance factors in the stem plug is accentuated by direct wounding of that tissue prior to inoculation.

Hallet & Sharrock (1993) also noted that resistance of the stem wound to infection took longer to develop in fruit with pedicels attached than in fruit picked in the normal manner (i.e. without pedicels). They found that reduced susceptibility in fruit without pedicels coincided with loss of an ability to absorb a coloured dye through the vasculature, whilst fruit with pedicels attached retained dye uptake ability but resistance still increased. The authors hypothesized that increased resistance through curing fruit without pedicels may be due to blockage of the vascular system or to a localised disruption of the water potential gradient, and that the much slower development of resistance in fruit with pedicels attached may involve a different mechanism. However, Experiment 3 showed that whole fruit water potential was not an important factor. (Localised water potential of the stem scar may be more relevant, but this is extremely difficult to measure.) Moreover, dye is transported through the phloem cells which are too narrow to carry *B. cinerea* spores (Hallet & Sharrock 1993), so the dye uptake observations may not have any bearing on resistance unless they also reflect accessibility to the larger xylem vessels.

Within the first 24 h, infection levels were similar in all three fresh wound treatments (Experiment 4, Part A). The trimmed pedicel treatment had the highest infection, presumably because the pathogen had successfully colonised the length of the pedicel and therefore had a higher infection potential by the time it encountered the stem plug zone, proposed site of kiwifruit defences. The drilled stem plug treatment resulted in slightly less infection than trimmed pedicels, which might have been due to removal of

sclerified tissue that physically impedes pathogen progress, and/or the partial removal of the site of kiwifruit chemical defence. Lowest infection was associated with the treatment in which the pedicel was completely removed. Creation of a stem scar by pedicel removal at the normal abscission zone may be important in inducing defence responses. At six weeks, there were significant differences between levels of infection resulting from inoculation of the various types of fresh wound. Inoculation of the trimmed pedicel was more efficacious than pedicel removal for the reasons described above. Drilling the stem scar resulted in the highest infection, possibly because the antifungal compounds which had accumulated in the stem plug over six weeks in response to the initial wound were removed. The depth of "resistant" tissue through which the mycelia had to grow was also reduced by the drilling.

Curing is the process of allowing time for plant wound repair. Wounding initiates defence responses in plants (Sommer 1982; Bostock & Stermer 1989; Brown 1989) which can inhibit pathogen infection, providing there is a sufficient time delay between wounding and inoculation to allow a host response. Poole & McLeod (1994) found that delaying inoculation after pedicel removal increased kiwifruit resistance to *B. cinerea* (Groups A and F, Table 3-3). Delays between inoculation and coolstorage also increased resistance (Groups B, D and E, Table 3-3), but a comparison of Groups B and C reveals that delay before inoculation was more important, because plant defences against pathogen attack are more likely to be effective if they are in place before the arrival of the pathogen. Although Poole & McLeod (1994) compared Group A with Groups B-E (Table 3-3), only the comparison with Group F is valid, since the results from groups which differ by more than one factor are confounded and would require multifactorial analysis.

Table 3-3: Effect of delays in pedicel removal, inoculation, and coolstorage on the incidence of rots developing in coolstorage in kiwifruit inoculated with *Botrytis cinerea* (1993). Percentage rots are presented with SEMs in parentheses (Table reproduced from Poole & McLeod 1994.)

Pedicel removal : delay after harvest (h)	0.1	3	3	3	3	0.1
Inoc. : delay after pedicel removal (h)	0	0	2	0	0	1
Coolstorage: delay after inoculation (h)	0.4	4	2	28	52	0.4
Harvest date/group	A	B	C	D	E	F
4 May 1993	88	59	5.2	16	11	-
	(3.2)	(3.5)	(2.8)	(4.6)	(4.8)	-
11 May 1993	85	33	12	61	36	-
	(4.1)	(1.3)	(8.4)	(3.5)	(9.3)	-
1 June 1993	82	-	-	-	-	53
	(5.0)	-	-	-	-	(6.0)

Initially it was thought that periods of greater than 24 h following pedicel removal at harvest were necessary to significantly reduce infection (Hallet et al. 1991; Pennycook & Manning 1992). More recent research indicates that curing effects occur within 24 h (Hallet & Sharrock 1993; Poole & McLeod 1994) and this is confirmed by Experiment 4, Part B results. In order to achieve high levels of infection in experimental work, it is therefore advisable to harvest fruit with pedicels attached and to remove them immediately prior to inoculation in order to delay the wound response.

There has been considerable debate over whether decreased infection in cured kiwifruit is caused purely by abiotic environmental factors such as reduced water permeability of the scar tissue, or by plant physiological changes, or a combination of both. Sharrock & Hallet (1992b) proposed that abiotic factors could be responsible for the desiccation and collapse of spores and hyphae on the surface of cured kiwifruit stem scars. However, their observation that spores in direct contact with the stem scar germinated poorly, whilst germination of spores not in direct contact was relatively unhindered also suggests the presence of some form of chemical inhibition. Brown & Barmore (1983) found that reduced infection associated with curing in citrus could not be attributed to abiotic factors such as lack of sufficient nutrients or water for growth. They observed more moisture in healed exocarp resistant to penetration by *Penicillium digitatum* than in susceptible uncured tissue.

Evidence suggests that the curing effect in kiwifruit may result from several defence mechanisms. Poole & McLeod (1992) found evidence of antifungal compounds in the stem plug with curing, but there were no structural changes, such as lignification, with curing. Bautista-Baños (1995) investigated the role of physical barriers in curing-induced kiwifruit defence against *B. cinerea.* She found no anatomical changes in the vascular system, but some thickening of the parenchyma cell walls in contact with *B. cinerea* hyphae. However, the primary route of *B. cinerea* through the stem plug is via the vascular strands (Sharrock & Hallet 1992b; Hallet & Sharrock 1993). Lignin and suberin were present, but there were no significant differences between uncured fruit and fruit cured at 0, 20 and 30°C (Bautista-Baños 1995). Only fruit cured at 10°C, the curing temperature which gave the lowest subsequent disease incidence, had significant increases in suberin and lignin (Bautista-Baños 1995). Whilst 10°C is the most effective curing temperature, curing at 20°C and extended periods at 0°C can also significantly reduce infection relative to uncured fruit (Hallet et al. 1991; Pennycook & Manning 1992). Therefore, physical barriers seem to play a role in curing but do not appear to be the major explanation for reduced infection.

McLeod & Poole (1994) investigated changes in chemical defences in response to inoculation immediately following stalk removal , and found that phenylalanine-ammonia lyase (PAL) began to increase immediately after pedicel removal and subsequent inoculation. They postulated that early resistance detected in kiwifruit involves phenylpropanoid metabolites. Significant increases in endochitinase activity occurred three days after inoculation and therefore may contribute to the latter phases of resistance development. Similar changes may occur in response to curing of the stem scar and need to be investigated. The role of chitinases in kiwifruit defence is examined later in this thesis.

Silicon addition to cucumbers (Chérif et al. 1992) and barley (Jiang et al. 1989) significantly reduces *Pythium* and powdery mildew infections respectively. Silicon accumulation in plant tissue surrounding pathogenic lesions has also been recorded in wheat and morning glory (Kunoh & Ishizaki 1975). Silicon is thought to influence plant susceptibility to fungal attack, either by acting as a mechanical barrier, or by inducing plant defence

mechanisms such as phenolics (Menzies et al. 1991), peroxidases and chitinases (Chérif et al. 1994). Recent research indicates that silicon-induced plant defences are the most likely reason for enhanced resistance (Chérif et al. 1992, 1994). Reduction of *B. cinerea* infection of kiwifruit stem scars by silicon application was attempted to provide indirect evidence of plant defence mechanisms (Experiment 6). The result was negative, but the absence of a treatment response is not conclusive proof that silicon is ineffective in inducing kiwifruit defence mechanisms. A definitive answer requires the use of a range of silicon concentrations under varying environmental conditions.

The results of our field trials show that host and inoculum factors influence the success of artificial inoculation experiments. This study has also highlighted the need to standardize experimental protocol to minimise variability and to achieve the high levels of disease incidence required in research. In general, young fruit should be inoculated with high spore loads to overcome any inherent defences associated with fruit maturity, and fruit should be harvested with pedicels attached to delay the host wound response. For example, a confounding factor was introduced into Experiment 1 when fruit were inadvertently exposed to different curing periods when they were harvested without pedicels. This complication was overcome in Experiment 2 by harvesting fruit with pedicels attached and removing them immediately prior to inoculation. An awareness of the weather before and during harvest is another important consideration. In Experiment 2, infection of both uninoculated and inoculated fruit was highest in fruit from the May 17 harvest. This suggests that there were higher spore numbers in the field on this day. Although the weather on May 17 was fine, it had been raining for the previous 24 h. Wet periods preceding fine weather are conducive to the formation and release of more spores (Brook 1990a). These fruit could have effectively received more inoculum and consequently had a higher disease incidence because their picking wounds were contaminated with spores carried on the fruit surface from the field at harvest. Maturity experiments, (eg. Experiment 2) should therefore involve at least four harvests since, with three harvests or less, an aberrant result on just one occasion can make it difficult to observe any trends. This example stresses the need to consider the effect of weather, particularly in trials that involve several harvests.

In the present study, large numbers of new infections appeared in all experiments within 8-12 weeks of coolstorage, apart from the yeast treatment in Experiment 5 where all infections had appeared by six weeks. This substantiates the current industry practice of delaying condition checking until ten weeks after harvest (Evans 1992; Hopkirk & Clark 1992).

At the time this study was initiated in 1992, preliminary work indicated that chitinases could be a significant factor in kiwifruit defence against *B. cinerea* (Sharrock & Hallet 1992b; Poole & McLeod 1992), and the remainder of this study was devoted to investigating the possible role of chitinases in the host resistance of the kiwifruit stem scar.

4 EXO- OR ENDOCHITINASES?

4.1 INTRODUCTION

4.1.1 Host Resistance In The Stem Plug

When I started this work in May 1992, several lines of evidence suggested that host mechanisms in kiwifruit stem plugs played an important role in resistance to *B. cinerea*, and the role of mechanisms associated with maturity and curing has already been discussed (Chapter 3, Section 3.5). Other factors indicative of host defences include the observation by Sharrock & Hallet (1992b) that *B. cinerea* spores were detected on 13% of picking scars, but only 1% developed aggressive rots. Their research also demonstrated that spore germination on cured fruit was relatively uninhibited when the spores were not in contact with the stem scar region, but spores in direct contact germinated poorly. Poole & McLeod (1992) found that rots were more likely to develop when the pericarp, rather than the stem plug, was inoculated. Natural infections occur primarily through penetration of damaged vasculature at the stem scar (Sharrock & Hallet 1992b), and the evidence cited above suggests that the plant defences are most active in the stem plug region.

4.1.2 Importance Of Chitinases In Kiwifruit Defence

Chitinases have been implicated in defence against fungal pathogens in many plants (Boller et al. 1983; Metraux & Boller 1986; Schlumbaum et al. 1986; Kurosaki et al. 1987; Fink et al. 1988; Kombrink et al. 1988; Ahl Goy et al. 1992), and research prior to May 1992 indicated that chitinase may be one of the most important factors in kiwifruit defence. Sharrock & Hallet (1992a) found that separation of growth stimulating nutrients and sugars from rotten stem plug extracts made it possible to detect inhibitors of *B. cinerea* germination. Poole & McLeod (1992) also extracted an inhibitor of *B. cinerea* mycelial expansion from the stem plug. Since plant defence mechanisms are often induced by wounding/curing and inoculation, kiwifruit responses in these situations were investigated. No evidence of physical barriers to growth in the stem plug was found (Poole & McLeod 1992; Sharrock & Hallet 1992a). Kiwifruit stem plug and

pericarp extracts did not inhibit *B. cinerea* enzymes such as polygalacturonases, pectinases, proteases, endoglucanses and methylesterases (Sharrock & Hallet 1992a), which have been associated with pathogenicity (Verhoeff 1980; Movahedi & Heale 1990). Poole & McLeod (1992) found higher concentrations of phenolics in the stem zone than in the pericarp, but toxicity of these compounds against *B. cinerea* was low. Equivalent amounts of lysozyme activity were found in the stem plug and pericarp, but there was little response to infection and curing. Actinidin was found predominantly in the pericarp rather than the stem plug, and levels were unaffected by curing (Sharrock & Hallet 1991). In contrast to these negative findings, exochitinase activity was greatest in the stem plug and increased in response to infection. There was, however, no increase in exochitinases with curing (Sharrock & Hallet 1992b). Poole & McLeod (1992) proposed that a reduction in infection caused by chitosan application could be related to chitinase induction. The role of chitinases in kiwifruit defence against *B. cinerea* consequently became the focus for the remainder of this thesis.

4.1.3 Plant And Fungal Chitinases

Preliminary studies on chitinases in kiwifruit measured exochitinase activity, but other research has indicated that this may not be the most appropriate activity to monitor. Exochitinases have been found in melon (Roby & Esquerre-Tugaye 1987b) and squash (Sharrock & Parkes 1990), but most plant chitinases are endo-acting (Boller 1985; Roberts & Selitrennikoff 1988; Broglie & Broglie 1994). Endochitinases have been recorded in numerous plant species, including soybean (Wadsworth & Zikakis 1984), pea (Mauch et al. 1988a), bean (Boller et al. 1983), potato (Kombrink et al. 1988), tomato (Joosten & de Wit 1989), and turnip (Conrads-Strauch et al. 1990). Results of measurements of plant chitinase levels using exochitinase assays only should therefore be viewed with caution (Boller & Mauch 1988).

A further complication in diseased tissue is that fungi can also produce chitinases. These are thought to be involved in physiological and morphological life cycle processes such as spore swelling, germination, sporangium development (Manocha & Balasubramanian 1994), and in response to mechanical injury (Sahai et al. 1993). Some workers consider that these enzymes are involved in apical extension of hyphae (Bartnicki-Garcia 1973;

Humphreys & Gooday 1984; Pedraza-Reyes & Lopez-Romero 1989), but this view is not universally accepted (Wessels 1986).

4.1.4 Enzyme Assays

Enzymes are biological catalysts. To follow the progress of an enzymatic reaction, the change in concentration of one of the reactants or products over time is monitored (Guibault 1970). This provides a measurement of the rate of catalysed reaction or "enzyme activity" (Bergmeyer 1974). Reaction rate can be influenced by factors such as enzyme concentration, temperature, incubation time, and substrate concentration. (Guibault 1970).

Valid measurement of enzyme activity requires that there is a definable, proportional relationship between the parameter being measured and time (Bergmeyer 1974). A relationship that is linear for at least part of the time is most desirable. With non-linear progress curves the reaction rate is continuously changing, making rate calculations extremely complicated. Moreover, the continuous removal and analysis of samples necessary to obtain reaction curves involves a considerable expenditure of time and effort (Bergmeyer 1974). It is good practice, therefore, to verify suitability and validity of an enzyme assay by testing for linearity, and to adapt the assay to the specific conditions of the system being studied to avoid wasted measurement of important samples through inadequate assay procedures.

4.2 OBJECTIVES

Exochitinase assays are discussed in this chapter, while endochitinase assays are evaluated in Chapter 5. The objectives of these Chapter 4 experiments were to:

1) test the suitability/validity of the Roberts & Selitrennikoff (1988) exochitinase assay,

2) determine if use of an exochitinase assay is adequate for measurement kiwifruit chitinase activity, and

3) distinguish between kiwifruit and *B. cinerea* chitinases.

4.3 MATERIALS AND METHODS

Unless otherwise stated, refer to Chapter 2, Section 2.2.4 for details of assay methods used in these experiments. Stem plug extracts used in these experiments were distinguished by the classification system described in Chapter 2, Section 2.2.2. For the purposes of this study, exo- and endochitinase activities are defined respectively by Kunz et al. (1992) and Bielka et al. (1984), as discussed in Chapter 1, Section 1.8.1, Part A.

4.3.1 Experiments 1-3

Title : Suitability of the Roberts & Selitrennikoff (1988) assay for measurement of kiwifruit exochitinase activity.

A Non-catalysed reaction (Experiment 1)

A modification of the exochitinase assay protocol in Chapter 2, Section 2.2.4, Part A was used to determine the amount of background absorbance associated with non-enzymic substrate hydrolysis at high pH. The high pH environment was created by the sodium tetraborate stopper used to preinhibit the enzyme reaction.

Modifications to the assay method were as follows:

1) A higher concentration of sodium acetate buffer (100 mM) was used instead of 50 mM, because a stronger buffer capacity was required to overcome the buffer capacity of ampholytes in the Rotofor fractions,

2) there were two duplicate wells per treatment,

3) samples were incubated for 2 h,

4) all wells in the microtitre plate were preinhibited and absorbances were measured both before and after the 2 h incubation, and

5) 100 µl of substrate was used.

The extracts tested comprised two water (no enzyme) controls and 20 fractions separated by a Rotofor separation from a bulk extract of diseased kiwifruit stem plugs pooled from fruit harvested on 4/5/92 from a commercial property in Patumahoe, South Auckland (Lat S 37° 11 Long E 174° 49), and on 5/5/92 from a Kumeu Research Station, West

Auckland (Lat S 36° 46 Long E 174° 33). Immediately after stalk removal, each fruit had been inoculated with 20 µl of a 1 x 10^5 spores/ml suspension. After nine weeks in coolstorage, infected stem plugs were extracted in approximately three volumes of water. The extract was dialysed overnight against water and 50 ml of extract plus Servalyte pH 3-10 ampholyte (1% final concentration) were placed in the Rotofor for 6 h and 20 min.

Results were not statistically analysed because increases in absorbance readings and variability in these readings were negligible in the Rotofor fractions and the water control.

B Linearity tests (Experiments 2-3)

The enzyme extract used for linearity tests (Experiments 2 and 3) was a bulk extract (92/N/I/D) of diseased kiwifruit stem plugs from Experiment 1 (Chapter 3, Section 3.3.1). The stem plugs, pooled from various harvest dates, were originally inoculated with 5,000 spores, and extracted in a 1:5 w/v ratio after 12 weeks coolstorage at 0°C. Water controls were also included in the linearity tests.

In Experiment 2, release of *p*-nitrophenol in an 8-fold dilution of the 92/N/I/D extract was measured at 405 nm after 1, 2, 3, 4 and 5 h incubation. Absorbance was plotted against incubation time.

For Experiment 3, release of *p*-nitrophenol in 6, 8, 10, 12 and 14-fold dilutions of 92/N/I/D extract was measured at 405 nm after 3 h incubation. Absorbance was plotted against enzyme concentration which was expressed as a percentage of undiluted 92/N/I/D extract.

4.3.2 Experiment No. 4

Title : Exo/endochitinase activity in kiwifruit leaves, 1992 season.

Kiwifruit leaves at various stages of expansion were harvested from T-bar vines in the Mt Albert Research Orchard, Auckland (Lat S 36° 52 Long E 174° 45) in early June 1992, and were couriered overnight in moist plastic bags to Palmerston North. Leaves

from Auckland were used because the leaves on vines at Massey University Fruit Crops Unit were already senescent at that time.

Leaves were randomly assigned to two groups of ten each. Leaves from one group were individually placed on moist filter paper, wrapped in aluminium foil and autoclaved for 15 min at 121°C to denature the plant enzymes, whilst the second group was not autoclaved. Initial attempts to inoculate intact leaves from both groups with a 2 mm diam. malt agar plug from a 10-14 day old sporulating colony of *B. cinerea* (K3), were unsuccessful, so the inoculation site was wounded with a sterile needle and the leaves reinoculated by placing one 5 mm diam. agar inoculum plug with the mycelial surface contacting the wound site, to the right of the central vein on the adaxial surface of each leaf (Fig. 4-1). All leaves were laid on fresh saturated filter paper, wrapped in aluminium foil and incubated at 20°C. After four days, 1:5 w/v extracts of leaf tissue from three different areas on each leaf (Fig. 4-1) were prepared.

Exochitinase activity in the extracts was measured using the Roberts & Selitrennikoff (1988) assay. The viscometric assay (Chapter 2, Section 2.2.4, Part B) was used to measure endochitinase activity. Exochitinase activity was expressed as nmol of *p*-nitrophenol released per minute per ml of crude extract, and endochitinase activity as percent decrease in viscosity relative to the enzyme-free control (refer Appendix II for formulae).

Exo- and endochitinase activity measurements were analysed separately. In both cases the experiment was considered as a randomised block design, where various steps were used to produce six extracts (or treatments):

1) Autoclaved, diseased,
2) autoclaved, adjacent diseased,
3) autoclaved, healthy,
4) live plant tissue, diseased,
5) live tissue, adjacent diseased, and
6) live tissue, healthy.

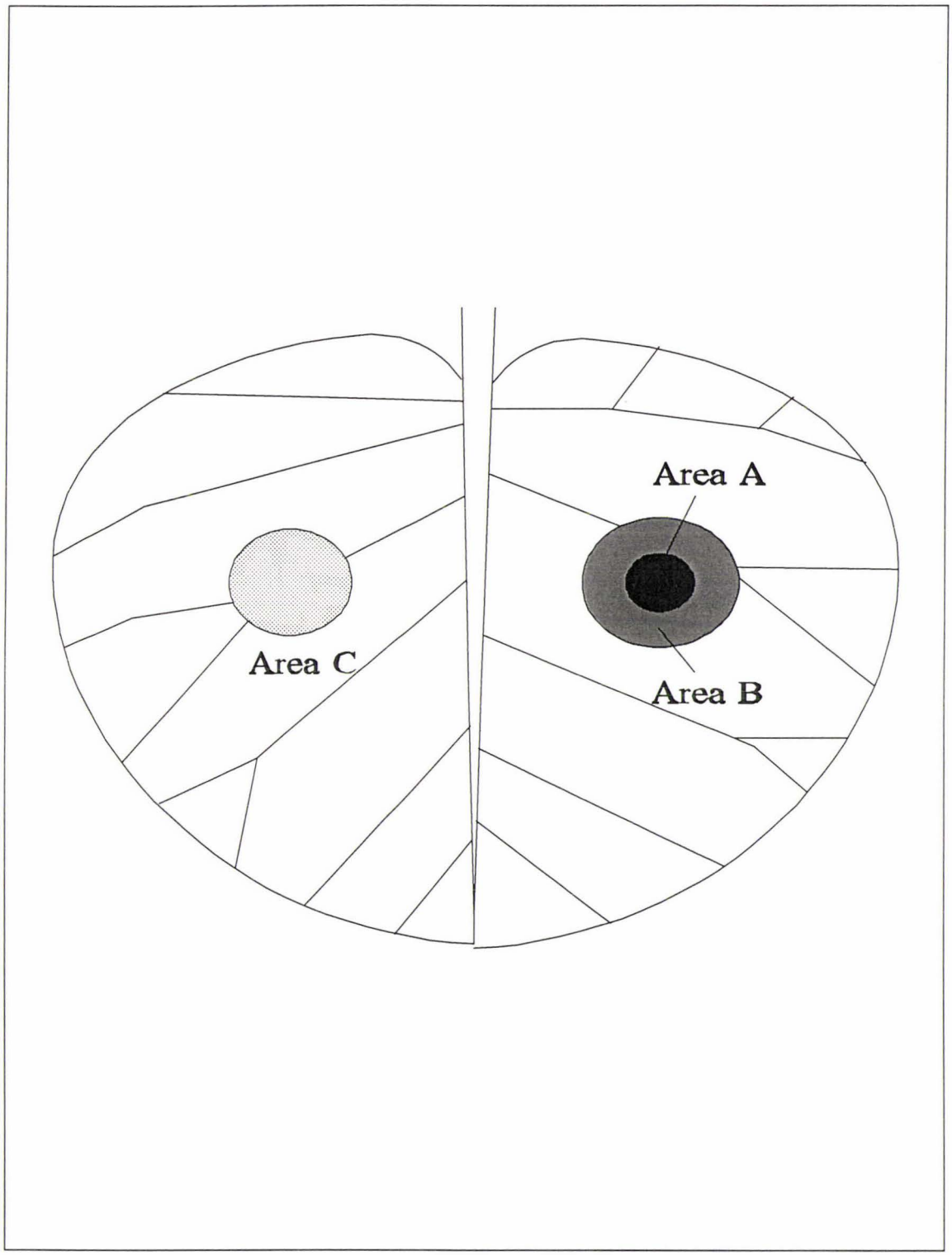


Figure 4-1. Sample sites for measurement of exo- and endochitinase activity in kiwifruit leaves. Area A = diseased inoculation site; Area B = leaf tissue immediately adjacent to the diseased lesion; Area C = healthy tissue remote from the lesion.

Six to nine replicates/treatment were used to monitor exochitinase activity. The number of replicates per treatment varied because it was not always possible to make extractions from all three areas on every leaf - in some cases the infection had progressed over the entire leaf, whilst on other leaves the amount of infected tissue was insufficient to produce an extract. For the endochitinase activity measurements there were three replicates (leaves) per treatment. The time consuming nature of this assay precluded measurement of more replicates. The exochitinase data was square root transformed, whilst the endochitinase data required a $\log_e$ transformation to satisfy the requirements of ANOVA (refer Chapter 2, Section 2.3.1). The two different transformations allowed the best approximation of the required conditions of ANOVA for each separate dataset.

4.3.3 Experiment No. 5

Title : Exochitinases in kiwifruit stem plugs from Experiment 1, Chapter 3, 1992 season.

On each of the harvest dates of Experiment 1 (Chapter 3, Section 3.3.1), 108 additional fruit were collected and divided randomly between three trays. Fruit of two trays were inoculated with 5,000 spores of *B. cinerea* per stem scar, while fruit in the third tray were not inoculated. After 12 weeks at 0 ± 0.3°C, ten replicates of 1:5 w/v stem plug extracts from individual fruit were obtained for each harvest/inoculum treatment combination.

Since not all the extracts could be produced on one day, days were treated as blocks and equal number of replicates from each treatment were extracted on each day. Only six diseased fruit were present in trays of inoculated fruit which had been collected on the third and final harvest date (25 May). Exochitinase activity in all the extracts was measured using the Roberts & Selitrennikoff (1988) assay. The experiment was carried out according to a randomised block design. Transformation of data was not required.

4.3.4 Experiment No. 6

Title : Exo- and endochitinases in kiwifruit stem plugs from Experiment 6, Chapter 6, 1993 season.

Exo- and endochitinase activity in three replicates from four different types of extract taken from Experiment 6 (Chapter 6, Section 6.3.7) were measured using the Boller et al. (1983) assay. Each replicate comprised a 1:5 w/v extract from a single stem plug. Stem plugs were randomly selected. They were snap frozen in liquid nitrogen and stored at -80°C until extracted.

Details of the four extract categories are summarised as follows:

1) Inoculated (5,000 spores/stem scar), stem plugs removed immediately after inoculation, i.e. 0 d coolstorage at 0°C,
2) inoculated (5,000 spores/stem scar), stem plugs removed after 3 d coolstorage at 0°C,
3) inoculated (5,000 spores/stem scar), healthy stem plugs removed after 6 wk coolstorage at 0°C, and
4) inoculated (5,000 spores/stem scar), diseased stem plugs removed after 6 wk coolstorage at 0°C.

(A 17 µl droplet of 0.25 M HCl was also applied to fruit in all four treatments immediately following pedicel removal and prior to inoculation, as part of the larger Experiment 6, Chapter 6 protocol, in order to further stimulate the host defence response.)

Exo- and endochitinase activity measurements were analysed separately. Exochitinase activity was $\log_e$ transformed. Transformation of the endochitinase data was not required.

4.4 RESULTS

4.4.1 Experiments 1-3

A Non catalysed reaction (Experiment 1)

Increases in background absorbance resulting from non-enzymic hydrolysis of the substrate at alkaline pH (pH ≈8.5) during the incubation period were negligible (Table 4-1).

Table 4-1: Increase in absorbance (mean ± SEM) in preinhibited enzyme and water controls in the Roberts & Selitrennikoff (1988) exochitinase assay following 2 h incubation at 37°C in 1992.

TREATMENT	INCREASE IN ABSORBANCE AT 405 nm
INFECTED ENZYME EXTRACT (samples 1-20)	0.005 ± 0.004
WATER CONTROL	0 ± 0

B Linearity tests (Experiments 2-3)

Linear regression provided a good fit for data shown in Fig. 4-2, A and B. (The 14-fold dilution (7.1% enzyme concentration) of 92/N/I/D extract in Fig. 4-2, B was treated as an outlier.) The assay can therefore be used to quantify exochitinase activity in kiwifruit.

4.4.2 Experiment No. 4

A Leaf exochitinase activity

Exochitinase activity was concentrated in diseased tissue of both living leaves and those autoclaved to destroy all plant enzymes (Fig. 4-3, A). Tissues from areas adjacent to the lesion, and from sites several cm from the lesion, had mean levels of exochitinase activity which did not differ significantly from the mean of the enzyme-free controls.

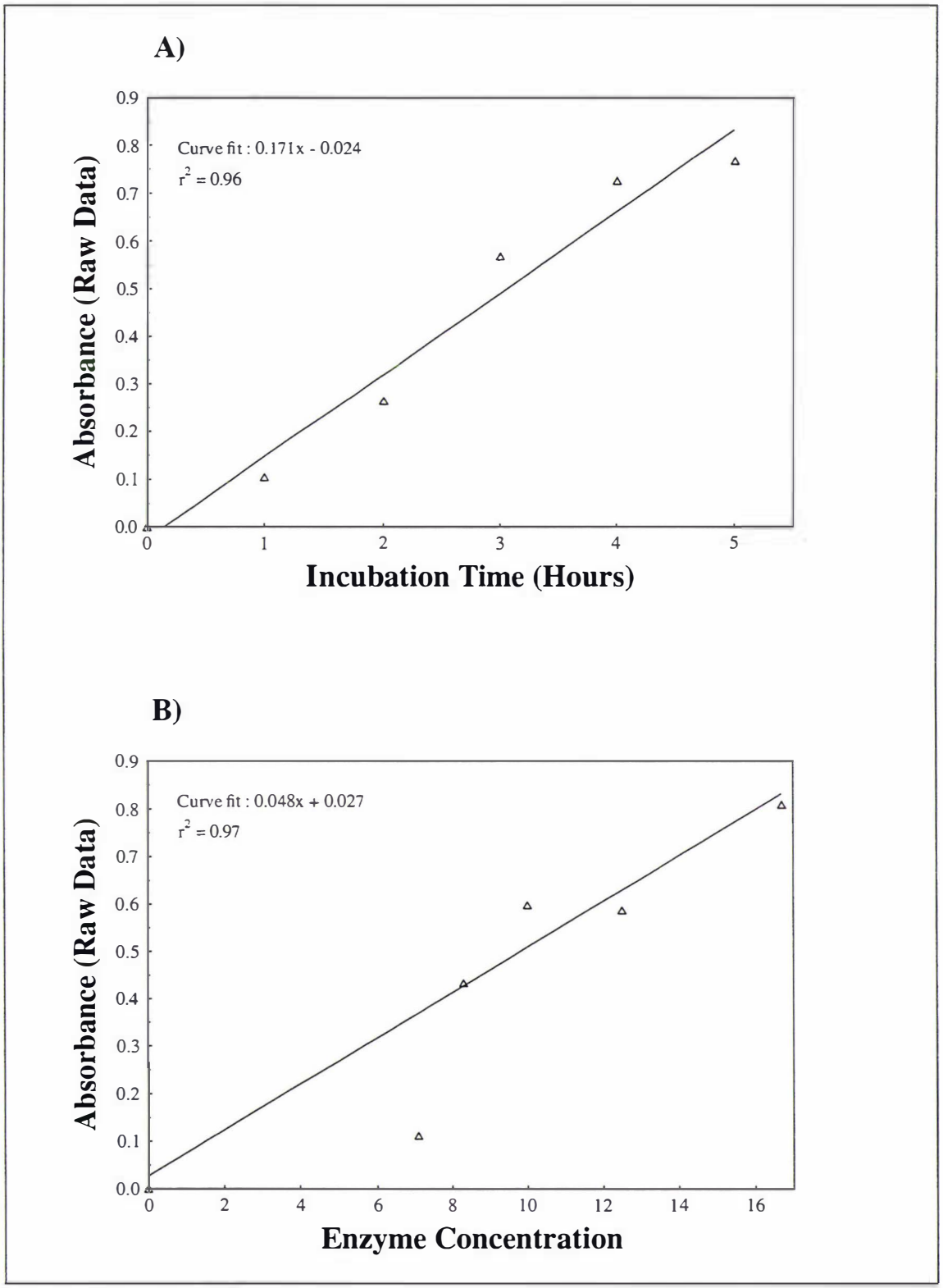


Figure 4-2. Linearity of *p*-nitrophenol absorbance (raw data) at 405 nm in the Roberts and Selitrennikoff (1988) assay, as affected by A) incubation time (hours) and B) enzyme concentration. Enzyme concentration is expressed as a percentage of undiluted, uncured, inoculated, diseased (92/N/I/D) kiwifruit stem plug extract.

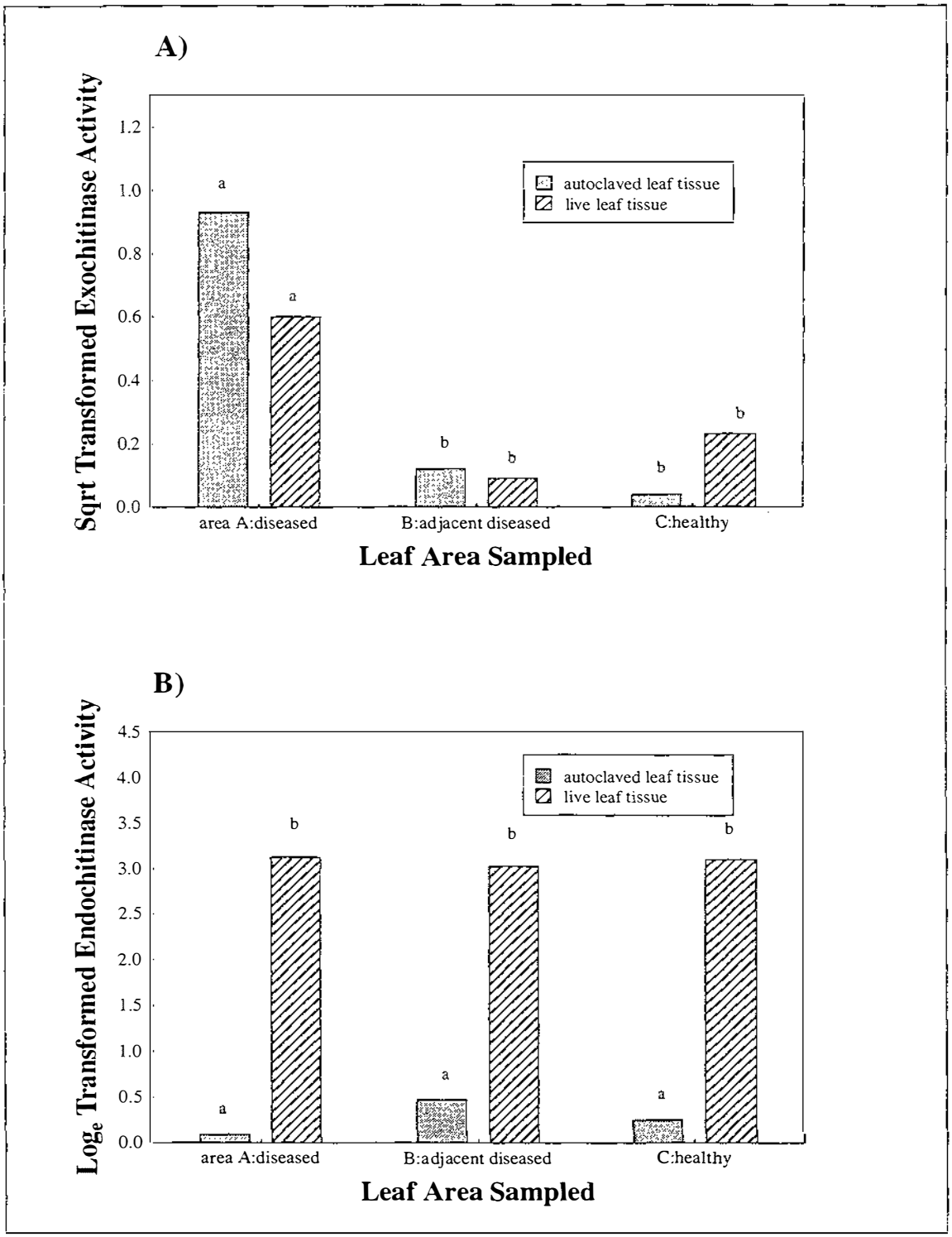


Figure 4-3. Chitinase activity from three separate areas of autoclaved and live kiwifruit leaves, assessed in 1992. A) exochitinase (square root transformed) and B) endochitinase ($\log_e$ transformed). Exochitinase activity is expressed as nmol of *p*-nitrophenol released per minute per ml of crude extract. Endochitinase activity is expressed as percent decrease in viscosity relative to the enzyme-free controls. a,b represent significant differences in Duncan's Multiple Range test (α=0.05).

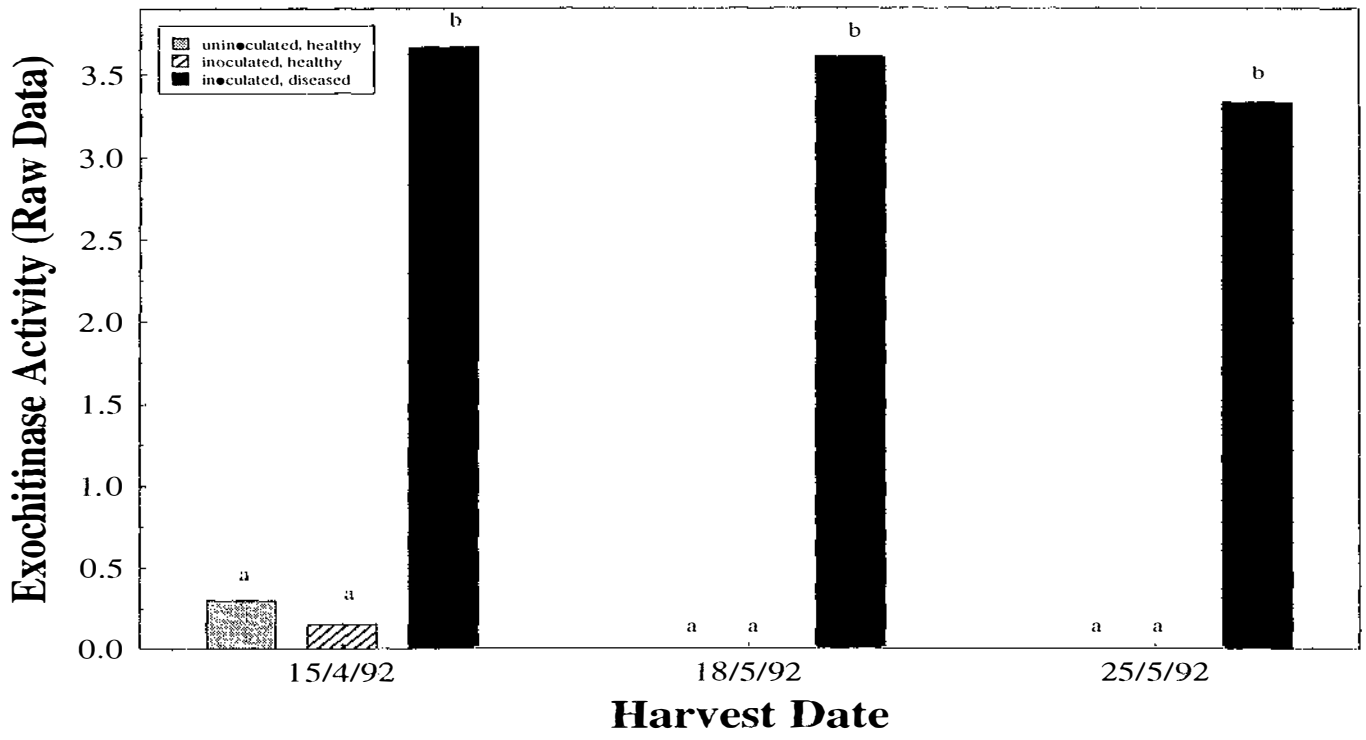


Figure 4-4. Exochitinase activity (raw data) of fruit in healthy and diseased stem plugs collected on three harvest dates in 1992 and not inoculated or inoculated (5,000 spores *B. cinerea* per stem scar), assessed after 12 weeks storage at 0 ± 0.3°C. Exochitinase activity is expressed as nmol of *p*-nitrophenol released per minute per ml of crude extract. a,b represent significant differences in Duncan's Multiple Range test (α=0.05).

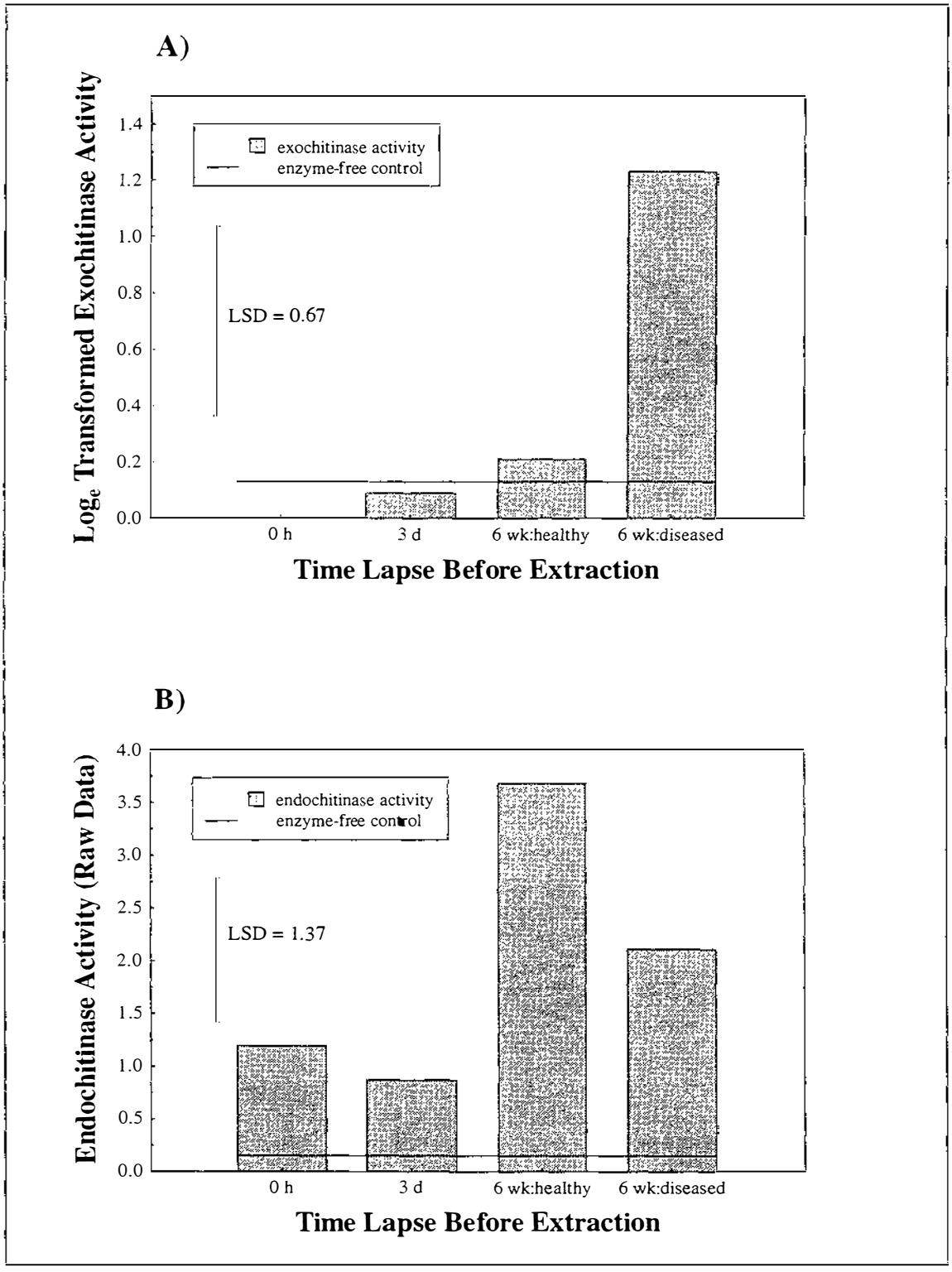


Figure 4-5. Chitinase activity in healthy and diseased kiwifruit stem plugs, assessed after 0 h, 3 d and 6 wk storage at 0 ± 0.3°C in 1993. A) exochitinase ($\log_e$ transformed) and B) endochitinase (raw data). Enzyme-free controls show averaged background noise. Exo- and endochitinase activities are expressed as nmol of N-acetylglucosamine released per minute per ml of crude extract. LSD = least significant difference.

B Leaf endochitinase activity

Endochitinase activity was only associated with live leaves and similar concentrations were found at all three sites (Fig. 4-3, B). Mean endochitinase activity in extracts of diseased and healthy areas of autoclaved leaves, where the plant component had been destroyed, did not differ from that of the enzyme-free controls (Fig. 4-3, B).

4.4.3 Experiment No. 5

Appreciable exochitinase activity was only associated with visibly infected stem plugs at all three harvests (Fig. 4-4). Harvest date had no significant effect on the level of exochitinase activity.

4.4.4 Experiment No. 6

A Stem plug exochitinase activity

Significant amounts of exochitinase activity were found only in diseased stem plugs (Fig. 4-5, A).

B Stem plug endochitinase activity

Endochitinase activity was found in all stem plugs, regardless of the length of time in coolstorage and whether the fruit was diseased or healthy (Fig. 4-5, B). Activity was higher in inoculated healthy compared to diseased stem plugs.

4.5 DISCUSSION

Negligible background activity associated with non-enzymic substrate hydrolysis (Experiment 1) together with a linear assay response (Experiments 2-3) confirmed that the Roberts & Selitrennikoff (1988) procedure was suitable for measurement of exochitinase activity in the kiwifruit system (refer Chapter 5, Section 5.5 for an evaluation of the various endochitinase assays).

Results from two seasons' data, using three assays on two different host tissues, showed that exochitinase activity was consistently associated with *B. cinerea* infection, while

endochitinase activity was associated with live plant tissue. Plant chitinases are generally endochitinases (Boller 1988; Graham & Sticklen 1994), bacterial chitinases are mainly exochitinases (O'Brien & Colwell 1987; Roberts & Selitrennikoff 1988), while fungal chitinolytic enzymes have exo- or endo- modes of action (Tracey 1955; Di Pietro et al. 1993; Harman et al. 1993; Hodge et al. 1995). Frändberg & Schnürer (1994) suggested that bacterial chitinases could exhibit endochitinase activity, but gave no conclusive evidence in support of this statement. Although bacterial endochitinases occur rarely in nature, they have been recorded on occasion (Greenwood et al. 1993). Much of the current literature on fungal chitinolytic systems does not clearly distinguish exo- and endochitinase activity. Hodge et al. (1995) found that exochitinase activity was predominant over endochitinase activity in five species of fungi. The reverse case was found to be true in a comprehensive review of fungal chitinases (Sahai & Manocha 1993). In the current study, the detection of exochitinase but not of endochitinase activity in killed, diseased tissue suggests that exochitinases are predominant in *B. cinerea.*

McLeod & Poole (1994) recorded exochitinase activity in both inoculated and uninoculated fruit. However, it is difficult to assess the significance of these results since the levels of exochitinase activity were small and variable. Also, there were no obvious trends in their data, and their measurements were made on fruit without visible signs of infection or pathogen growth. Sharrock & Hallet (1992a) found that exochitinase activity (with two exceptions) was only associated with visibly decayed fruit, four weeks or more after inoculation. In our study, no definite exochitinase activity was detected in kiwifruit tissue without visible signs of infection. Studies on the role of chitinases in kiwifruit defence against *B. cinerea* should therefore focus on endo- rather than exochitinase activity.

Defence mechanisms other than chitinase appeared responsible for "resistant" fruit in Experiment 6, which were inoculated but did not develop infection. Chitinase activity was higher in inoculated healthy than in diseased stem plug extracts in this experiment, but this trend was not observed in other trials (see Experiments 1 and 6, Chapter 6, Section 6.4.1 and 6.4.6. Note that the presence of fungal exochitinases, which were not

distinguished by the Molano et al. (1977) assay, may be a confounding factor in these Chapter 6 experiments). Increases in phenylpropanoid metabolites are frequently observed as an initial response of plants to injury and infection, and probably contributed to early resistance in kiwifruit also (McLeod & Poole 1994).

Having established the need to measure endochitinase activity, selection of a reliable assay to quantify activity in the kiwifruit system was the objective of the next piece of research.

5 ENDOCHITINASE ASSAY DEVELOPMENT

Chitinase activity can be measured by turbidometric, fluorometric, colorimetric, viscometric or radiometric methods. Although numerous assays are available, quantitative measurement is fraught with difficulty because the natural substrate of chitinases is insoluble, rendering kinetic measurements difficult (Cabib 1987). Moreover, heterogenous particle sizes in substrate suspensions can produce highly variable results, and many of the available methods are inconvenient or cumbersome (Roberts & Selitrennikoff 1988; Hackman & Goldberg 1964; Molano et al. 1977). Measurement of kiwifruit chitinase activity presents special challenges because chitinases comprise only a fraction of the extremely small amount of total protein present in stem plugs, and activity is induced to a lesser extent than in other crops. For example, ten to thirty-fold increases in chitinase enzymes in tobacco were observed following four days exposure to 20 µl/l ethylene (Keefe et al. 1990). Inoculation of pea pods with *Fusarium solani* increased activity of a chitinase isoform sixty-fold in 28 h (Mauch et al. 1988a), and up to six hundred-fold increases in chitinase were recorded in lesions of cucumber infected by *Colletotrichum lagenarium* (Metraux & Boller 1986). A total protein content of 1.4 mg/ml was present in a crude extract of beans (Boller et al. 1988), and application of 10 µl/l ethylene over 24 h increased activity thirty-fold (Boller et al. 1983). These examples are in contrast to a three-fold increase in kiwifruit chitinase activity one week after inoculation with *B. cinerea* (McLeod & Poole 1994), and to an average mean total protein content of 0.10 ± 0.01 mg/ml (mean ± SEM) measured in crude extracts used in the current study. In the search to find a procedure sufficiently sensitive to quantitatively measure endochitinase activity in kiwifruit, three of the seven assays tested were considered worthy of further development to the specific requirements of the kiwifruit system.

The overall purpose of this work was to select assays that would meet one or both of the following requirements:

1) quantitative measurement of endochitinase activity and resolution of the concentration differences normally encountered in the kiwifruit system,
2) quick and simple detection of the presence/absence of endochitinases in the large numbers of fractions generated by chromatography procedures used in the process of protein purification.

5.1 SECTION 1 - CALCOFLUOR PETRI DISH ASSAY

5.1.1 Introduction

Trudel & Asselin (1989) detected the presence of chitinases in glycol chitin gels by staining with Calcofluor white. The fluorescent stain attached to undegraded glycol chitin, but unbound stain was washed from enzyme-digested areas which did not then fluoresce under UV light. Sharrock & Gill (unpubl. data) adapted this method as a qualitative test for the presence of kiwifruit chitinase activity by placing kiwifruit extracts into wells within petri dishes containing glycol chitin agar. After incubation they added Calcofluor white to visualize non-fluorescent zones of substrate degradation around the wells (refer Chapter 2, Section 2.2.4, Part C for assay details). For the assay to be used quantitatively, there must be a definable relationship (preferably linear) between diameter of the reaction zone and enzyme concentration. The relationship between assay sensitivity and resolution must also be established.

5.1.2 Materials And Methods

The basic assay protocol is given in Chapter 2, Section 2.2.4, Part C with specific modifications described below. All stem plug extracts were diluted with NaOAc extraction buffer.

Stem plug extracts used in the course of this work were classified by the system described in Chapter 2 (Section 2.2.2). Exact concentrations of these extracts were unknown, so results are expressed as a percentage of the undiluted extract (100%).

A Linearity test

Stem plugs of uninoculated, healthy fruit pooled from the various harvest dates of Experiment 1, Chapter 3 (Section 3.3.1), and bulk extracted in five volumes of NaOAc buffer after 12 weeks coolstorage at 0 ± 0.3°C, were used to prepare chitinase extract 92/N/U/H. Aliquots (30 µl) of neat extract, 5, 10, 20, 50 and 100-fold dilutions of this extract were incubated for 24 h in 0.01% glycol chitin agar plates. Water (30 µl) was included as a negative control. Radii of degraded glycol chitin regions, measured as described in Chapter 2 (Section 2.2.4, Part C), were plotted against $\log_{10}$ percentage enzyme concentration.

B Sensitivity versus resolution

i 2-5 h incubation

The ability of the Calcofluor petri dish assay to resolve a range of concentration differences normally encountered in the kiwifruit system was determined by assaying, in triplicate, neat, two and five-fold dilutions of 94/C/U/H extract following two and five hours incubation. This 94/C/U/H extract was produced from uninoculated, healthy stem plugs of fruit harvested with attached pedicels on 23 May 1994 from Levin, which were cured stem scar uppermost in open trays for one week at 20°C following pedicel removal, and then stored at 0 ± 0.3°C for 12 weeks. After this time, stem plugs were removed from healthy fruit and extracted in three volumes of NaOAc. The extract was stored at -80°C until required.

Anion exchange fractions of 94/C/U/H extract were used to test assay sensitivity with two and five hours incubation. These fractions were produced by exchanging 94/C/U/H extract into 20 mM Tris-HCl buffer, pH 8, using a 6,000 MWCO Bio-Rad Econo-Pac P6 cartridge (Chapter 2, Section 2.2.8, Part B), and injecting 2 ml of the resultant eluate onto an equilibrated Bio-Rad Econo-Pac Q anion exchange column. Adsorbed protein fractions were eluted after 15 min washing with a 0-1 M linear gradient of sodium chloride in 20 mM Tris-HCl, applied over 50 min. The flow rate was 1 ml/min and fractions were collected every 2 min. Fractions selected for use in the Calcofluor sensitivity test were fractions 1 and 17, comprising basic and acidic chitinases respectively, and 6 and 8 which contained negligible protein.

Radii of degraded glycol chitin areas were measured, and means and SEMs were calculated.

ii 5-48 h incubation

Plates containing 0.01, 0.05 or 0.1% glycol chitin concentrations were prepared. Neat extract, 2, 5, 10 and 50-fold dilutions of 94/C/U/H extract were incubated for 5 or 48 h. The dilution range tested encompassed concentration differences normally encountered in the kiwifruit system, through to extremely low enzyme concentrations (eg. 2% 94/C/U/H extract) that would not be detected in other assays. Water and extraction buffer negative controls, and anion exchange fractions 1 and 8 of undiluted 94/C/U/H extract (Chapter 5, Section 5.1.2, Part B, (i)), were also included to monitor assay sensitivity. All samples were assayed in triplicate. Radial means and SEMs were calculated.

5.1.3 Results

A Linearity test

There was a linear relationship between the radius of degraded glycol chitin enzyme areas and log enzyme concentration (Fig. 5-1). Glycol chitin degradation was not observed in negative controls (data not shown).

B Sensitivity versus resolution

i 2-5 h incubation

The contrast between degraded and undegraded areas of glycol chitin was much less distinct and radial measurements were consequently more variable in plates that had been incubated for 2 h compared to those which received 5 h incubation.

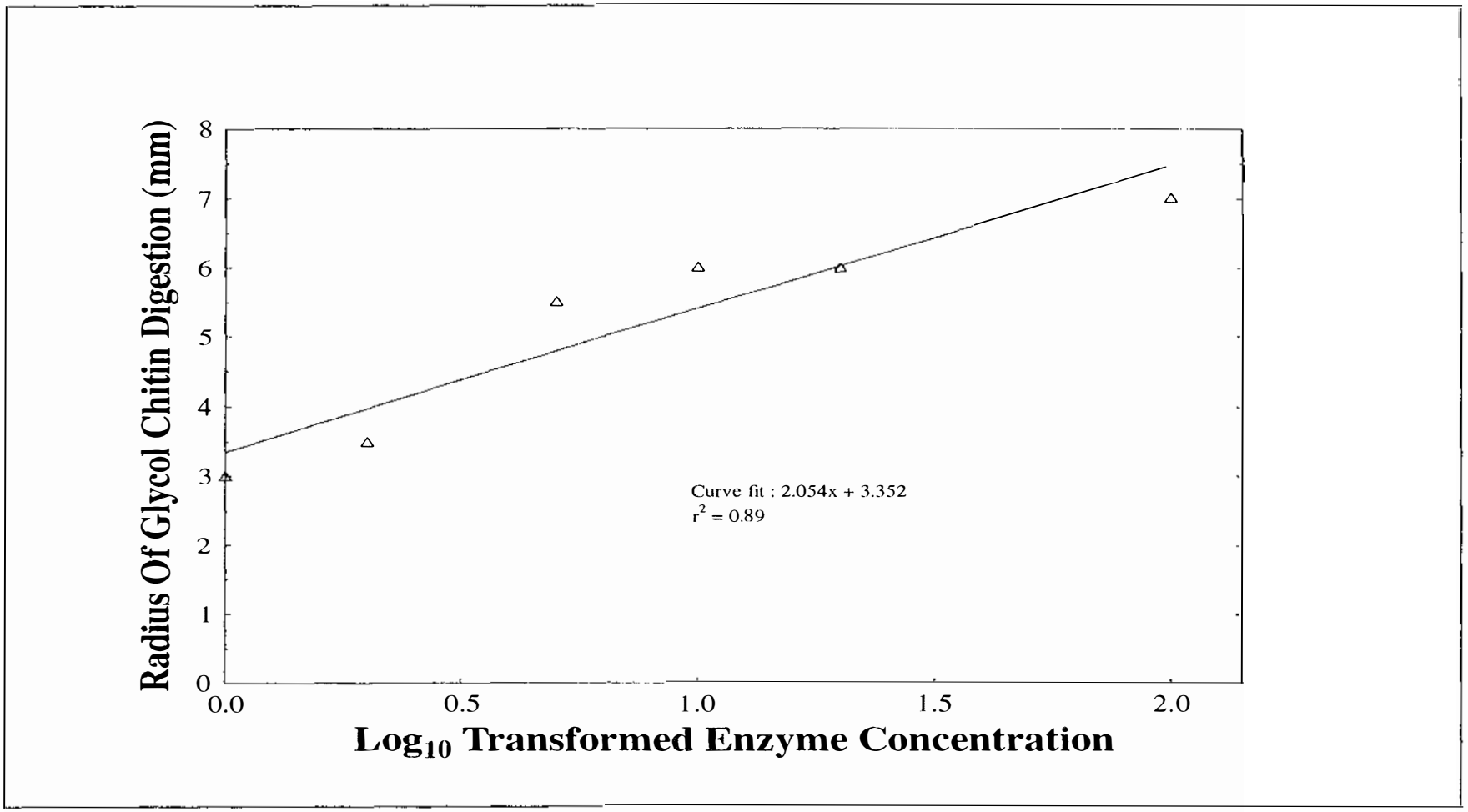


Figure 5-1. Relationship between area of glycol chitin substrate degradation (mm) and enzyme concentration in the Calcofluor assay after 24 h incubation at 37°C in 1993. Enzyme concentration is expressed as a percentage of undiluted, uncured, uninoculated, healthy kiwifruit stem plug extract (92/N/U/H).

Table 5-1: Chitinase activities (means ± SEM) of kiwifruit stem plug extracts, expressed as radial extension (mm) of enzyme-digested regions on glycol chitin plates after 2 and 5 h incubation at 37°C in 1994.

TEST SAMPLE	STAIN RADIAL EXTENSION (mm) AFTER 2 HOURS	5 HOURS
Concentrations of cured, uninoculated, healthy extract (94/C/U/H extract)		
100% conc. (undiluted)	2.5 ± 0.0	3.9 ± 0.1
50% conc. (2-fold dilution)	2.0 ± 0.1	3.7 ± 0.1
20% conc. (5-fold dilution)	1.8 ± 0.4	3.3 ± 0.2
Anion exchange fractions of undiluted 94/C/U/H extract		
Fraction 1 (basic chitinase)	0.7 ± 0.7	3.3 ± 0.0
Fraction 6 (negligible protein)	0.0 ± 0.0	0.0 ± 0.0
Fraction 8 (negligible protein)	0.0 ± 0.0	0.0 ± 0.0
Fraction 17 (acidic chitinase)	0.0 ± 0.0	2.0 ± 0.3

Two hours incubation was insufficient to detect chitinase activity in the anion exchange fractions, but 5 h incubation made it possible to detect activity in anion fractions 1 and 17 (Table 5-1). Radii of enzyme digested regions from extracts differing five-fold in concentration were only just resolved in the 5 h incubation (Table 5-1).

ii 5-48 h incubation

Enzyme digestion of glycol chitin was not found in water and extraction buffer negative controls (data not shown), regardless of substrate concentration and length of incubation.

An increased substrate concentration did not increase the stain intensity of undegraded regions, and therefore did not improve the contrast between degraded and undegraded areas. Radii of digested regions decreased with increased glycol chitin after 5 h incubation, but this trend was less apparent in samples that had been incubated 48 h (Table 5-2).

Radial measurements were larger, but margins of undegraded areas were less defined and radial measurements more variable when incubation increased from 5 to 48 h, regardless

of glycol chitin concentration (Table 5-2). After 5 h, ten-fold concentration differences were clearly resolved at all three glycol chitin concentrations, but after 48 h much larger concentration differences were required, particularly at the lower glycol chitin concentrations (Table 5-2).

After 5 h, the glycol chitin assay was highly sensitive and detected extremely low levels of activity in 2% 94/C/U/H extract that were not measurable in the Boller et al. (1983) and Molano et al. (1977) assays. With extended 48 h incubation, assay sensitivity increased as chitinase activity was detected in cation exchange fraction 8, where the protein content was virtually nil.

Table 5-2: Chitinase activities (means ± SEM) of kiwifruit stem plug extracts, expressed as radial extension (mm) of enzyme-digested regions on 0.01, 0.05 and 0.1% glycol chitin plates after 5 and 48 h incubation at 37°C in 1994.

TEST SAMPLE	% GLYCOL CHITIN	STAIN RADIAL EXTENSION (mm) AFTER 5 HOURS	48 HOURS
Concentration of 94/C/U/H extract			
100% conc. (undiluted)	0.01	3.8 ± 0.0	5.4 ± 0.6
50% conc. (2-fold diln)	0.01	3.6 ± 0.2	5.3 ± 0.7
20% conc. (5-fold diln)	0.01	2.8 ± 0.1	4.3 ± 0.1
10% conc. (10-fold diln)	0.01	2.5 ± 0.0	4.4 ± 0.4
2% conc. (50-fold diln)	0.01	2.0 ± 0.1	4.6 ± 0.3
Anion exchange fractions of 94/C/U/H undiluted extract			
Fraction 1 (basic chitinase)	0.01	3.5 ± 0.3	7.4 ± 0.1
Fraction 8 (negligible protein)	0.01	0.0 ± 0.0	3.9 ± 0.3
Concentration of 94/C/U/H			
100% conc. (undiluted)	0.05	3.3 ± 0.2	5.7 ± 0.3
50% conc. (2-fold diln)	0.05	3.1 ± 0.3	5.8 ± 0.6
20% conc. (5-fold diln)	0.05	2.8 ± 0.2	4.4 ± 0.4
10% conc. (10-fold diln)	0.05	2.3 ± 0.2	4.6 ± 0.6
2% conc. (50-fold diln)	0.05	0.9 ± 0.5	4.2 ± 0.5
94/C/U/H Anion exchange fractions			
Fraction 1 (basic chitinase)	0.05	2.8 ± 0.2	5.9 ± 1.2
Fraction 8 (negligible protein)	0.05	0.0 ± 0.0	1.9 ± 1.0
Concentration of 94/C/U/H			
100% conc. (undiluted)	0.1	2.9 ± 0.2	8.3 ± 0.6
50% conc. (2-fold diln)	0.1	2.5 ± 0.2	5.9 ± 0.6
20% conc. (5-fold diln)	0.1	1.5 ± 0.8	6.0 ± 0.1
10% conc. (10-fold diln)	0.1	0.8 ± 0.4	5.2 ± 0.4
2% conc. (50-fold diln)	0.1	0.0 ± 0.0	4.8 ± 0.3
94/C/U/H Anion exchange fractions			
Fraction 1 (basic chitinase)	0.1	2.6 ± 0.2	6.2 ± 0.2
Fraction 8 (negligible protein)	0.1	0.0 ± 0.0	0.8 ± 0.8

5.2 SECTION 2 - STAGES IN THE MODIFICATION OF THE COLORIMETRIC ASSAY OF BOLLER ET AL. (1983)

5.2.1 Introduction

Measuring the liberation rate of N-acetylglucosamine from chitin by assays such as the colorimetric assay of Boller et al. (1983) is one of the most widely used methods of quantifying chitinase activity (Stirling et al. 1979). The principle and methodology of this assay are described in detail in Chapter 2 (Section 2.2.4, Part D). Briefly, incubation of insoluble chitin with chitinases releases soluble intermediate chain length chitin oligomers, and N-acetylglucosamine dimers and monomers. The oligomeric products are then further degraded into monomers by snail gut enzyme during a second incubation step. In a third step, dimethylaminobenzaldehyde (DMAB) reagent reacts with the monomers to produce a coloured product.

Preliminary measurements of chitinase activity in crude infected kiwifruit extracts produced low, variable absorbances (0.05 ± 0.01). The effect of substrate concentration on absorbance was tested in an initial attempt to improve sensitivity. Although increased substrate concentration elevated the final absorbance, it was impractical for large scale testing as the substrate became too viscous to dispense accurately. Alternative methods of increasing sensitivity and reducing variability were required.

A First and second incubations

An increased concentration of crude infected kiwifruit extract had no significant effect on final absorbance, indicating that either the enzyme concentration was non-limiting or that the length of the first incubation period was too long, allowing adequate time for even the smallest amount of enzyme to digest all available substrate. Ideally, the length of the first incubation should be short enough for the reaction to be in a linear phase so that differences in enzyme activity can be resolved, whilst the second incubation should go to completion, allowing all oligomers produced by chitinolytic digestion to be converted into N-acetylglucosamine. These incubation conditions needed to be optimised for the kiwifruit system.

B Reduction in variability

The previous investigations highlighted the need to address the problem of variability. In the original protocol, the first reaction was terminated by removal of insoluble, undegraded substrate by centrifugation. However, if small particles of undegraded substrate are not precipitated by centrifugation, chitinases in the supernatant could continue digestion on beyond the first incubation, thereby contributing to variable results. The use of Millipore tubes with in-built filters to remove substrate was considered, but the tubes were not recyclable, making the cost prohibitive. Denaturing chitinases by boiling, before centrifugation to remove substrate, would ensure the end of the first reaction, but it was necessary to determine:

1) whether boiling could cause non-enzymic breakdown of chitin polymers, and
2) whether snail enzyme involved in the second incubation could degrade chitin polymers.

Other potential causes of variability investigated were the degree of variability associated with individual batches of snail gut enzyme, and methods of shaking to improve mixing and hence enzyme access to the substrate.

C Increasing sensitivity

Since it was not possible to increase substrate concentration, different substrates which might offer more accessible enzyme attachment sites were tested in a further attempt to increase sensitivity.

5.2.2 Materials And Methods

The general assay protocol is given in Chapter 2, Section 2.2.4, Part D, with specific modifications described within this section. In earlier experiments, the initial reaction mix included all the components listed in Chapter 2 (Section 2.2.4, Part D) which, together with the addition of 0.2 ml of water, is Sharrock's (unpublished) modification of the Boller et al. (1983) assay. This water diluted the active components and hence was omitted in later experiments, resulting in a 3.6-fold increase in absorbance.

Unless otherwise stated, uncured, inoculated, diseased kiwifruit stem plug extract (92/N/I/D), prepared as described in Chapter 4 (Section 4.3.1, Part B), was used in all the Boller et al. (1983) assay experiments. On specified occasions, *Streptomyces griseus* chitinase (Sigma C-6137, 1 mg/ml in 10 mM NaOAc buffer) was used as a standard to check the assay procedure.

Microscopic examination of the substrate revealed diverse particle sizes, so the suspension was agitated on a magnetic stirrer during aliquot removal to ensure representative sampling.

Throughout the developmental process, filtered fresh solutions of reactants were used to reduce variability. Measurement of absorbances with an ELISA plate reader would have offered the advantage of simultaneous measurement of large numbers of samples, but it was rejected since a spectrophotometer, with its longer pathlength measurements, was more accurate.

A First and second incubations

There were four incubation condition experiments, each with two replicates per treatment. Data transformations were not required since the required conditions of ANOVA (normality and homogeneous variances) were already satisfied (refer Chapter 2, Section 2.3.1).

i Length of the first incubation

Activity of standard reaction mixes (including 0.2 ml water) was determined after first incubation durations of 0, 1, 2, 3, 4 or 5 h. Treatment effect on absorbance was analysed as a completely randomised design (CRD).

ii Length of the second incubation

Absorbance of reaction mixes (containing 0.2 ml of water) was measured after a standard first incubation of 2 h, and second incubations of 0.5, 1, 1.5, 2, 2.5, 3 or 3.5 h. Absorbance data was analysed as a CRD.

iii pH and temperature of the first incubation

Using standard volumes (0.1 ml) of pH 4, 4.5, 5 or 5.5 NaOAc buffers in the initial reaction mix, (no 0.2 ml water), the first incubation was conducted at 25, 30 and 37°C. The experiment was analysed as a factorial design.

iv Length and temperature of the second incubation

Standard reaction mixes, without the 0.2 ml water, received the standard first incubation, then the second incubation was carried out at 37 or 42°C for 0, 1, 2, 3 or 4 h. A factorial design was used.

B Reduction in variability

For each of the three experiments described in this section, absorbance data was analysed as a CRD. A square root transformation was necessary in each case to satisfy the assumptions of ANOVA.

i Heat stability of the substrate

Substrate aliquots were boiled for 1, 3 and 10 minutes, then cooled to room temperature. Activity was assayed in reaction mixes containing the usual NaOAc buffer, *S. griseus* chitinase, 0.2 ml water and boiled or untreated substrate.

ii Variability of snail gut enzyme batches

Activity was assayed in standard reaction mixes, with 0.2 ml water and *S. griseus* chitinase, incubated in the normal manner, but with three different batches of snail gut enzyme or without snail gut enzyme. The ability of snail enzyme to attack chitin polymers was tested by omitting the first incubation step.

iii Variability of different mixing methods

Standard reaction mixtures with 0.2 ml water were agitated in Eppendorf tubes and flat-bottomed cryovials (Nalgene) attached in vertical and horizontal positions throughout the first incubation. This was followed by the standard assay procedure.

C Increasing sensitivity

Three new colloidal chitin substrates were produced using a modification of the method of Shimahara & Takiguchi (1988) using bleached crab shell chitin (Sigma), washed Sigma crab shell chitin, and undyed, coarsely ground cuttlefish chitin (Calbiochem). The modified protocol for colloidal chitin substrate preparation is given in Appendix III.

Crab shell chitin was washed by first mixing 10 g of chitin in 175 ml cold HCl (Analar, BDH). After 12 h at 4°C, the mixture was filtered through glass wool into 1 l of cold ethanol (Analar, BDH), centrifuged at 8,700*g* for 20 minutes and resuspended in 1 l of water. The centrifuge and water resuspension steps were repeated a further five times to bring the chitin to a neutral pH. Finally water was removed under vacuum filtration and the chitin lyophilized to dryness over 48 h.

Under standard assay conditions, the new substrates were tested alongside the "old" substrate (reacetylated chitosan, described in Chapter 2, Section 2.2.3, Part B) using 100, 50 and 20% uncured, uninoculated, healthy 93/N/U/H extract. This extract was obtained from stem plugs pooled from various harvest dates of Experiment 2, Chapter 3 (Section 3.3.2) and extracted in five volumes of NaOAc buffer after 12 weeks coolstorage at 0 ± 0.3°C.

Treatment effect on absorbance was analysed as a split plot experiment, where the three extract concentrations were treated as main plot treatments and the four substrates as split plot factors. Data transformation was not required.

5.2.3 Results

A First and second incubations

i Length of the first incubation

ANOVA indicated that the length of incubation had a significant effect on absorbance (P=0.021). Absorbance increased with incubation durations of up to 2 h, but beyond this increases in absorbance were not significant (Fig. 5-2, A).

ii Length of the second incubation

The effect of duration of the second incubation on absorbance was non-significant (P>0.05).

iii pH and temperature of the first incubation

Only the effect of temperature was significant (P=0.0301). Irrespective of pH, absorbance readings from samples incubated at 37°C were significantly higher than those in samples incubated at 25°C, whilst absorbances at 30°C were intermediate between the two (Fig. 5-2, B). However, the temperature effect was reversed when this experiment was repeated, i.e. absorbance was highest at 25°C, followed by 30°C and then 37°C.

iv Length and temperature of the second incubation

Only the length of the second incubation had a significant effect on absorbance (P=0.0077). However, whilst differences were statistically significant, they were biologically meaningless. Absorbance fluctuated with incubation time in an unpredictable manner, indicative of the highly variable nature of the assay. For example, absorbance after 1 h was significantly higher than absorbance after 3 h (Fig. 5-2, C). Fluctuations in absorbance were also observed in the previous experiment where the length of the second incubation was investigated (Section 5.2.3, Part A, (ii)), but these fluctuations occurred at different points in time and were not significant.

Appreciable absorbance was measured in diseased stem plug extracts at 0 h (Fig. 5-2, C).

v Overall results from first and second incubation experiments

Omission of water from the initial reaction mix increased absorbances from the 0-0.15 range encountered in Fig. 5-2, A to the 0.25-0.55 range found in the later experiments (Fig. 5-2, B and C).

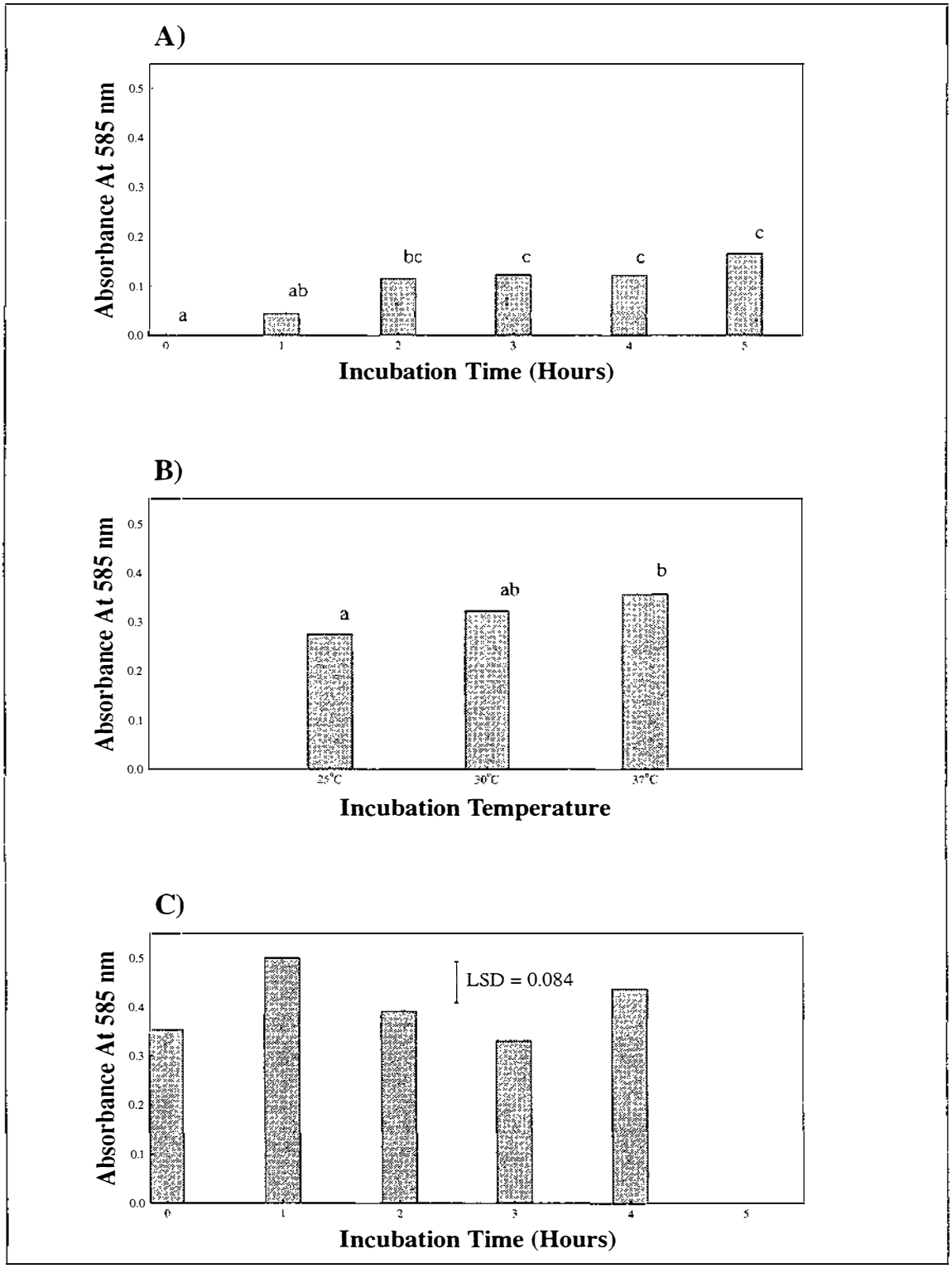


Figure 5-2. Absorbance (raw data) of uncured, inoculated, diseased kiwifruit stem plug extract (92/N/I/D) in the Boller et al. (1983) assay, as influenced by A) first incubation time, B) first incubation temperature and C) second incubation time, measured in 1993. LSD = least significant difference. a,b,c represent significant differences in Duncan's Multiple Range test (α=0.05).

B Reduction in variability

i Heat stability of the substrate

The enzyme-free control had a significantly lower absorbance than that of all other treatments (P=0.0001). There were no significant differences between treatments where substrate was boiled for up to ten minutes, or not boiled (Fig. 5-3, A).

ii Variability of snail gut enzyme batches

Absorbances in all treatments (excluding the treatment with no first incubation) were significantly higher than the "enzyme-free control" which lacked chitinase (P=0.0001 and Fig. 5-3, B). Absorbance was significantly affected by different snail gut enzyme batches (Fig. 5-3, B). There was also appreciable absorbance in the treatment without snail gut enzyme (Fig. 5-3, B).

iii Variability of different mixing methods

Choice of mixing method significantly affected absorbance (P=0.0097). Mixing samples in flat bottomed cryovials was more effective than using tapered Eppendorf vials, and best results were achieved when the tubes were vertically orientated (Fig. 5-3, C)

iv Summary of reduction in variability

Overall absorbances were higher in the two experiments where commercial *S. griseus* chitinase was used to degrade chitin (Fig. 5-3, A and B), than in the experiment where kiwifruit chitinase was used (Fig. 5-3, C).

There was less variation in this series of three experiments (Fig. 5-3) than in earlier experiments (Fig. 5-2).

C Increasing sensitivity

The significant interaction term (P=0.0001) in the ANOVA indicated that the effect of enzyme concentration on absorbance varied with the type of substrate used (Fig. 5-4, A). Absorbance increased with enzyme concentration for all the substrates derived from

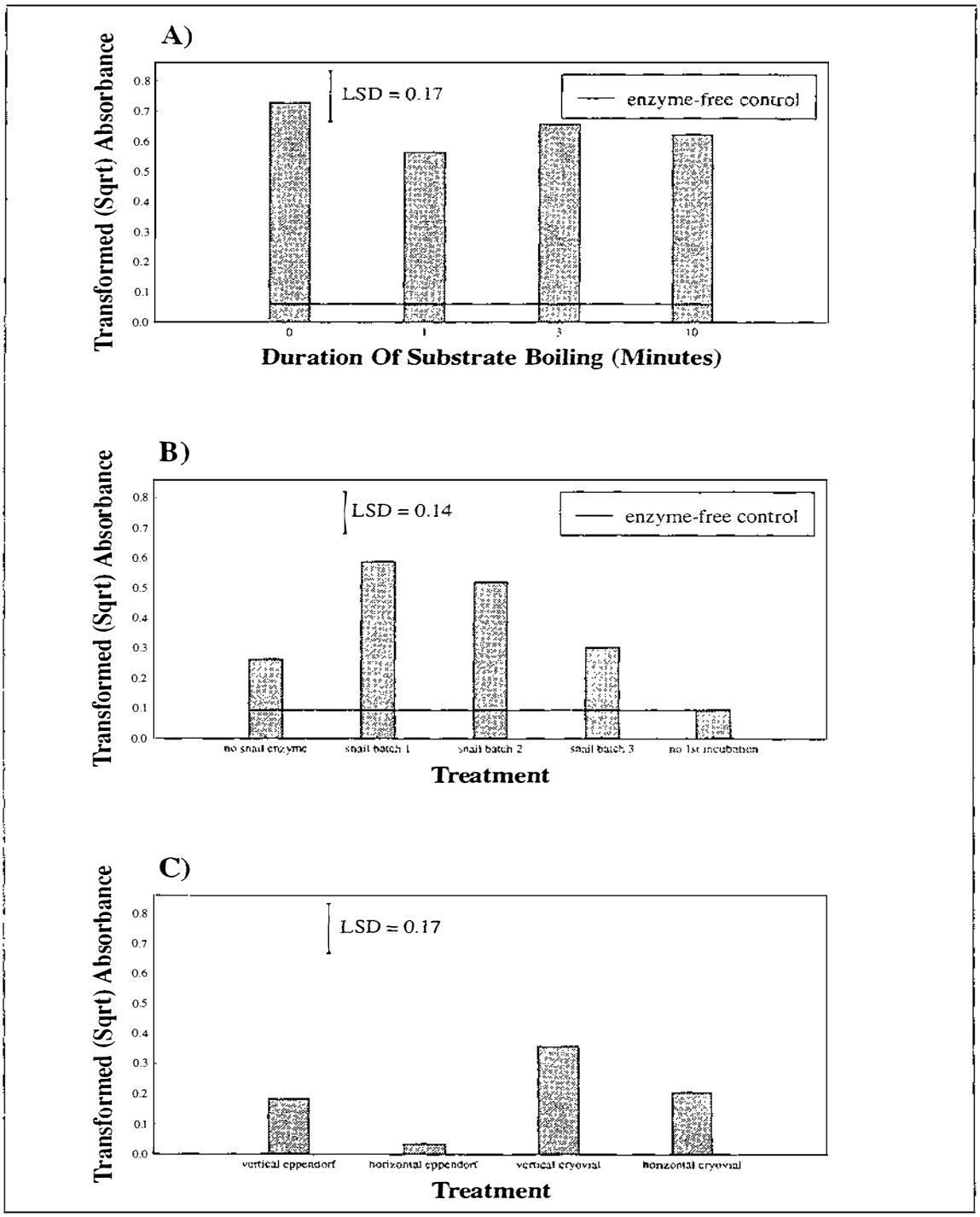


Figure 5-3. Absorbance (square root transformed) at 585 nm in the Boller et al. (1983) assay, as affected by A) substrate heat stability, B) presence/absence of different batches of snail gut enzyme, and C) variability of different mixing methods, measured in 1993. *S. griseus* chitinase was used in A) and B), and uncured, inoculated, diseased kiwifruit stem plug extract (92/N/I/D) was substituted for *S. griseus* chitinase in C). The enzyme-free controls in A) and B) show averaged background noise. Batch 3 of snail gut enzyme was used in the "no 1st incubation" treatment in B). LSD = least significant difference.

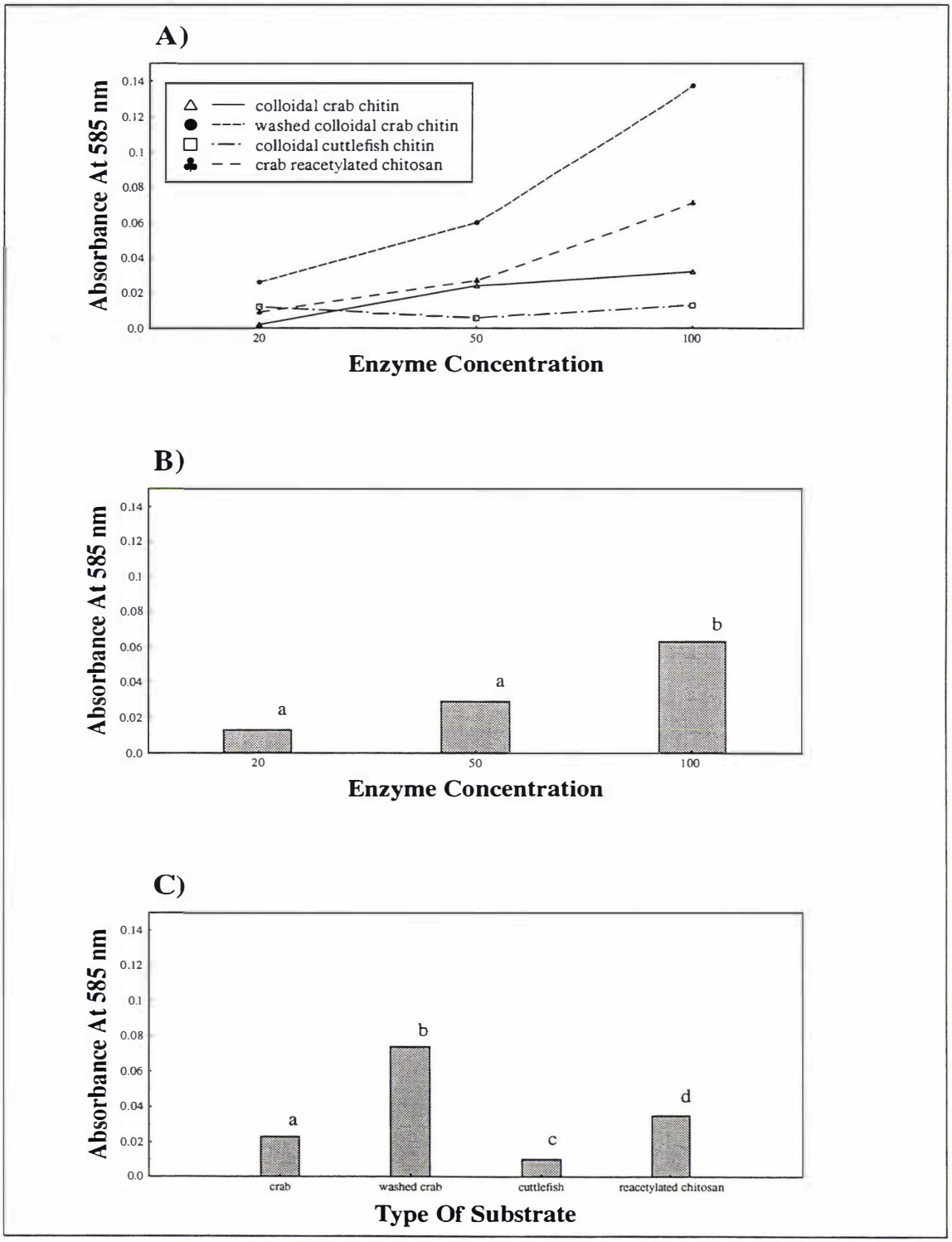


Figure 5-4. Absorbance (raw data) of uncured, uninoculated, healthy kiwifruit stem plug extract (93/N/U/H) in the Boller et al. (1983) assay, as affected by A) interaction between enzyme concentration and type of substrate, B) enzyme concentration averaged over substrate type, and C) substrate type averaged over enzyme concentration, measured in 1994. Enzyme concentration is expressed as a percentage of undiluted 93/N/U/H extract. a,b,c represent significant differences in Duncan's Multiple Range test (α=0.05).

crab but not for the colloidal cuttlefish chitin (Fig. 5-4, A). LSD values were not included on the interaction graph (Fig. 5-4, A), as the data was unbalanced (and Duncan's Multiple Range test only applies to main effects).

Type of substrate (P=0.0001) and enzyme concentration (P=0.025) main effects were also both significant. Absorbances of the 20 and 50% concentrations of 93/N/U/H extract were both low, but that of the undiluted extract was significantly higher (Fig. 5-4, B).

A significantly different absorbance was associated with each type of substrate (Fig.5-4, C). Chitin sourced from crab was a more suitable substrate than that from cuttlefish. Acetylated chitosan gave higher absorbances than unwashed colloidal chitin, but washed colloidal chitin was the most hydrolysed substrate (Fig. 5-4, C).

5.3 SECTION 3 - RADIOMETRIC ASSAY OF MOLANO ET AL. (1977)

5.3.1 Introduction

Despite the extensive modifications to the Boller et al. (1983) assay, it was considered too variable and insensitive to accurately quantify kiwifruit endochitinase activity. The Molano et al. (1977) radiometric assay, with modifications described by Cabib (1988), is regarded as the most sensitive chitinase assay available (Cabib 1987; Wirth & Wolf 1990) and is the assay of choice for most researchers (Roberts & Selitrennikoff 1988). Assay conditions such as incubation length and temperature require optimisation for the kiwifruit system.

The low energy and short range emission of tritium particles are impossible to measure with 100% efficiency by liquid scintillation (Oldham 1968). Counting efficiency is often less than 30% due to chemical and colour quenching, a process in which molecules absorb UV without re-emission. Construction of a quench curve, to allow correction for internal quenching, is essential to determine absolute activity in samples.

5.3.2 Materials And Methods

The general assay protocol is given in Chapter 2 (Section 2.2.4, Part E), with specific modifications described below.

Spurious counts may result from floating particles of undegraded chitin which are not successfully removed by centrifugation (Cabib 1988). To minimise this problem, an additional filtration step, using Alltech filters, was included in the protocol (refer Chapter 2, Section 2.2.4, Part E).

Stem plugs were chosen to produce extracts with low and high chitinase activities:

1) Low chitinase activity extract (93/N/I/H)
Uncured fruit pooled from various harvest dates of Experiment 2, Chapter 3 (Section 3.3.2) were inoculated with 5,000 spores of *B. cinerea* per stem scar immediately after pedicel removal. Stem plugs free of infection following 12 weeks coolstorage at 0 ± 0.3°C were extracted in five volumes of NaOAc buffer.

2) High chitinase activity extract (94/C/U/H)
Section 5.1.2, Part B (i) describes production of this cured, uninoculated, healthy stem plug extract.

A Assay optimisation

i Length of incubation

Activity was assayed, in triplicate, in tubes containing 0, 50 or 100% low activity kiwifruit extract (93/N/I/H) diluted with NaOAc buffer, agitated at 25°C for 3, 6, 9 or 18 h. Treatment effect on chitin solubilization, as measured by disintegrations per minute (dpm), was analysed as a split plot experiment, where the three extract concentrations were treated as main plot treatments and the four incubation times as split plot factors. A square root transformation was necessary to satisfy the assumptions of ANOVA.

ii Temperature of incubation

Incubation temperature optimisation was investigated by incubating three replicate tubes

of 0, 50 or 100% 93/N/I/H extract at 25, 30 or 37°C for 18 h. Data were analysed as a split plot experiment, with extract concentration as the main plot treatment and temperature as the split plot factor. Data were square root transformed.

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

Tubes with 0, 17, 33, 67 or 100% low (93/N/I/H) or high (94/C/U/H) activity extract were incubated at 25°C for 18 h. Disintegrations per minute (raw data) were plotted against enzyme concentration (raw data) to determine if a relationship existed between these two factors.

The ability of the assay to resolve differences in enzyme concentration was determined by statistical analysis. Data from the two extracts were analysed as two separate CRD experiments. No transformation was required for the 93/N/I/H dpm data, but the 94/C/U/H dpm were $\log_e$ transformed to satisfy the assumptions of ANOVA.

B Quench curve correction

Machine (Beckman LS2800) counting efficiency was determined by reading counts per minute (cpm) in five Wheaton glass scintillation vials, each containing 9.92 ml of ASCII scintillant (Amersham), and 0.08 ml of a calibrated external standard - tritium labelled toluene (Amersham). After correction for time-based decay, the external standard had a radioactive concentration of 2.97 x 10^6 dpm per millilitre of toluene at 20°C. The degree of quenching associated with coloured enzyme extracts was assessed by repeating counts, following addition of 0, 0.02, 0.04, 0.08 or 1.6 ml of low activity 93/N/I/H extract. The extract was selected because of its strong yellow coloration. Data were used to generate a quench correction curve so that cpm could be converted to dpm.

5.3.3 Results

A Assay optimisation

i Length of incubation

There was no significant interaction between enzyme concentration and incubation time ($P>0.05$).

Samples incubated 18 h had significantly higher dpm than those incubated 3-9 h (P=0.0014 and Fig. 5-5, A).

Disintegrations per minute increased non-linearly with increasing enzyme concentration (P=0.0001 and Fig. 5-5, B). Some radioactivity was present in the enzyme-free sample (Fig. 5-5, B).

ii Temperature of incubation

The significant interaction term in the ANOVA (P=0.0121) indicated that the effect of incubation temperature on chitin solubilization varied with enzyme concentration (Fig. 5-6, A). Temperature had no effect on dpm in the enzyme-free (0% extract) samples. With 50% extract, 30°C produced the highest dpm followed by 25°C and 37°C. This order was reversed for 100% extract, hence the significant interaction (Fig. 5-6, A).

The incubation temperature main effect was not significant (P>0.05).

Disintegrations per minute increase non-linearly with increased enzyme concentration (P=0.0001 and Fig. 5-6, B). All three enzyme concentrations were significantly resolved (Fig. 5-6, B).

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

Assay progress curves were plotted on the same graph (Fig. 5-7) using unanalysed (raw) data. A quadratic relationship between enzyme concentration and dpm existed for both high (94/C/U/H) and low (93/N/I/H) activity extracts, although the curve was steeper for the high activity extract (Fig. 5-7). With enzyme concentrations below 40%, curves for both high and low activity extracts were sufficiently close to a straight line to be of use for determining enzyme activity (Fig. 5-7).

All concentrations of low activity extract (93/N/I/H) produced significantly different dpm (P=0.0003).

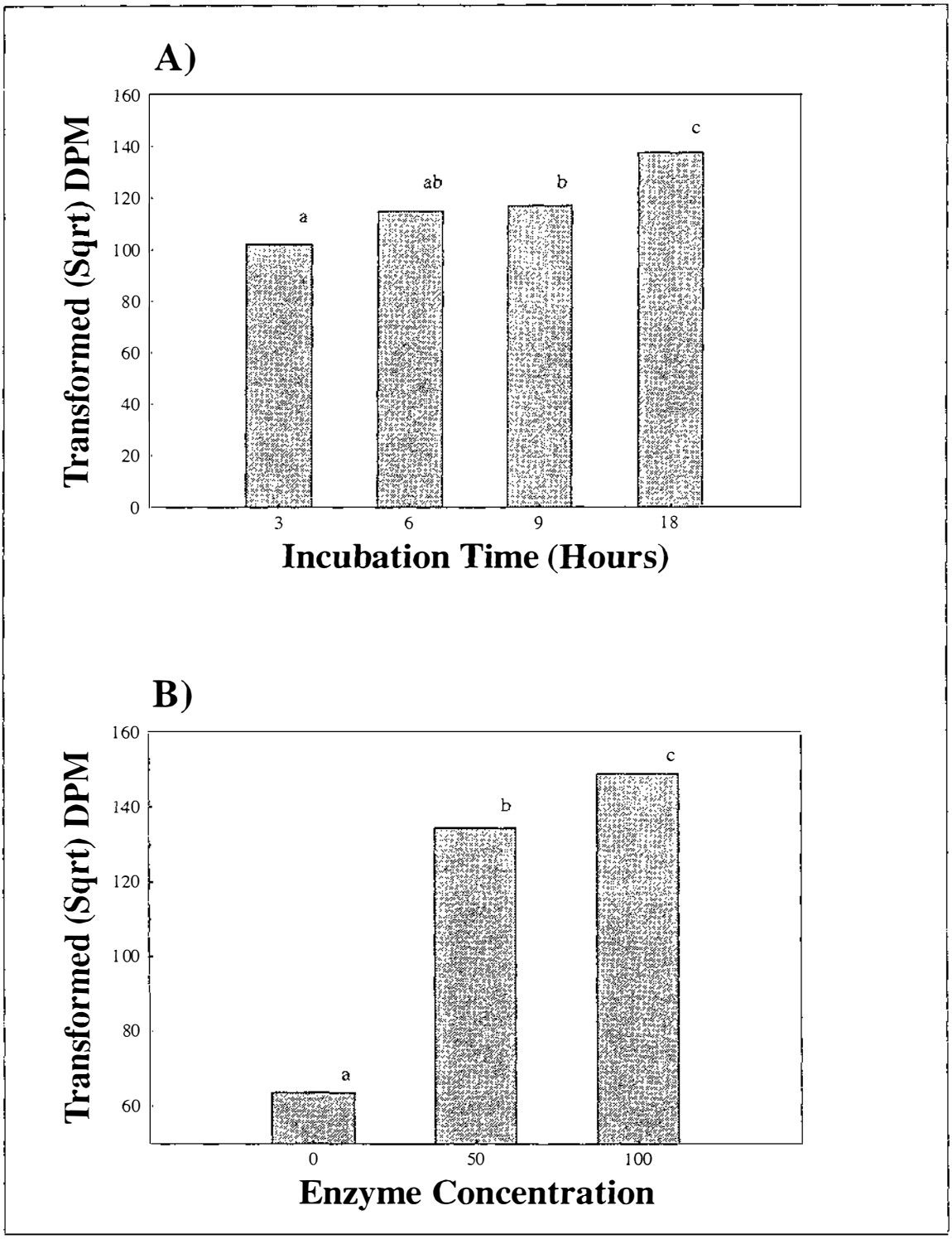


Figure 5-5. Number of disintegrations per minute (square root transformed) of uncured, inoculated, healthy kiwifruit stem plug extract (93/N/I/H) in the Molano et al. (1977) assay, as affected by A) incubation time averaged over enzyme concentration, and B) enzyme concentration averaged over incubation time, measured in 1994. Enzyme concentration is expressed as a percentage of undiluted 93/N/I/H extract. a,b,c represent significant differences in Duncan's Multiple Range test (α=0.05).

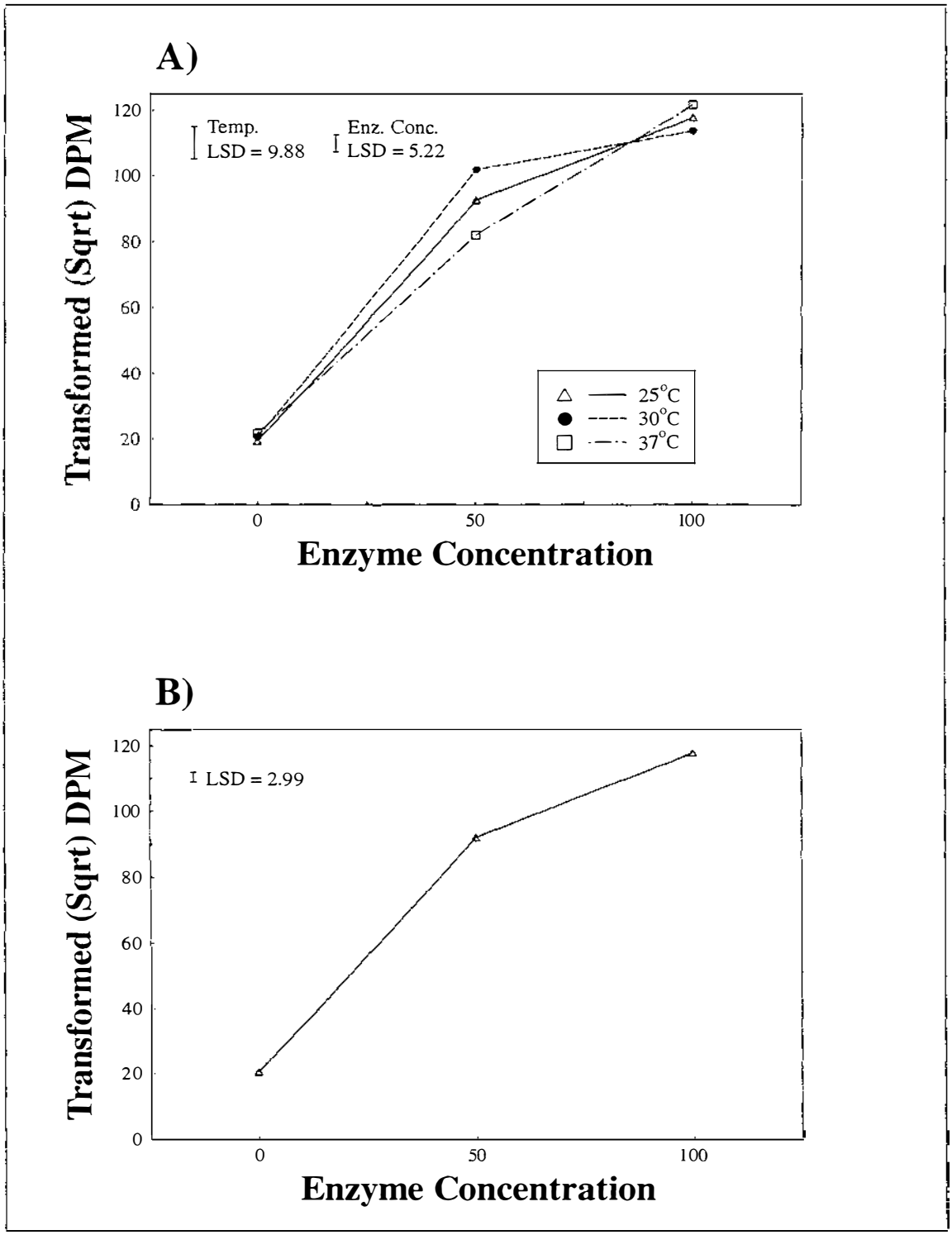


Figure 5-6. Number of disintegrations per minute (square root transformed) of uncured, inoculated, healthy kiwifruit stem plug extract (93/N/I/H) in the Molano et al. (1977) assay, as affected by A) interaction between enzyme concentration and temperature, and B) enzyme concentration averaged over temperature, measured in 1994. Enzyme concentration is expressed as a percentage of undiluted 93/N/I/H extract. LSD = least significant difference.

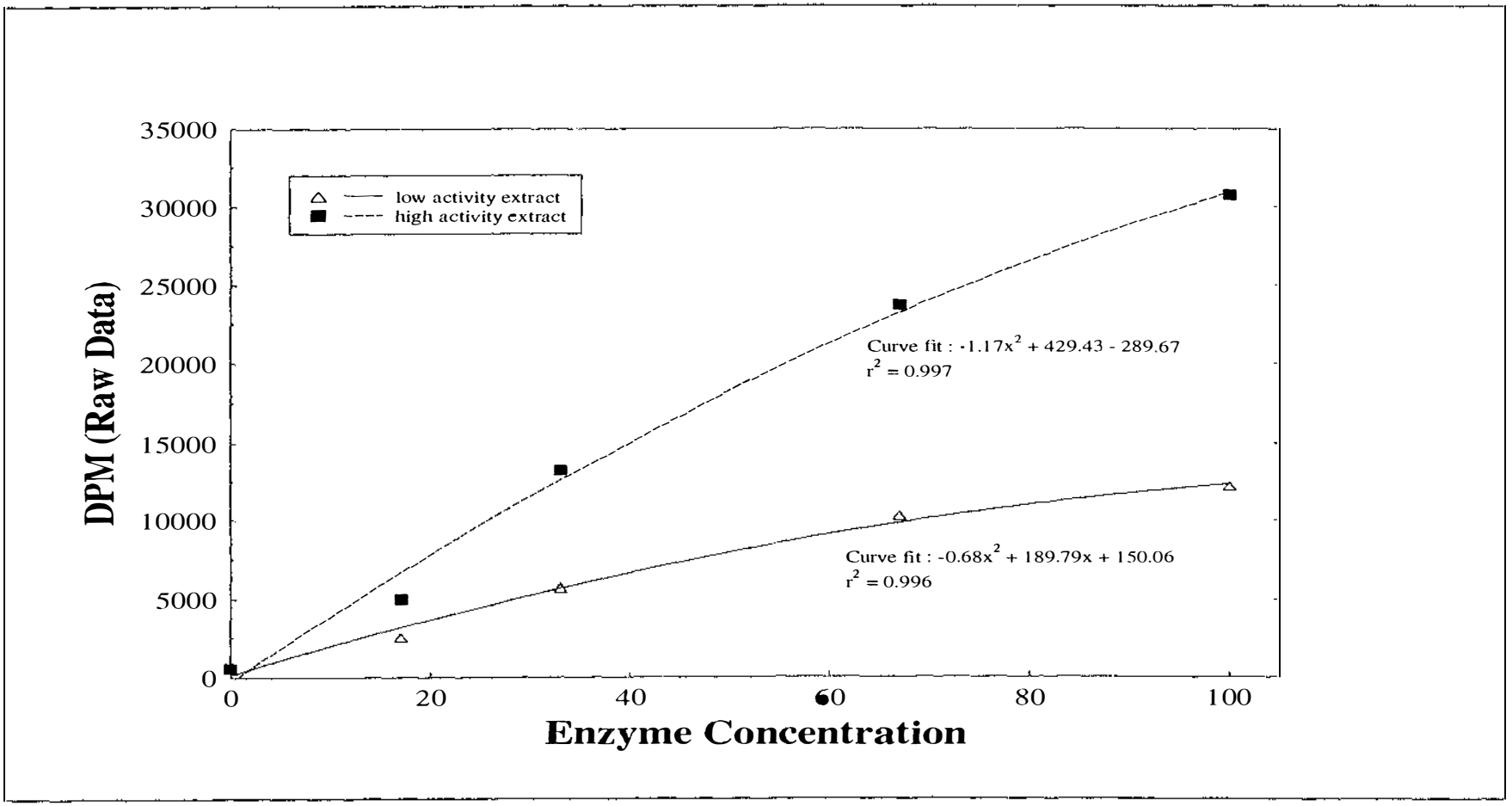


Figure 5-7. Relationship between enzyme concentration and number of disintegrations per minute (raw data) of low activity, uncured, inoculated, healthy kiwifruit stem plug extract (93/N/I/H) and high activity, cured, uninoculated, healthy (94/C/U/H) kiwifruit stem plug extracts in the Molano et al. (1977) assay, measured in 1994. Enzyme concentration is expressed as a percentage of undiluted 93/N/I/H or 94/C/U/H extract.

High activity extract data required $\log_e$ transformation. Low concentrations (0-40%) of 94/C/U/H extract were readily differentiated (P=0.0003), but there were no significant differences in dpm at the higher concentrations.

B Quench curve correction

Colour quenching caused a relatively constant 10% reduction in counting efficiency (Table 5-3) within and beyond the range of concentrations normally encountered.

Table 5-3: Percent counting efficiency (mean ± SEM) in heavily colour quenched and unquenched tritium labelled toluene, using uncured, inoculated, healthy extract (93/N/U/H) as a quench.

SAMPLE	COUNTING EFFICIENCY
Unquenched	39.7 ± 0.8
Quenched	30.3 ± 0.5

5.4 SECTION 4 - ASSAY COMPARISONS

5.4.1 Introduction

Having optimised the assays to the kiwifruit system, the Boller et al. (1983) assay was initially compared with other non-radio assays in current use, then subsequently with the Molano et al. (1977) radio assay.

5.4.2 Materials and Methods

A Comparison of the Boller et al. (1983), viscometric and Calcofluor assays

One extract with low and one with high chitinase activity were used in this experiment:

1) Low activity extract (93/N/U/H)

This extract was produced by the same procedure as the 92/N/U/H extract (Section 5.1.2, Part A), except that stem plugs were sourced from Experiment 2, Chapter 3 (Section 3.3.2).

2) High activity extract (92/C/U/H)

As for the 94/C/U/H extract (Section 5.1.2, Part B, (i)), except that fruit were harvested on 4 May 1992 from Patumahoe, South Auckland.

Chitinase activity was measured in the three assays, using neat extract, two and five-fold dilutions of the high and low activity extracts. Calcofluor assay samples were incubated overnight for 17 h. Chitinase activity was expressed as nmol/min/ml, percent decrease in viscosity, and radial glycol chitin degradation (mm) for the colorimetric assay of Boller et al. (1983), viscometric and Calcofluor assays respectively. Samples were then ranked from greatest through to least chitinase activity.

B Comparison of the Boller at al. (1983) and Molano et al. (1977) assays

Endochitinase activity in three replicates of five treatment samples from Experiment 6, Chapter 6 (Section 6.3.7) was measured by the Molano et al. (1977) and Boller et al. (1983) assays and was expressed as ng/min/ml or nmol/min/ml respectively.

Treatments (Table 5-4) were chosen to represent a range of chitinase activities, and are given in expected order of greatest to least activity as pre-determined by the radiometric assay of Molano et al. (1977). The five samples were as follows:

Table 5-4: Kiwifruit stem plug extracts used in a comparison of the Boller et al. (1983) and Molano et al. (1977) assays.

TEST SAMPLE	TREATMENT					
	Inoculated*	Cured†	HCl‡	Chitosan§	Diseased	Coolstorage¶
A	+	+	+	+	+	+
B	+	-	+	-	+	+
C	+	-	+	-	-	+
D	+	-	+	-	-	-
E	-	-	-	-	-	-

* Following pedicel removal, inoculated fruit received 5000 *B. cinerea* spores/stem plug.

† The curing treatment, where applied, comprised 72 h at 20°C after inoculation.

‡ HCl (0.25 M) was applied in a 17 µl droplet to sample stem plugs after inoculation and prior to coolstorage.

§ Chitosan (10 mg/ml) in 17 µl of 0.25 M HCl applied immediately after inoculation. and prior to curing and coolstorage.

¶ Fruit were stored for 6 wk at 0 ± 0.3°C.

5.4.3 Results

A Comparison of the Boller et al. (1983), viscometric and Calcofluor assays

All three assays ranked the samples in the same order, with one exception: the Calcofluor assay ranked 100% 93/N /U/H first equal instead of third (Table 5-5).

Table 5-5: Kiwifruit stem plug extracts ranked in order of descending chitinase activity, as determined by measurements in the Boller et al. (1983), viscometric and Calcofluor assays in 1994, where rank 1 = highest measured activity and rank 6 = lowest activity measurement.

TEST SAMPLE	CHITINASE ACTIVITY RANKING BY BOLLER	VISCOMETRIC	CALCOFLUOR
Various concentrations of low activity 93/N/U/H extract			
100% conc. (undiluted)	3	3	1=
50% conc. (2-fold dilution)	5	5	4
20% conc. (5-fold dilution)	6	6	5
High activity 92/C/U/H extract			
100% conc. (undiluted)	1	1	1=
50% conc. (2-fold dilution)	2	2	2
20% conc. (5-fold dilution)	4	4	3

Table 5-6 shows that the Calcofluor assay was the most sensitive (significant activity was detected in 20% 93/N/U/H extract), but had the poorest resolution (it did not distinguish between different concentrations of 92/C/U/H extract). The Boller et al. (1983) and viscometric assays resolved all three concentrations of both extracts (Table 5-6).

Table 5-6: Chitinase activities (means ± SEM) in kiwifruit stem plug extracts measured by the Boller et al. (1983) assay, viscometric and Calcofluor assays in 1994.

TEST SAMPLE	CHITINASE ACTIVITY MEASURED BY BOLLER (nmol/min/ml)	VISCOMETRIC (% decrease in viscosity)	CALCOFLUOR (mm)
Various concentrations of low activity 93/N/U/H extract			
100% conc. (undiluted)	2.0 ± 0.1	9.5 ± 2.1	3.0 ± 0.1
50% conc. (2-fold dilution)	0.7 ± 0.0	3.5 ± 1.8	2.5 ± 0.0
20% conc. (5-fold dilution)	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.1
High activity 92/C/U/H extract			
100% conc. (undiluted)	4.3 ± 0.1	35.7*	3.0 ± 0.0
50% conc. (2-fold dilution)	3.5 ± 0.6	22.0	2.8 ± 0.3
20% conc. (5-fold dilution)	1.3 ± 0.2	6.8	2.6 ± 0.0

* No replication due to sample shortage.

B Comparison of the Boller et al. (1983) and Molano et al. (1977) assays

Rankings in both assays were similar (Table 5-7). Significant activity was detected in sample E by the Molano et al. (1977) but not by the Boller et al. (1983) assay. Variability within treatments, as assessed by SEM, ranged from 0-33% of the mean in the Boller et al. (1983) assay and 15-59% in the Molano et al. (1977) assay (Table 5-7).

Table 5-7: Chitinase activities (means ± SEM) in kiwifruit stem plug extracts measured by the Boller et al. (1983) and Molano et al. (1977) assays, ranked from highest activity (rank=1) through to lowest activity (rank=5) in 1994.

TEST SAMPLE	BOLLER ASSAY Activity (nmol/min/ml)	Ranking	MOLANO ASSAY Activity (ng/min/ml)	Ranking
Sample A	6.5 ± 0.8	1	662.4 ± 136.1	1
Sample B	2.1 ± 0.2	3	474.5 ± 157.7	2
Sample C	3.7 ± 0.5	2	440.9 ± 261.3	3
Sample D	1.2 ± 0.4	4	72.3 ± 41.9	4
Sample E	0.0 ± 0.0	5	41.1 ± 6.3	5

C Overall assay comparisons

The Boller et al. (1983), Molano et al. (1977), Calcofluor and viscometric assays all gave comparable sample rankings, but differed in other aspects such as sensitivity, resolution, reliability and ease of use (Table 5-8). Reliability of the Boller et al. (1983) assay was ranked as poor because results were often not repeatable, as shown by the effect of pH and temperature of the first incubation (refer Section 5.2.3, Part A, (iii)).

Table 5-8: Ranking* of four chitinase assays, with regard to sensitivity, resolution, reliability and ease of use.

CHARACTERISTIC	CHITINASE ASSAY			
	BOLLER	MOLANO	VISCOMETRIC	CALCOFLUOR
Sensitivity	B	A	B	A
Resolution	B	B	B	C
Reliability†	C	B	B	B
Ease of use	C	B	C	A

* A=excellent, B=satisfactory, C=poor.
† Reliability relates to inherent variability in the assay as well as to the ability to produce consistent results from repeated measurements.

5.5 DISCUSSION

After examination of seven different chitinase assays, this research demonstrated that the radiometric assay of Molano et al. (1977) was the most reliable, accurate assay for quantitative measurement of kiwifruit chitinase activity, while the Calcofluor assay was more suited to qualitative detection. The Calcofluor petri dish assay was highly sensitive, and could be easily used to screen large sample numbers for the presence of chitinase, but it had poor resolution, as illustrated by the shallow gradient of the linear relationship between substrate degradation and $\log_{10}$ enzyme concentration (Section 5.1.3, Part A). This poor resolution is a feature of circular gel diffusion-based assays in which a fixed amount of enzyme encounters ever-increasing amounts of substrate as it moves away from the central point of application. The rate of radial diffusion consequently decreases

because the enzyme must hydrolyse the substrate it encounters before it can migrate further outwards. Literature on the quantitative measurement of chitinase activity in kiwifruit is scarce, but the work of McLeod & Poole (1994) showed that differences in chitinase activity in diseased and healthy fruit were less than five-fold. The Calcofluor assay was unable to distinguish such concentration differences (Section 5.1.3, Part B (i) and (ii) and Section 5.4.3, Part A), but this assay is still useful for qualitative assessments because of its sensitivity and ease of use.

Use of glycol chitin as a substrate in petri dish assays has not been recorded in the literature to date. Conventional plate assays use colloidal chitin, which is relatively insensitive (O'Brien & Colwell 1987; Wirth & Wolf 1990; Frändberg & Schnürer 1994). Glycol chitin is a specific endochitinase substrate (Pan et al. 1991), and Trudel & Asselin (1989) found that it was incorporated more uniformly into gels than colloidal chitin. Its use in combination with the Calcofluor white stain in the present study raised the sensitivity of the plate assay method above that of the Boller et al. (1983) colorimetric assay and viscometric assay (see Section 5.4.3, Part A). The best compromise between sensitivity and resolution was achieved by a 5 h incubation of plates containing 0.01% glycol chitin. Shorter incubation (2 h) allowed insufficient time for enzymes to diffuse through the agar. Resolution decreased with prolonged incubation (48 h) because lower enzyme concentrations had enough time to digest a comparable region of surrounding substrate to that of the higher enzyme concentrations. However, protracted incubation enabled detection of a minute level of enzyme activity in the 94/C/U/H anion exchange fraction no. 8, due to extreme sensitivity of the assay. It is impractical to attempt further purification of such low levels of activity, as losses at consecutive stages of protein purification can equal the initial level of activity. To screen extracts in protein purification work, therefore, the sensitivity of the Calcofluor assay needs to be reduced by use of a shorter incubation period (5 h for the kiwifruit system).

Results from Section 5.4.3, Part A showed that resolution and sensitivity of the viscometric assay was moderate and comparable with that of the Boller et al. (1983) colorimetric assay. This is in agreement with the findings of Stirling et al. (1979), Boller & Mauch (1988) and Ohtakara (1988), although Evrall et al. (1990) and Cabib (1987)

described the viscometric assay as insensitive. Anomalies between the findings of these scientists could be explained by the wide variation which exists between different batches of substrate and their subsequent susceptibility to chitinolytic degradation (Koga & Krager 1983; Ohtakara 1988). Glycol chitin offers the advantage of being soluble, but there is some concern that this artificial substrate no longer resembles chitin and non-specific reactions may occur (Hackman & Goldberg 1964; Cabib 1987). Moreover, decrease in substrate viscosity is a function of enzyme concentration, but the rate of decrease may vary with other factors, such as the mode of substrate preparation, thereby rendering viscometric methods unsuitable for absolute measurements of chitinase activity (Jeuniaux 1966). In the present study, five samples could be processed in duplicate per day, compared with 20 samples assayed in triplicate for the Molano et al. (1977) radioassay. Without exception, other workers consider the viscometric assay too time consuming to process large numbers of samples (Stirling et al. 1979; Ohtakara 1988, Boller & Mauch 1988; Boller 1992).

Adaptation of the Boller et al. (1983) colorimetric assay to the specific requirements of the kiwifruit system improved sensitivity and reliability. Section 5.2.3, Part A, (i) results showed that a non-linear phase was entered, and resolution consequently decreased, when the length of the first incubation exceeded 2 h. Section 5.2.3, Part A, (ii) and (iv) indicated that further hydrolysis by the snail gut enzymes occurred readily, and 1 h provided sufficient time for complete hydrolysis of soluble oligomers produced during the first incubation. Snail gut enzymes were unable to hydrolyse substrate polymers, as shown by negligible absorbance when the first incubation step was omitted (Section 5.2.3, Part B (ii)). High absorbance at 0 h in Section 5.2.3, Part A (iv) suggested that a major product of chitinase digestion in this experiment was N-acetylglucosamine, which was probably released by *B. cinerea* exochitinases present in the diseased 92/N/I/D extract. (The Kunz et al. (1992) definition of exochitinase activity is used here.) The presence of appreciable absorbance in the "no snail enzyme" treatment of Section 5.2.3, Part B (ii), coupled with a significant increase in absorbance following snail gut enzyme addition, suggested that both exo- and endochitinase activities were present in the *S. griseus* crude extract. Although bacterial chitinases are predominantly exochitinases, endochitinase activity has been recorded on occasion (refer Chapter 4,

Section 4.5).

The wide pH and temperature tolerance of the first and second incubations, expressed in the results from Section 5.2.3, Part A, (iii) and (iv), reflected the crude nature of the kiwifruit and snail gut extracts used for Boller et al. (1983) assay optimisation. These extracts contained a wide range of isoforms, all with different pH and temperature optima. In this study, the mean pH of the third incubation was 9.3. Reissig et al. (1955) found that the pH of this step, which they optimised at 8.9, had a critical influence on colour development. This pH allows for optimal buffering capacity of borate buffer (capacity is maximised within ± 0.5 pH unit of borate pKa 9.3).

Enzyme concentration was non-limiting, but the increase in sensitivity when higher substrate concentrations were used, the benefits of improved mixing of substrate and enzyme (see Section 5.2.3, Part B, (iii)), and the influence of different substrates (Section 5.2.3, Part C), indicated that accessibility to substrate binding sites was a limiting factor in the assay. Cabib (1987) noted that nascent chains of chitin were more readily digested than native chitin, and proposed that restricted enzyme access to the highly aggregated chains of native chitin might hinder efficient hydrolysis. Progress curves of the Boller et al. (1983) assay are non-linear (Boller & Mauch 1988). The typical quadratic shape of assay progress curves might also be explained by substrate accessibility and heterogeneity (Molano et al. 1977). Initially hydrolysis sites on the exterior of substrate molecules are readily available and substrate digestion is proportional to enzyme concentration. With further degradation of different parts of heterogenous substrate molecules, hydrolysis sites remaining are less accessible, so substrate digestion slows and the non-linear portion of the progress curve is entered. In Section 5.2.3, Part C, regenerated chitin, produced by reacetylation of chitosan, was more efficient as a substrate than colloidal chitin, unless the chitin was washed. Molano et al. (1977) obtained the same result. Regenerated chitin is highly hydrated (Cabib 1987) and this may facilitate enzyme access and ease of digestion. Washing the chitin substrate might remove impurities that interfere with, or restrict access to, enzyme binding sites. It is also possible that soluble monomers/dimers which could cause end-product inhibition of chitinase activity are removed by the washing.

Despite improvements to Boller et al. (1983) assay performance, greater sensitivity and repeatability were required. High variability is an inherent problem of many chitinase assays because of the heterogeneous nature of substrates (Cabib 1987), but the problem is exacerbated in the Boller et al. (1983) assay in which the three incubation steps increase the chances of non-representative sampling and experimental error (Saborowski et al. 1993).

Many other factors contribute to the variability of the Boller et al. (1983) assay. The first incubation is terminated by centrifugation to remove unreacted chitin (Boller et al. 1983). Inadvertent stirring of the sediment after centrifugation could contaminate the supernatant with undigested chitin, allowing kiwifruit chitinases (which were either bound to the chitin pellet or present in the supernatant) to continue digestion beyond the intended termination of the first incubation. This creates spurious results. Section 5.2.3, Part B, (i) showed that the chitin substrate was heat stable. This made it possible to incorporate a boiling step after the first incubation to denature kiwifruit chitinases and prevent further reaction. This protocol modification was unique to this study and overcame the aforementioned problem, thereby reducing assay variability.

Section 5.2.3, Part B, (ii) showed that there was considerable variation between different batches of snail gut enzyme. This was because the extracts were impure and contained variable amounts of different enzymes. Boller & Mauch (1988) noted that snail gut extract could even contain endochitinases, which further contribute to variable results if unreacted substrate is not completely removed after the first incubation.

Instability of the coloured end product of the Boller et al. (1983) assay is another factor contributing to variability. Reissig et al. (1955) found that colour faded at a rate of 1.5% every five minutes after 20 minutes at 37°C. This loss was reduced to 0.5% every five minutes by transferring the samples to room temperature after the final incubation, but still represents a sizeable loss when there are large numbers of samples to process.

Many assays are unable to distinguish exo- and endochitinase activity (Tronsmo & Harman 1993), and one advantage of the Boller et al. (1983) assay is its ability to

measure both types of activity (Boller et al. 1983; McLeod & Poole 1994). The assay's ability to measure exochitinase activity hinges on two assumptions. Firstly, that only monomeric products are released by exochitinase activity (Boller 1988; Boller & Mauch 1988) and, secondly, that these are detected specifically by the method of Reissig et al. (1955). However, Robbins et al. (1988) reported that some exochitinases release dimeric products. In addition, Domard & Vasseur (1991) demonstrated colorimetric determination of N-acetylglucosamine by the method of Reissig et al. (1955) was non-specific. Although it was most sensitive to the monomer, it also measured all soluble N-acetylglucosamine oligomers to a lesser extent. These findings bring into question the ability of the Boller et al. (1983) assay to clearly differentiate exo- and endochitinase activity (Tronsmo & Harman 1993). A more reliable means of distinguishing exo- and endochitinases is to use separate assays which are specific for each type of activity, for example, the colorimetric assay of Roberts & Selitrennikoff (1988) for exochitinases and a viscometric assay for endochitinases.

In Section 5.4.3, Part B, the Molano et al. (1977) assay was found more sensitive than the Boller et al. (1983) assay, although both ranked samples in a similar order. Boller & Mauch (1988) found that both assays yielded equivalent results, providing chitinase activities in test samples were above the detection limit of the Boller et al. (1983) assay. Many samples tested in the current study were close to this limit. The Molano et al. (1977) assay is the most rapid and sensitive quantitative assay in common use (Cabib 1987; Wirth & Wolf 1990). More rapid quantification can be achieved using infrared reflectance spectroscopy (Roberts et al. 1994), but this technique requires a special spectrophotometer, the cost of which prohibits its use in most laboratories.

For the kiwifruit system, optimal counts in the Molano et al. (1977) assay were achieved by incubating samples at 25°C for 18 h. Incubation periods of up to 24 h are not uncommon for this assay (Saborowski et al. 1993). In the present study, counts were present in enzyme-free blanks (Section 5.3.3, Part A (i) and (ii)). Barrett-Bee & Hamilton (1984) observed high blank values, particularly at high substrate concentrations. These researchers used filtration instead of centrifugation to remove unreacted substrate. They considered that filtration produced more reproducible results

because the fineness of the precipitate allowed some trailing on the meniscus when centrifugation was used. They proposed that small radiolabelled fragments passing through the filter and appearing in the filtrate were the cause of high blanks. The protocol employed in the present study included both filtration and centrifugation, but very fine substrate particles could have escaped both stages and appeared in the blanks. Inadequate removal of non-specific label during substrate preparation can also cause high blanks, but this was unlikely here because the substrate was extensively washed until filtrate counts were negligible. Another possible explanation for high blanks is substrate leaching - release of non-covalently bound material and non-enzymic deacetylation of the chitin over time. Rewashing the substrate after extended periods of storage should remove these leachates. Alternatively, compensation for the substrate leaching effect can be achieved by subtracting the dpm of the enzyme-free blank from the sample dpm.

High variation was associated with the Molano et al. (1977) assay (Section 5.4.3, Part B). Cabib (1988) noted that spurious counts were found occasionally, and considered that these were probably caused by small chitin particles floating on the surface of the supernatant. Insolubility and heterogeneity of the substrate may contribute to variable results. However, results in this study were consistently repeatable, despite the high variation. Roberts et al. (1994) also attested to the reliability of this assay.

In the present study, linearity was observed at low enzyme concentrations, but overall the relationship between radioactivity and enzyme concentration was non-linear. Molano et al. (1977), Barret-Bee & Hamilton (1984) and Ludwig-Müller et al. (1994) also observed an early linear phase followed by a non-linear stage in the radioassay. Non-linearity of the Molano et al. (1977) assay is often cited as a disadvantage. However, if samples are appropriately diluted to a point where results will be on the linear portion of the curve, inter-sample comparisons should not pose any problems. Pitfalls of the assay are that:

1) it is not specific for chitinase, since labelled acetyl groups may also be cleaved off the substrate by chitin deacetylase (Stirling et al. 1979), and
2) the assay does not distinguish between exo- and endochitinase activities (Tronsmo

& Harman 1993).

Overall the rapidity, reliability and sensitivity of the radioassay made it the best quantitative method for this project. The selection and development of a viable assay for measurement of chitinase activity removed the main obstacle to gathering information on the role of chitinases in kiwifruit defence against *B. cinerea*.

6 CORRELATIVE EVIDENCE FOR THE ROLE OF CHITINASES IN KIWIFRUIT DEFENCE AGAINST *B. CINEREA*

6.1 INTRODUCTION

6.1.1 Collection Of Correlative Evidence

Preliminary studies indicated that chitinases contributed to kiwifruit stem scar resistance to *B. cinerea* (Chapter 4, Section 4.1.2), but more detailed research is required for confirmation, and the collection of correlative evidence plays an important role in this process. This evidence is necessary to justify the need for protein purification, which can be time consuming and expensive. Direct evidence for an antifungal role of chitinases can then obtained by purifying kiwifruit chitinases and testing their antifungal activity against *B. cinerea* in vitro. In this study two approaches were used to gather correlative evidence:

1) Measurement of chitinase levels in experimental treatments that reduced infection
- the aim of this exercise was to look for correlation between resistance to infection and increased chitinase activity.

2) Elicitor application to increase chitinase activity
- another approach to gathering correlative evidence was to artificially stimulate chitinase activity by elicitor application in order to observe the effects on resistance. Chitinases are pathogenesis-related (PR) proteins (Graham & Graham 1991), which by definition means that the proteins are present in negligible concentrations in healthy plants and their synthesis is induced in response to pathological or stress-related situations (Cutt & Klessig 1992). Plant chitinases have been induced by the stress hormone ethylene (Boller et al. 1983; Ishige et al. 1993), pathogen infection (Mauch et al. 1984; Métraux & Boller 1986; Roby & Esquerre-Tugaye 1987a; Tuzun et al. 1989; Conrads-Strauch et al. 1990; Rasmussen et al. 1992), animal predation (Bronner et al. 1991), temperature

shock (Van Damme et al. 1993), exposure to necrotizing salt solutions (Métraux & Boller 1986), wounding (Bronner et al. 1991; Van Damme et al. 1993), and by the application of elicitors such as fungal cell wall fragments (Kurosaki et al. 1987) and other chitin derivatives including chitosan (Hirano et al. 1990a; Wilson et al. 1994).

6.1.2 Chitinase Levels And Fruit Maturity

As discussed previously (Chapter 3, Section 3.5), most crops become more susceptible to infection as they mature, but for kiwifruit the opposite is true. In tobacco, increased resistance with ageing was correlated with an increase in chitinase activity (Wyatt et al. 1991). The effect of kiwifruit maturation on endogenous chitinase levels is unknown.

6.1.3 Chitinase Levels And Curing

Curing of wounded tissue has been associated with an increase in host resistance in a wide range of plants (Ismail et al. 1978; Ben-Yehoshua et al. 1988; Morris et al. 1989), including kiwifruit (Pennycook & Manning 1992; Poole & McLeod 1994). Plants often initiate defence mechanisms such as the synthesis of morphological barriers in response to wounding (Eckert 1978), and curing is thought to decrease infection by allowing time for wound repair and/or reducing water permeability at wound sites (refer Chapter 3, Section 3.5). McLeod & Poole (1994) found that endochitinase activity increased in fruits which were inoculated at harvest and then held for 0-168 h at 22°C before sampling, but it was unclear whether the increase in chitinases was in response to inoculation, to curing, or to a combination of these two.

6.1.4 Use Of Chitosan As An Elicitor

Chitosan, a partially deacetylated derivative of chitin, is an ideal elicitor because it has been shown to coordinately increase host resistance and chitinase activity in strawberries (El Ghaouth et al. 1992c), cucumber (El Ghaouth et al. 1994a), and peas (Hadwiger et al. 1984). Chitosan can also induce host resistance through stimulation of phytoalexins (Kendra & Hadwiger 1984; Cuero et al. 1992; Notsu et al. 1994), defence proteins such as β-1,3-glucanases (Liénart et al. 1993), induction of proteinase inhibitors (Sequeira 1990), and formation of structural barriers (Pearce & Ride 1982; Kauss et al. 1989; Benhamou & Thériault 1992; El Ghaouth et al. 1994b). Moreover, chitosan is fungicidal

against a wide range of fungi (Allan & Hadwiger 1979; Stössel & Leuba 1984; El Ghaouth et al. 1992b), including *B. cinerea* (El Ghaouth et al. 1992b). Coating horticultural produce with chitosan can reduce weight loss and respiration, thereby prolonging product shelf life (El Ghaouth et al. 1992a). Chitosan is a natural biodegradable product, regarded as safe for consumption (Yalpani et al. 1992), as indicated by feeding trials with domestic animals (Wilson et al. 1994). Chitosan has hypolipidemic activity in rabbits and hens, i.e. it reduces cholesterol (Hirano et al., 1990b). All these characteristics make chitosan application a potentially attractive tool for reducing postharvest infections of kiwifruit whilst at the same time providing correlative evidence of the role of chitinases in defence.

Preliminary work by Poole & McLeod (1992) showed that topical application of chitosan reduced *B. cinerea* infection in kiwifruit, although it was unknown whether this reduction was attributable to the fungistatic properties of chitosan, or to a stimulation of host defences, or a combination of both. The authors did not measure chitinase activities in response to chitosan application. The optimal concentration for application of chitosan was not determined.

Chitosan is insoluble in water but soluble in dilute acid (Foster & Webber 1960). It is typically dissolved in 0.25 M HCl (Benhamou & Thériault 1992; El Ghaouth et al. 1992c), although more recently 0.04 M HCl has been successfully used (El Ghaouth et al. 1994a). The effect of an acidic solvent in the kiwifruit/chitosan system is not known. Preliminary trials in this study established that the lowest solvent concentration which solubilised 10 mg/ml of chitosan was 0.04 M for HCl, acetic, formic and lactic acids. Salicylic and citric acids were not used because they were poor solvents for chitosan. The use of different solvents merits further investigation.

6.1.5 Chitinases In Storage

Intense activity during the kiwifruit harvest season frequently made it necessary to store samples at -80°C before assessment of chitinase activity. It was therefore important to assess long term stability of chitinase activity in crude and partially purified stem plug extracts.

6.2 OBJECTIVES

The objectives of these experiments were to examine the:

1) relationship between fruit maturity at harvest and endogenous chitinase levels,
2) effect of curing on kiwifruit chitinases,
3) conditions which would allow chitosan to be used most effectively,
4) effect of chitosan application on disease and endogenous chitinase levels, and
5) stability of chitinase extracts in long term storage for future experiments.

6.3 MATERIALS AND METHODS

Unless stated otherwise, chitinase activity in kiwifruit stem plug extracts in all experiments of this chapter was measured using the modification of the Molano et al. (1977) assay described in Chapter 2 (Section 2.2.4, Part E).

6.3.1 Experiment No. 1

Title : Fruit maturity and endogenous chitinase activity - 1993 harvest.

Stem plugs of ten healthy and ten diseased fruit from each of the four harvest dates and the 0 and 25,000 inoculum levels in Experiment 2, Chapter 3 (Section 3.3.2) were excised following 12 wk storage at 0 ± 0.3°C. In addition, 10 stem plugs were excised immediately in the field (i.e. 0 h after harvest) on each of the four harvest dates in Experiment 2, Chapter 3.

The stem plugs were snap frozen in liquid nitrogen and stored at -80°C until they were extracted. Crude extracts of single stem plugs were prepared as described in Chapter 2 Section 2.2.1, using five volumes of 0.1 M NaOAc extraction buffer, pH 5.8. Extracts were stored at -80°C until chitinase activity was measured.

The extracts were considered as the experimental units and the manner in which these

extracts were originally obtained (split plot design) was no longer relevant. A randomised block design was subsequently adopted for processing the extracts. As day-to-day variations in the assay were apparent (data not shown), and since it was impossible to process all samples within a day, activity measurements were blocked over several days.

A square root transformation was necessary to satisfy the assumptions of ANOVA (refer Chapter 2, Section 2.3.1). Linear contrasts were used to make specific treatment comparisons.

6.3.2 Experiment No. 2

Title : Curing and endogenous chitinase activity - 1994 season.

Fruit with attached pedicels, harvested on 23 May 1994 from Levin, were randomly divided into two groups. Immediately after pedicel removal, the first group was packed and placed into 0°C storage. The second group was cured, stem scar uppermost, in open trays for one week at 20°C before transfer to 0°C. After nine weeks at 0°C, four single stem plugs from healthy fruit in each treatment were extracted in five volumes of NaOAc buffer. Chitinase activity in the extracts was measured using the modification of the Molano et al. (1977) assay described in Chapter 2 (Section 2.2.4, Part E), except that 40 µl of test extract and 25 µl of NaOAc extraction buffer were used. Fruit were stored at 0°C for nine weeks before extraction to eliminate diseased fruit. This meant that any increases in chitinase activity could be attributed to curing, rather than to pathogen ingress and resultant fruit responses.

The experiment was carried out according to a completely randomized design (CRD) and data transformation was not necessary.

The indirect ELISA protocol listed in Chapter 2, Section 2.2.9, Part A was also used to compare cured and uncured extracts. In initial trials sugar beet (CH2, CH4 and SP), cucumber and bacterial (*Janthinobacterium lividum*) chitinase antibodies were serially diluted (1 in 200 to 1 in 6,400), but in later experiments a single dilution (1000-fold)

of CH4 was used.

Samples (5 µl) of cured and uncured extract were added to 20 µl of Laemmli (1970) sample buffer and heated as described in Chapter 2, Section 2.2.6, Part C. In addition, 50 µl of both extracts, previously exchanged into 50 mM Tris-HCl buffer, pH 8, were lyophilised to dryness and redissolved in 20 µl of Laemmli (1970) sample buffer in order to produce 12.5-fold concentrates. (Buffer exchange was necessary to overcome problems of band distortion associated with the high acetate concentration.) Original and concentrated samples were applied to the precast Bio-Rad Tris-HCl Mini-Protean II Ready gel (T=15%, C=2.6). After 45 min electrophoresis at 200 V, using Laemmli (1970) electrode buffer, pH 8.3 (Appendix I), the gel was blotted onto a nitrocellulose membrane and reacted with a 500-fold dilution of sugar beet CH4 antibody (refer Chapter 2, Section 2.2.9, Part B for Western blot protocol details).

To qualitatively compare banding patterns, replicate samples (400 µl) of cured and uncured extracts were lyophilised and resuspended in 30 µl and 20 µl of NaOAc extraction buffer respectively. Serva pI 3-10 test mixture (1 µl diluted three-fold in Milli-Q water), 5 µl of uncured and 4 µl of cured concentrates were applied to a precast 150 µm 125 x 125 mm pH 3-10 Servalyt Precote IEF gel and focused for 3.5 h at 4 W. Once focusing was complete, a 0.01% (w/v) glycol chitin gel (Chapter 2, Section 2.2.6, Part B) was overlaid on the IEF gel for 1 h, prior to 2 h incubation in a humid chamber at 37°C, followed by staining with 0.01% (w/v) Calcofluor white (Sigma) to visualise chitinase activity. The IEF gel was silver stained (refer Chapter 2, Section 2.2.6, Part E and Appendix V for stain protocols) to show total proteins.

6.3.3 Experiment No. 3

Title : Curing and total protein content of kiwifruit stem plug extracts - 1994 season.

Total protein in the Experiment 2 extracts was measured using the 8-80 µg/ml Bradford (1976) assay, as described in Chapter 2, Section 2.2.5.

The experiment was carried out according to a CRD and data transformation was not

required.

6.3.4 Preparation For Chitosan Experiments

Crude crab-shell chitosan (Sigma) was purified as described by El Ghaouth et al. (1992c). Chitosan was ground to a fine powder in a Waring blender and then passed through a 500 μm sieve (Ende Colts (filters) Ltd). A 2 g batch of this powder was dissolved in 24 ml of 0.25 M HCl, and undissolved particles were removed by 15 min centrifugation at 10,000*g*. The viscous solution was then brought to neutral pH by addition of 2.5 M NaOH. Precipitated chitosan was collected by centrifugation as before, and washed twice by resuspending the precipitate in deionised water and recentrifuging. The washed precipitate was then lyophilised to dryness (24-48 h).

Unless stated otherwise, purified chitosan solutions were prepared by dissolving the lyophilised powder (10 mg/ml) in 0.25 M HCl, and adjusting the pH to 5.6 with 2 M NaOH (El Ghaouth et al. 1992c).

6.3.5 Experiment No. 4

Title : Chitosan application and susceptibility to B. cinerea infection - 1993 harvest.

Forty boxes of 36 fruit with attached pedicels were randomly harvested from pergola-trained vines in a commercial orchard in Levin on 24 May 1993. The boxes were randomly divided into four groups of ten boxes, constituting four blocks. These were further divided into five sets of two boxes each, and one "main plot" treatment was randomly assigned to each set of two boxes. The main plot treatments were as follows:

1) uninoculated - solvent - chitosan (U)
2) uninoculated + solvent + chitosan (U + S + C)
3) inoculated - solvent - chitosan (I)
4) inoculated + solvent - chitosan (I + S)
5) inoculated + solvent + chitosan (I + S + C)

(To minimise costs, it was originally intended to use data from the positive control fruit

in Experiment 5 for an "uninoculated + solvent - chitosan" (U + S) treatment. It was subsequently realised that this would not have been valid as the Experiment 5 protocol did not include the "split plot" temperature treatments.)

The five main plot treatments were applied immediately following pedicel removal at 4 h after harvest. A 17 µl droplet containing 5,000 *B. cinerea* spores was placed on each stem scar of fruit in inoculated treatments, whilst uninoculated fruit received one droplet of 0.01% Tween 20. Once this first droplet had dried (about 1 h), a second droplet containing 10 mg/ml chitosan in pH adjusted 0.25 M HCl (treatments 2 and 5) or 0.25 M HCl only (treatment 4) or just 0.01% Tween 20 (treatments 1 and 3) was applied. Fruit were packed once the second droplet was dry. Application of the five main plot treatments was completed 7 h after harvest.

Two split plot temperature treatments, 0 or 20°C, were next applied for 72 h to one of the boxes from each set.

Once treatment application was completed, all boxes were moved into the 0°C store and disease incidence was assessed 12 wk after harvest.

The data were analysed as a split plot experiment. A square root transformation was necessary to satisfy the assumptions of ANOVA.

6.3.6 Experiment No. 5

Title : Chitosan concentration and susceptibility to B. cinerea infection - 1993 harvest.

Harvest details were the same as for Experiment 4. Six boxes of 36 fruit were assigned to each of four blocks, each block comprising a separate position in the coolstore.

Each box within a block then received a different treatment. The six treatments were as follows:

1) uninoculated + solvent - chitosan (U + S)

2) inoculated + solvent - chitosan (I + S)

treatments 3) - 6) inoculated + solvent + chitosan (I + S + C)

3) I + S + C with 10 mg/l chitosan

4) I + S + C with 100 mg/l chitosan

5) I + S + C with 1 mg/ml chitosan

6) I + S + C with 10 mg/ml chitosan

As in Experiment 4, the spore suspension droplet was applied first, followed by the chitosan droplet. Treatments 1 and 2 were intended as positive and negative controls respectively. Treatments 3-6 comprised four different chitosan solutions, each differing in concentration 10 -fold. After treatment application, fruit were packed, left at ambient temperature for 38 h to allow for chitosan induction of plant defences, and then placed in a 0°C coolstore. Percent infection was assessed after 12 wk storage at 0 ± 0.3°C.

Data were analysed as a randomised block design and no transformation was required.

6.3.7 Experiment No. 6

Title : Chitosan application and endogenous chitinase activity - 1993 harvest.

In addition to the fruit used in Experiment 4, an extra four boxes per treatment combination, plus ten extra untreated fruit, were collected for measurement of chitinase activity. Inoculum/chitosan and temperature treatments were applied to the boxes of fruit in the same manner as described in Experiment 4.

Stem plugs from the ten untreated fruit were excised immediately in the field (i.e. 0 h after harvest). Ten stem plugs from treated fruit were randomly removed at 7 h, 1, 2, 3, or 6 d, and 6 wk after harvest from the four boxes/treatment combination. Sampling at 1, 2 and 3 days coincided with application of the temperature treatment, whilst the 7 h sample marked the completed application of the inoculum/chitosan droplets. Infections were visible in the 6 wk samples, so, where possible, ten diseased and ten healthy fruit were collected from each treatment combination.

Stem plug snap freezing and crude extract preparation were the same as that described in Experiment 1 of this Chapter. Chitinase activity measurements were blocked over several days.

The extracts were considered as the experimental units. Since it was impossible to process all samples within a day, activity measurements were blocked over several days using a factorial randomised block design. Extracts from each different sample time were processed as a separate batch.

The large number of interactions between different experimental factors resulted in a highly complex statistical model. To avoid this problem, data from each sample time after harvest were analysed separately. Consequently, the statistical significance of treatment comparisons between different sample times could not be assessed, although visual comparisons were achieved by plotting the data from various sample times together on one graph (see Fig. 6-6 and 6-7). Statistical results from each sample time after harvest were summarised rather than presented individually.

6.3.8 Cautionary Note

After set up of the 1993 chitosan trials (Experiments 4-6), it was subsequently discovered that the chitosan treatments (U+S+C and I+S+C) had been applied in pH adjusted solvent, but the pH in the solvent only treatments (U+S and I+S) had not been adjusted to pH 5.6. Consequently, differences in disease incidence and chitinase activity between these treatments might have been due to either the effect of solvent pH, or chitosan addition, or a combination of both. The 1994 trial (Experiment 7) was designed to distinguish these factors.

6.3.9 Experiment No. 7

Title : Different chitosan solvents and susceptibility to infection - 1994 harvest.

Fruit with attached pedicels were collected from pergola-trained vines in Levin on 23 May 1994. Treatment combinations were applied immediately after pedicel removal, approximately 3 h after harvest. Apart from four boxes of uninoculated controls, 84

boxes of 36 fruit each were inoculated with 5,000 *B. cinerea* spores per stem scar. Four boxes were reserved as inoculated controls and one of five acidic solvents - 0.25 M HCl (for a direct comparison with the previous experiments), 0.04 M HCl, 0.04 M acetic, 0.04 M lactic or 0.04 M formic acid - was applied to the rest of the inoculated fruit. Acidic solvent was either applied on its own or in combination with chitosan, using both pH unadjusted solvent and solvent in which the pH was adjusted to 5.6 with 2 M NaOH. This gave a total of 22 treatment combinations. Fruit were packed, left at 20°C for three days to allow for chitosan elicitation of plant defence mechanisms, and then were transferred to 0°C. Percent infection was assessed after 12 wk at 0 ± 0.3°C.

The experiment was carried out according to a randomised block design, where each of the treatment combinations was applied to one box of fruit in four blocks, each block comprising a separate position in the coolstore. Data transformation was not required. Treatment comparisons were obtained by linear contrasts.

6.3.10 Experiments 8-9

Title : Extended storage of stem plug extracts and chitinase activity - 1994 season.

A Stem plug extracts from uncured fruit (Experiment 8)

Chitinase activity in the four uncured kiwifruit extracts from Experiment 2 were measured immediately after extraction (time 0), as described in Experiment 2. Each extract was then divided in two, and one half was stored at -20°C and the other half at -80°C. Activity measurements were made at three month intervals for one year.

The data were assessed as a completely randomized, repeated measures design and transformation was not required.

B Stem plug extracts from cured fruit (Experiment 9)

Experiment 8 was repeated using the cured extracts from Experiment 2.

6.4 RESULTS

Unless otherwise stated, chitinase activity is expressed as ng of substrate (tritiated chitin) solubilised per minute per ml of crude extract.

6.4.1 Experiment No. 1

All stem plugs, regardless of harvest date, inoculum loading and disease status, contained significant amounts of chitinase activity relative to the enzyme-free control (P=0.0001). There was no appreciable difference in the amount of initial enzyme activity for any of the four harvest dates (Fig. 6-1). Endochitinase activity in uninoculated, healthy stem plugs had increased significantly after 12 wk of coolstorage, and increases were greater in the later-harvested fruit (Fig. 6-1) which had higher mean soluble solids contents (refer Chapter 3, Section 3.3.2) and were also more resistant to infection (Chapter 3, Section 3.4.2).

After 12 wk storage at 0 ± 0.3°C, there was no consistent relationship between chitinase activity and disease status of the inoculated tissue (Fig. 6-2).

6.4.2 Experiment No. 2

Curing had no significant effect on total chitinase activity, after nine weeks in the coolstore (P>0.05 and Table 6-1).

Preliminary ELISA showed that there was no reaction between kiwifruit chitinase and the bacterial and cucumber antibodies. Binding of the CH2 and SP antibodies was weak and did not consistently differentiate cured and uncured extracts (data not presented). In contrast, the CH4 antibody exhibited strong binding. Reaction of CH4 with the cured extracts produced significantly higher absorbances than the uncured extracts, and these results were consistently observed on different days and over a range of antigen dilutions (Fig. 6-3, A and B). The Western blot showed that CH4 had bound to a single 30 kDa protein in the cured and uncured extracts, and that the level of this protein appeared to increase with curing (Plate 6-1). An identical result was obtained when the Western blot was repeated on a separate occasion (data not shown). The CH4 antibody did not cross

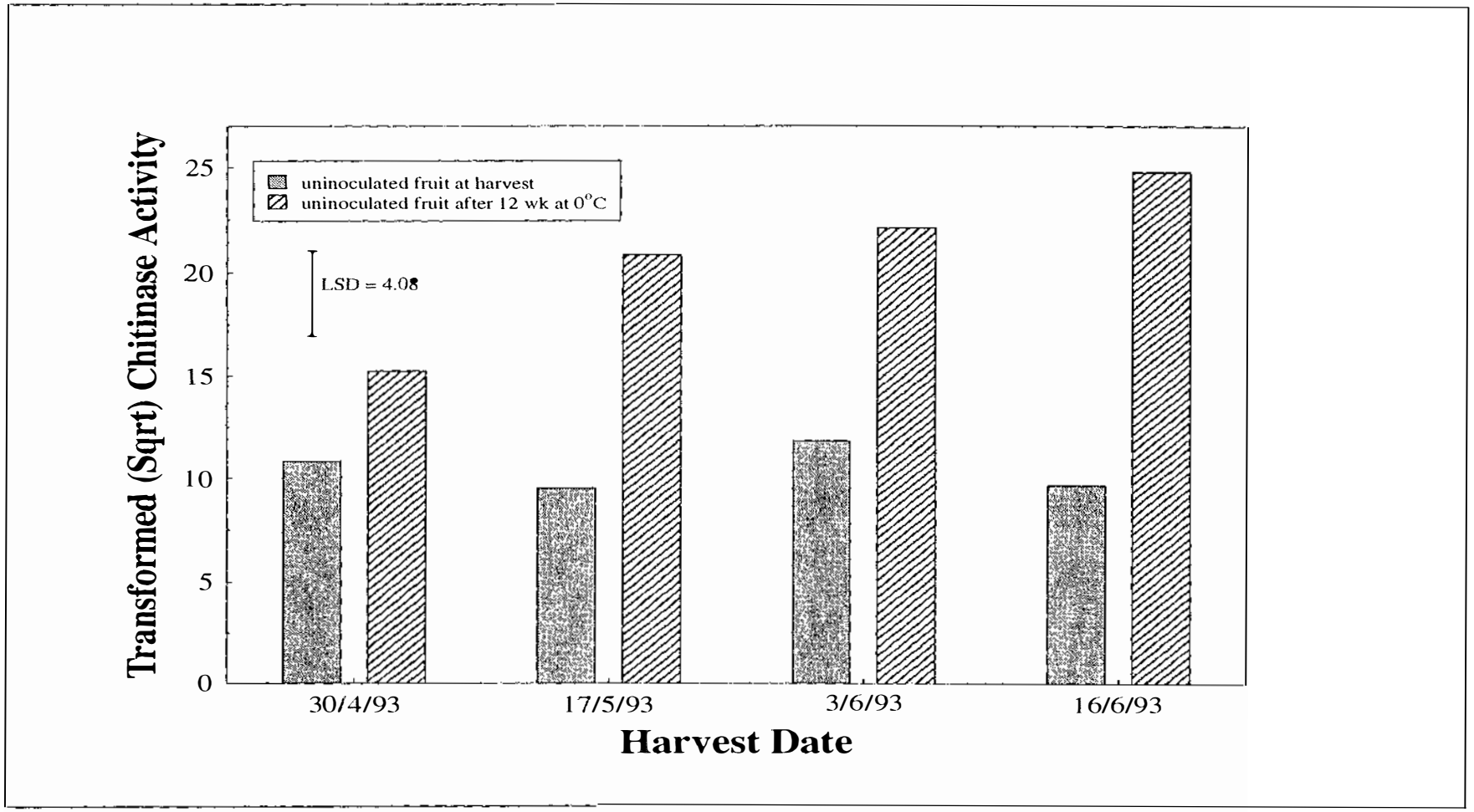


Figure 6-1. Chitinase activity (square root transformed) in uninoculated, healthy kiwifruit, assessed before and after 12 wk storage at 0 ± 0.3°C in 1993. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. LSD = least significant difference.

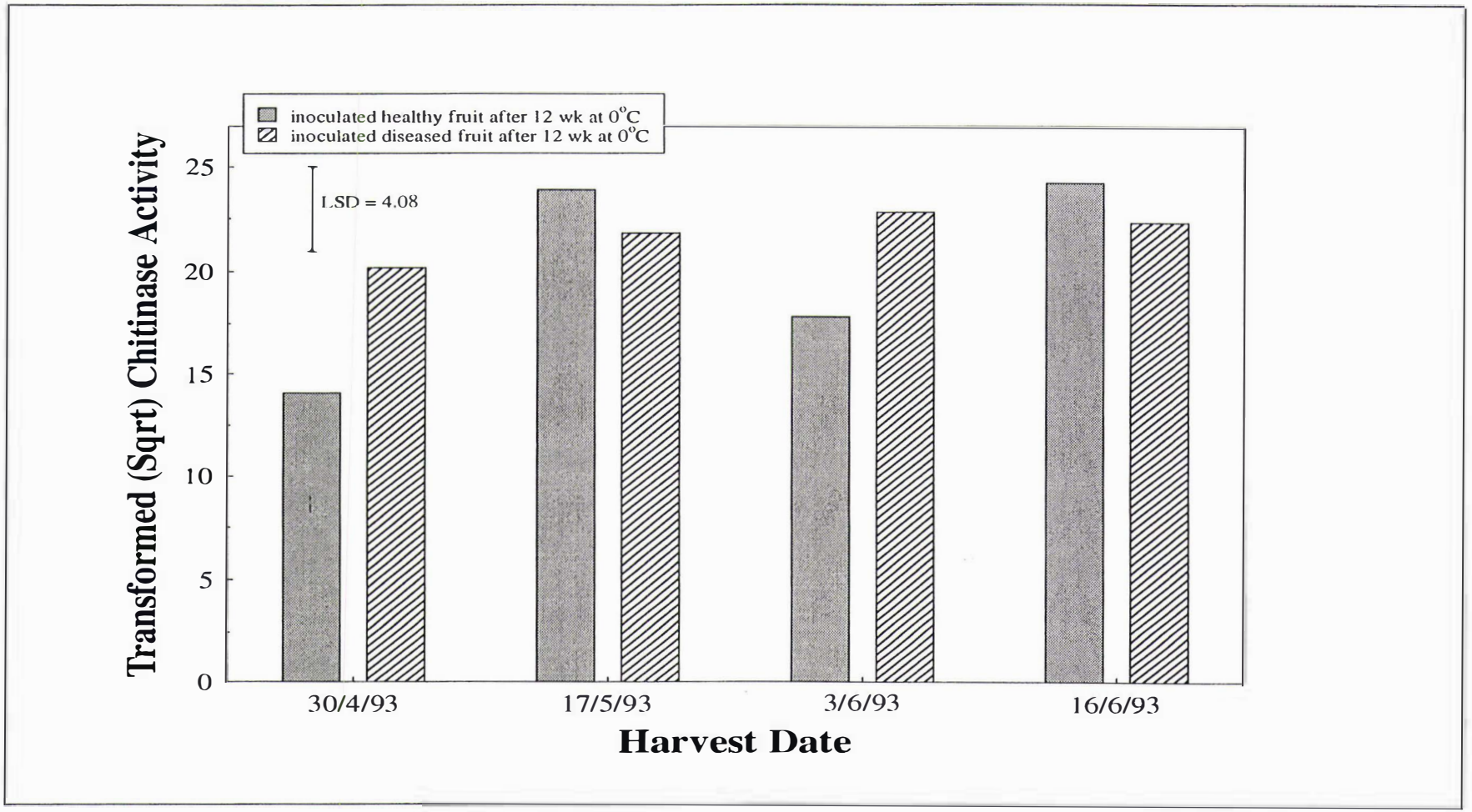


Figure 6-2. Chitinase activity (square root transformed) in healthy and diseased kiwifruit inoculated with 5,000 spores of *B. cinerea* per stem scar before storage, assessed before and after 12 wk storage at 0 ± 0.3°C in 1993. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. LSD = least significant difference.

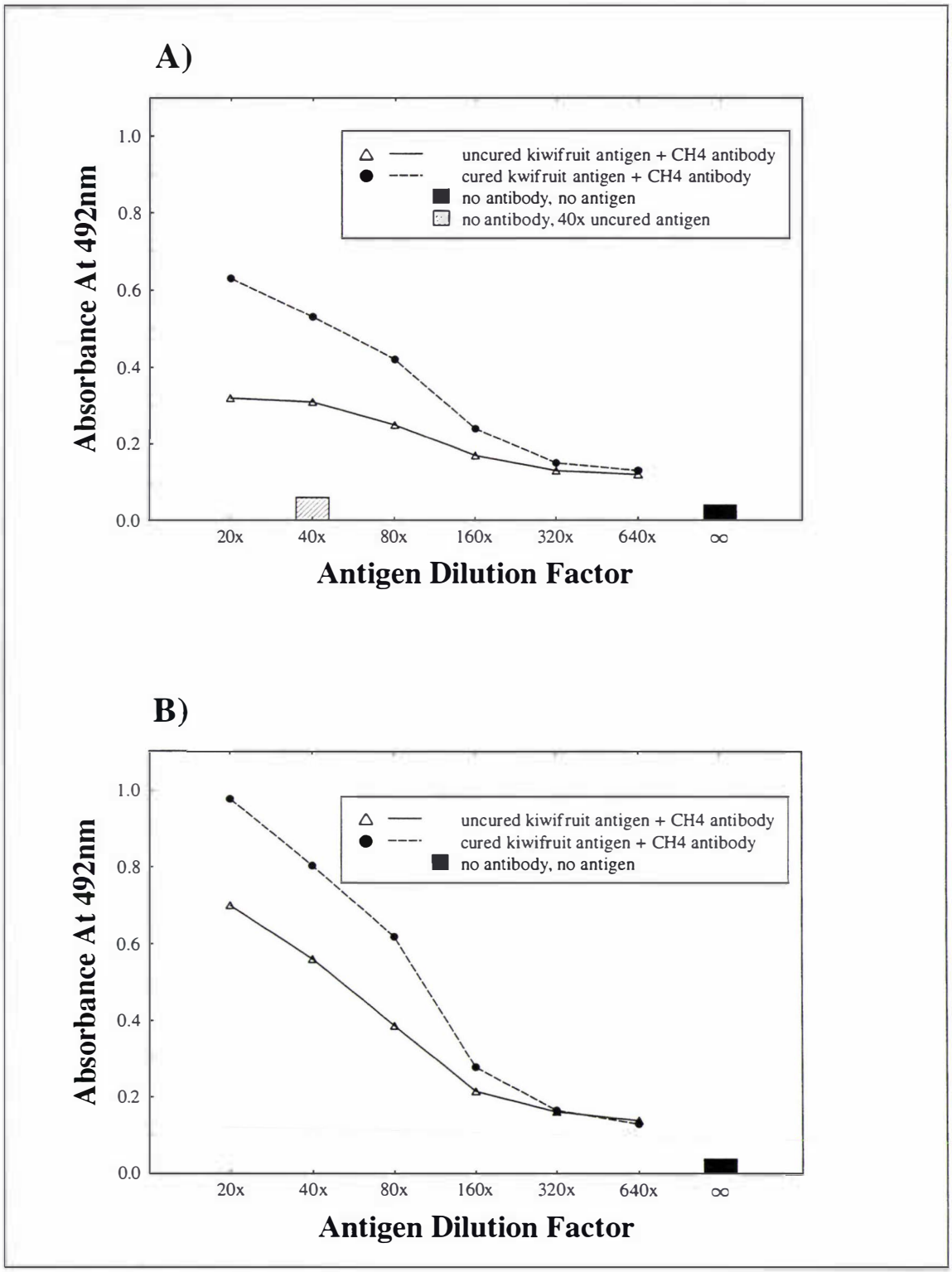


Figure 6-3. Absorbance values (raw data) from ELISA tests of polyclonal sugar beet chitinase CH4 antibody against kiwifruit antigen. A) 31 July 1995, no replication, B) 2 August 1995, mean of two replicates per treatment. Antigen comprised crude, healthy, cured 94/C/U/H and uncured 94/N/U/H kiwifruit stem plug extracts. CH4 antibody was diluted 1000-fold in PBS-Tween.

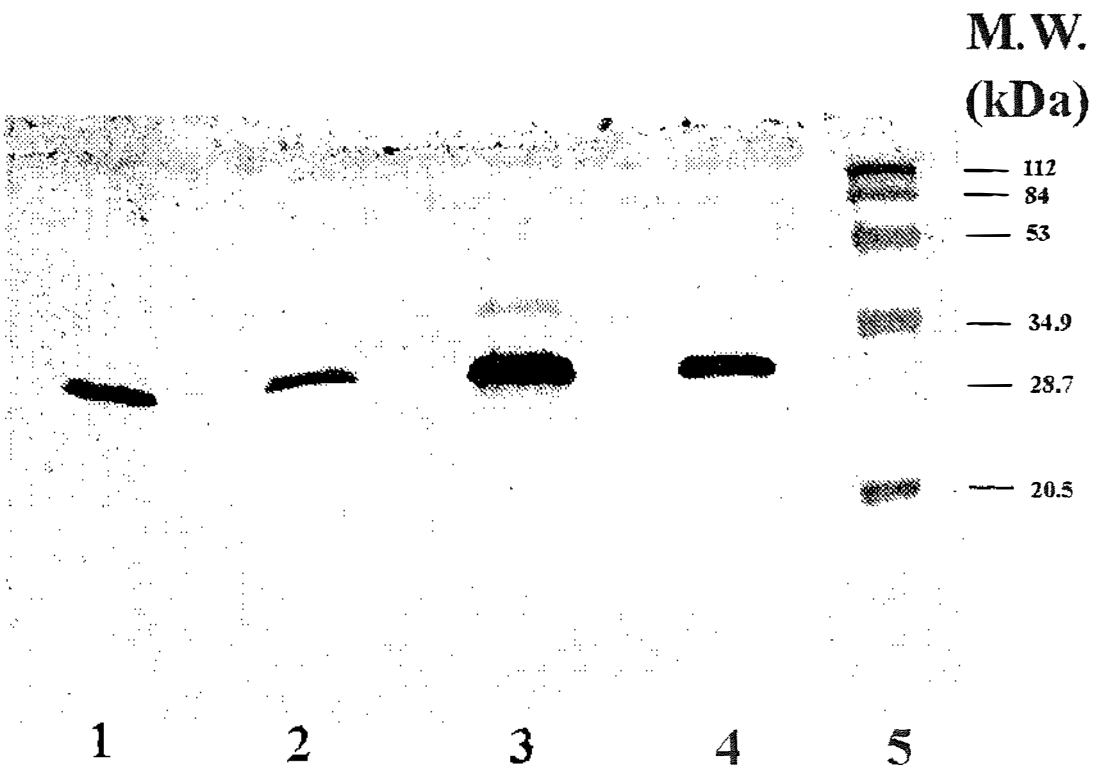


Plate 6-1. Western analysis of proteins in crude, cured and uncured kiwifruit stem plug extracts, treated with CH4 antibody raised against a sugar beet basic chitinase. Lane 1, 12.5-fold concentrate of uncured 94/N/U/H extract; lane 2, original uncured extract; lane 3, 12.5-fold concentrate of cured 94/C/U/H extract; lane 4, original cured extract; lane 5, molecular weight standards. Each lane contained 5 µl of extract. CH4 antibody was diluted 500-fold in PBS-Tween.

react with a partially purified extract of bean chitinase (data not shown).

The total protein and chitinase activity banding patterns were identical for cured and uncured extracts (Plate 6-2, A and B). Most proteins in the extracts were acidic, but there was one basic chitinase with a pI of 9 (Plate 6-2, A and B).

Table 6-1: Chitinase activity (mean ± SEM) in stem plug extracts of cured and uncured, uninoculated healthy fruit, after 9 weeks coolstorage at 0°C in 1994.

TREATMENT	CHITINASE ACTIVITY (ng/min/ml)*
Uncured	392.3 ± 34.0
Cured	485.8 ± 46.8
LSD† = 141.4, n‡ = 4	

* ng of tritiated chitin solubilised per minute per ml of crude extract.
† least significant difference.
‡ sample number.

6.4.3 Experiment No. 3

Protein content was significantly greater in cured fruit than in uncured fruit (P=0.0382 and Table 6-2).

Table 6-2: Total protein (mean ± SEM) in stem plug extracts of cured and uncured, uninoculated healthy fruit, after 9 weeks coolstorage at 0°C in 1994.

TREATMENT	TOTAL PROTEIN (μg/ml of extract)
Uncured	71.7 ± 2.0
Cured	81.2 ± 3.0
LSD* = 8.8, n† = 4	

* least significant difference.
† sample number.

6.4.4 Experiment No. 4

There was no significant "interaction" (refer Chapter 2, Section 2.3.4, Part C) between the inoculum/chitosan and temperature treatments on infection (P=0.1455).

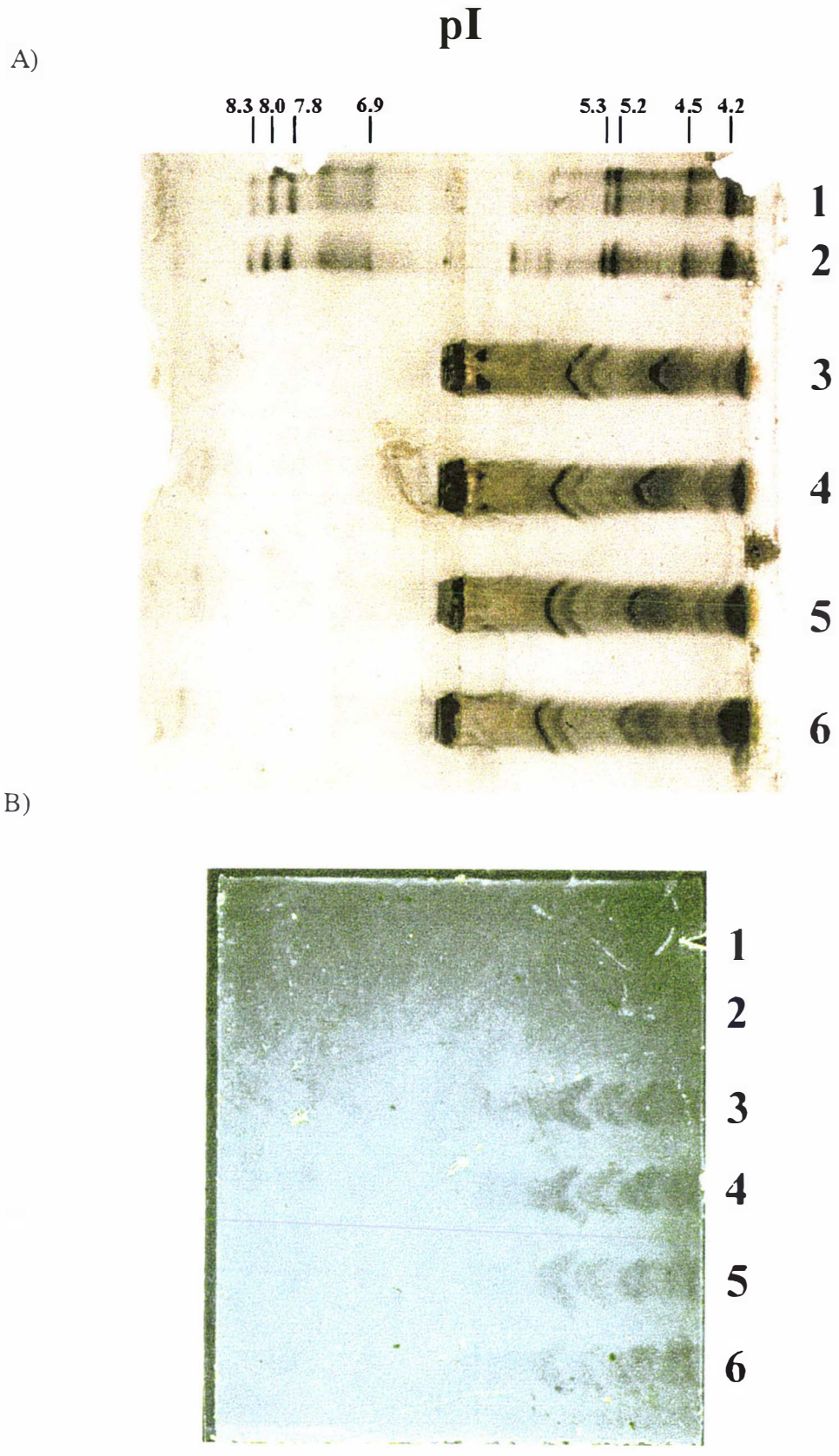


Plate 6-2. Total proteins and corresponding chitinase activities of cured and uncured crude kiwifruit stem plug extracts as detected by silver staining on a pH 3-10 isoelectrofocusing polyacrylamide gel (A) and Calcofluor staining of the associated overlay gel with a glycol chitin substrate (B). Lanes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

Interpretation of the "main effects" (refer Chapter 2, Section 2.3.4, Part C) was meaningful. Irrespective of temperature, inoculum/chitosan treatments had a pronounced effect on disease incidence (P=0.0001). There was a significant increase in infection when solvent and chitosan were applied to uninoculated fruit (Fig. 6-4, A). Infection increased significantly when the fruit was inoculated, and acid solvent addition caused a further significant increase in infection (Fig. 6-4, A). When a combination of solvent and chitosan was used on inoculated fruit, infection was significantly less than in the solvent only treatment, but did not differ markedly from inoculated (but otherwise untreated) fruit.

Percent infection was lower at 20°C than at 0°C, regardless of the inoculum/chitosan treatment (P=0.0137 and Fig. 6-4, B), and this was particularly the case in the two treatments containing chitosan (the overall interaction data were not significant and are not presented here).

6.4.5 Experiment No. 5

The infection level in the inoculated+solvent treatment (negative control) was significantly higher (P=0.0001) than in all the other treatments which had similar levels of infection (Fig. 6-5). Infection levels in the chitosan treatments were comparable with the positive uninoculated+solvent control. There was no benefit in increasing the chitosan concentration within the range tested (Fig. 6-5). (Note that the pH of the "U+S" and "I+S" control treatments was ≈1, compared to pH 5.6 for all other treatments containing chitosan.)

6.4.6 Experiment No. 6

Chitinase activity was plotted against time after harvest, for up to 6 d after harvest in Fig. 6-6, and up to 6 wk after harvest in Fig. 6-7. Data from each sampling time after harvest were analysed separately, producing six ANOVA's and 12 different LSD values. The large number of LSD's prevented their inclusion on Fig. 6-6 and 6-7 and results of the statistical analysis are summarised on page 187.

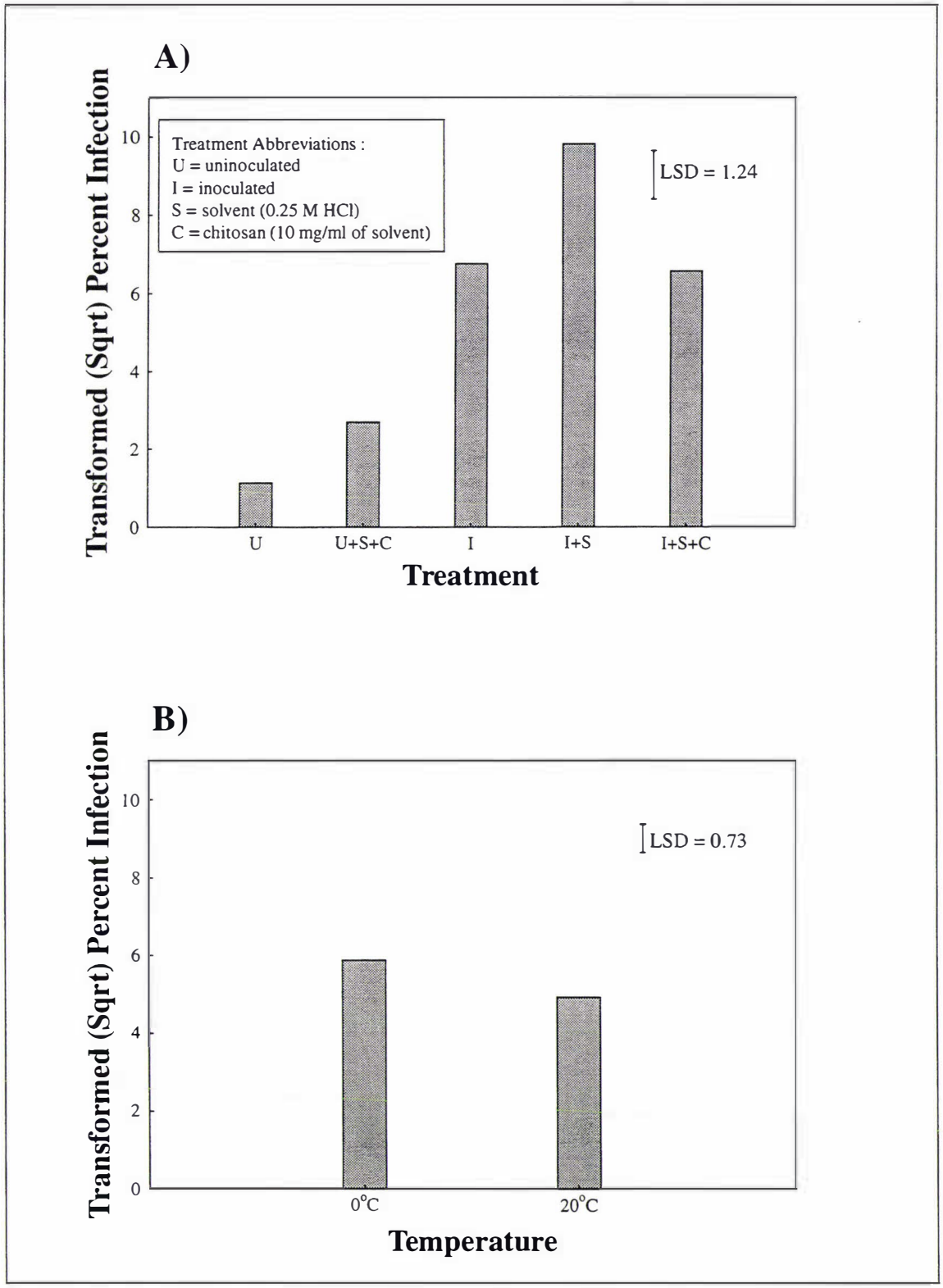


Figure 6-4. Percent infection (square root transformed) of fruit after 12 wk storage at 0 ± 0.3°C in 1993, as influenced by A) inoculation, solvent and/or chitosan application (averaged over elicitation temperature), B) elicitation temperature (averaged over inoculation, solvent and/or chitosan application). LSD = least significant difference.

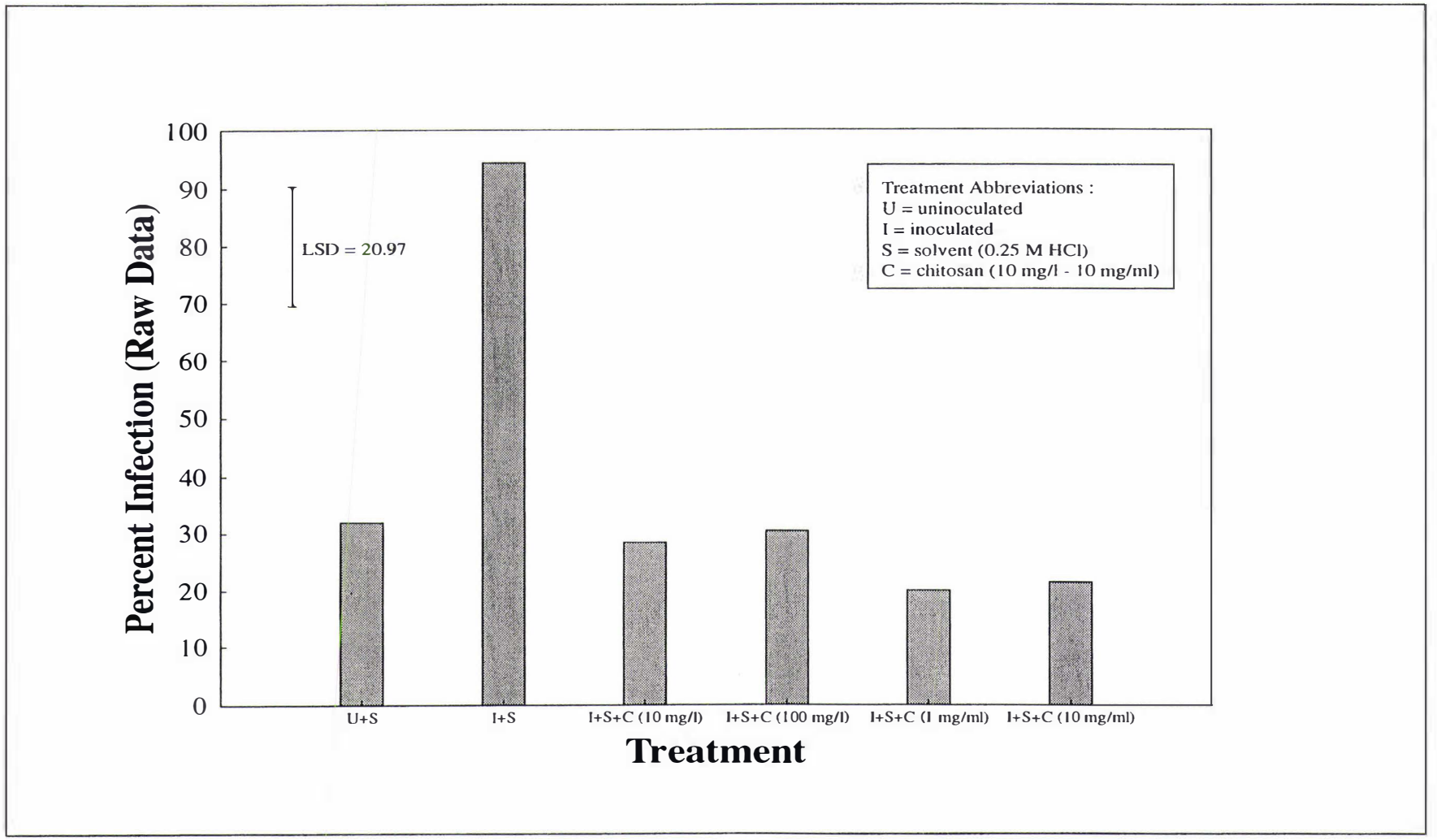


Figure 6-5. Relationship between chitosan concentration and percent infection (raw data) of fruit, after 12 wk storage at 0 ± 0.3°C in 1993. LSD = least significant difference.

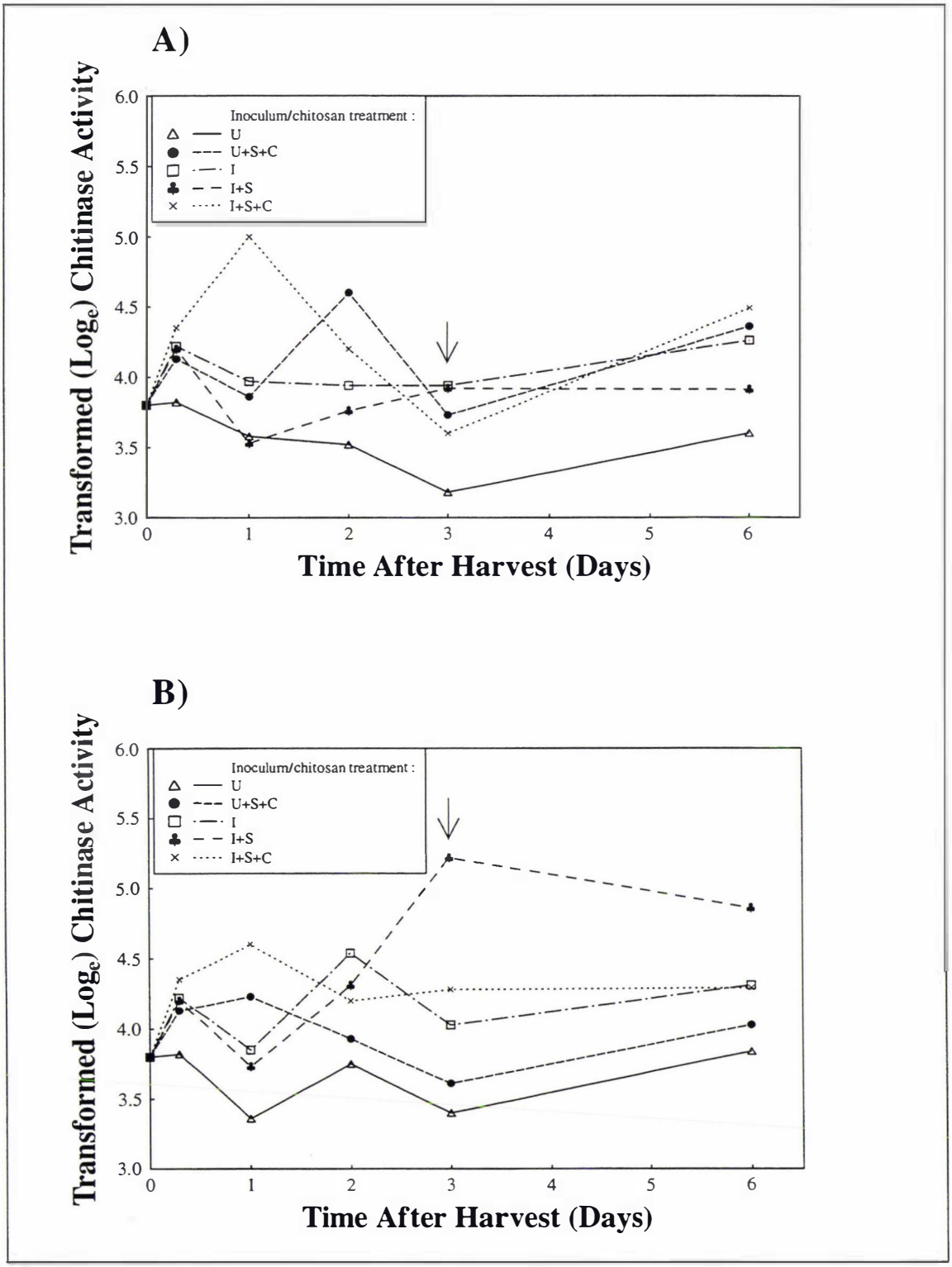


Figure 6-6. Chitinase activity (log_e transformed) of fruit, as influenced by inoculation, solvent and/or chitosan application, and after 0, 1, 2 or 3 days "curing" exposure to A) 0°C or B) 20°C. Stem plugs were extracted from the fruit 0-6 days after harvest. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. ↓ = end of curing period.

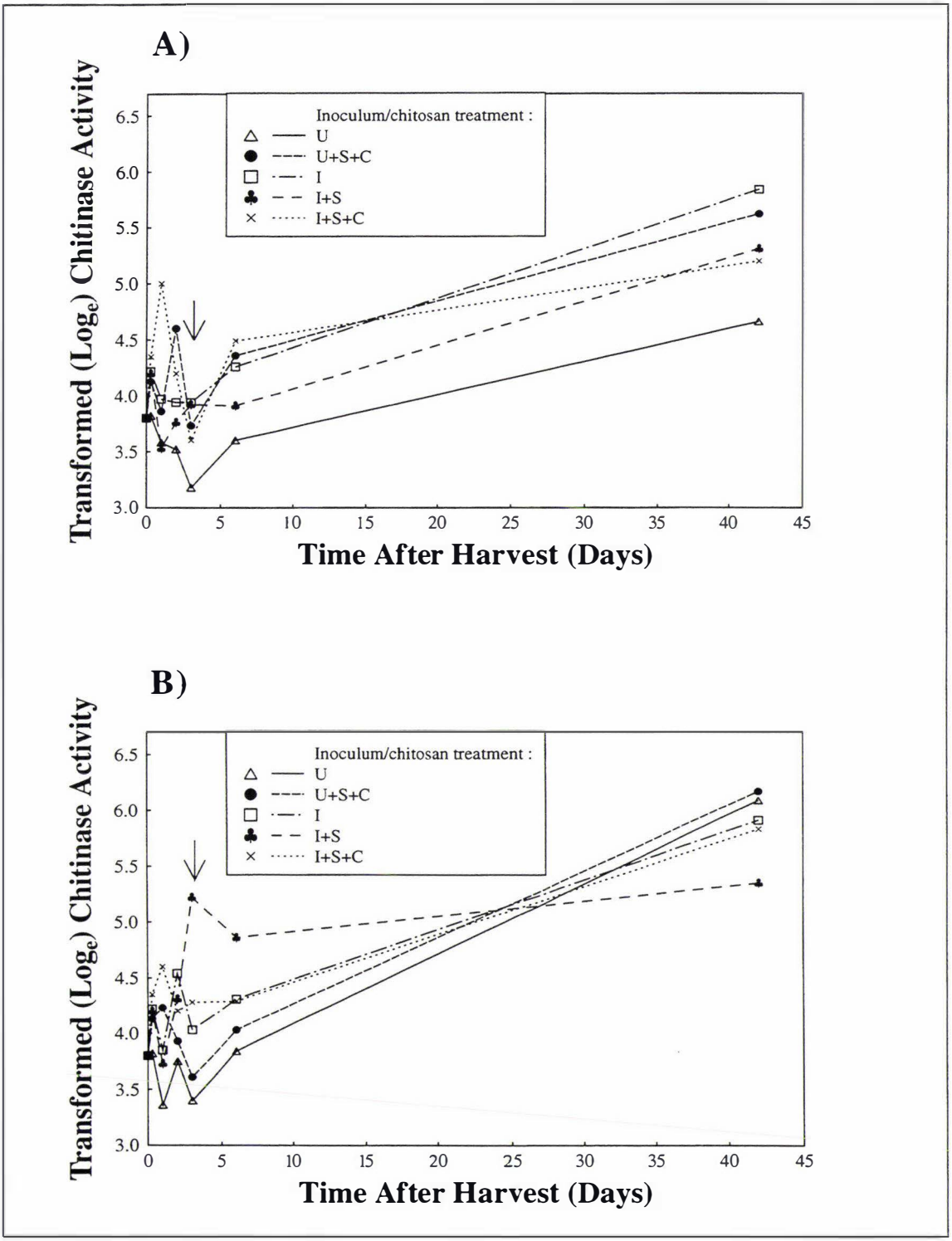


Figure 6-7. Chitinase activity (log_e transformed) of fruit, as influenced by inoculation, solvent and/or chitosan application, and after 0, 1, 2 or 3 days "curing" exposure to A) 0°C or B) 20°C. Stem plugs were extracted from the fruit 0-42 days after harvest. Only data from healthy stem plug extracts are presented. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. ↓ = end of curing period.

There were significant "interactions" between inoculum/chitosan and temperature treatments for the stem plugs excised 2 and 3 d after harvest. At these two sample times, exposure to 20°C for 72 h elicited more chitinase activity in several of the inoculum/chitosan treatments than the same period at 0°C, but the situation was reversed for the U+S+C treatment (day 2) and U+S+C and I treatments (day 3). However, the biological relevance of these observations is questionable, because interaction patterns were not consistent at each sampling time after harvest and there were large fluctuations in the data over days 1-6.

The temperature "main effect" was an increase in chitinase activity with increased temperature, although this difference was only significant in stem plugs excised 3 d and 6 wk after harvest.

When the five inoculum/chitosan treatments were ranked according to chitinase activity, the ranking differed at each sampling time after harvest, and large unpredictable fluctuations in the activity measurements were found in all treatments at both "curing" temperatures (Fig. 6-6). Despite these inconsistencies, there were some general trends worth noting.

During the six days of assessment, chitinase activity in uninoculated fruit did not rise above the level found at harvest (Fig. 6-6). However, treatments involving inoculation, solvent and/or chitosan application appeared to stimulate chitinase production. These increases in chitinase activity occurred within 7 h of harvest, and became significant 1 d or more after harvest at both "curing" temperatures (Fig. 6-6). Activity in the I+S+C treatment was generally associated with one of the two highest chitinase activities at each sampling time (Fig. 6-6).

By 6 wk after harvest there were appreciable increases in chitinase activity, which were unrelated to either inoculum/chitosan or temperature treatments. These increases were also found in uninoculated (U) fruit (Fig. 6-7).

Overall, chitinase activity in stem plugs excised six weeks after harvest was significantly

greater in diseased than in healthy fruit (Table 6-3). (Note that the 6 wk data presented in Fig. 6-7 are from healthy stem plug extracts only.)

Table 6-3: Chitinase activity (log_e transformed means ± SEM) in various healthy and diseased stem plug extracts of cured, uninoculated and inoculated fruit, with and without the addition of acidic solvent and chitosan, after 6 weeks coolstorage at 0 ± 0.3°C in 1993.

EXTRACT DISEASE STATUS	CHITINASE ACTIVITY (ng/min/ml)*
Healthy	5.6 ± 0.1
Diseased	6.0 ± 0.1
P† = 0.0007	

* ng of tritiated chitin solubilised per minute per ml of crude extract.
† linear contrast P-value.

6.4.7 Experiment No. 7

Inoculation significantly increased infection (Fig. 6-8, A). Solvent application further increased infection relative to the inoculated control, but these increases were only significant when 0.04 M formic acid and 0.25 M HCl were used (Fig. 6-8, A).

The unadjusted solvents varied from pH 1 to pH 3. Adjusting solvent pH to 5.6 reduced disease incidence to a level comparable with that in the inoculated (but otherwise untreated) control (Fig. 6-8, B).

When chitosan was added to pH unadjusted solvent, a significant decrease in infection occurred relative to when the pH unadjusted solvent was applied on its own. Application of chitosan in pH adjusted solvent did not have a significant effect on disease incidence (Fig. 6-8, B).

6.4.8 Experiment No. 8

The "main effect" of storage duration, and the "interaction" between storage duration and temperature on chitinase activity were not significant (P>0.05). There was, however, a general reduction in chitinase activity of extracts stored at -20°C compared with -80°C

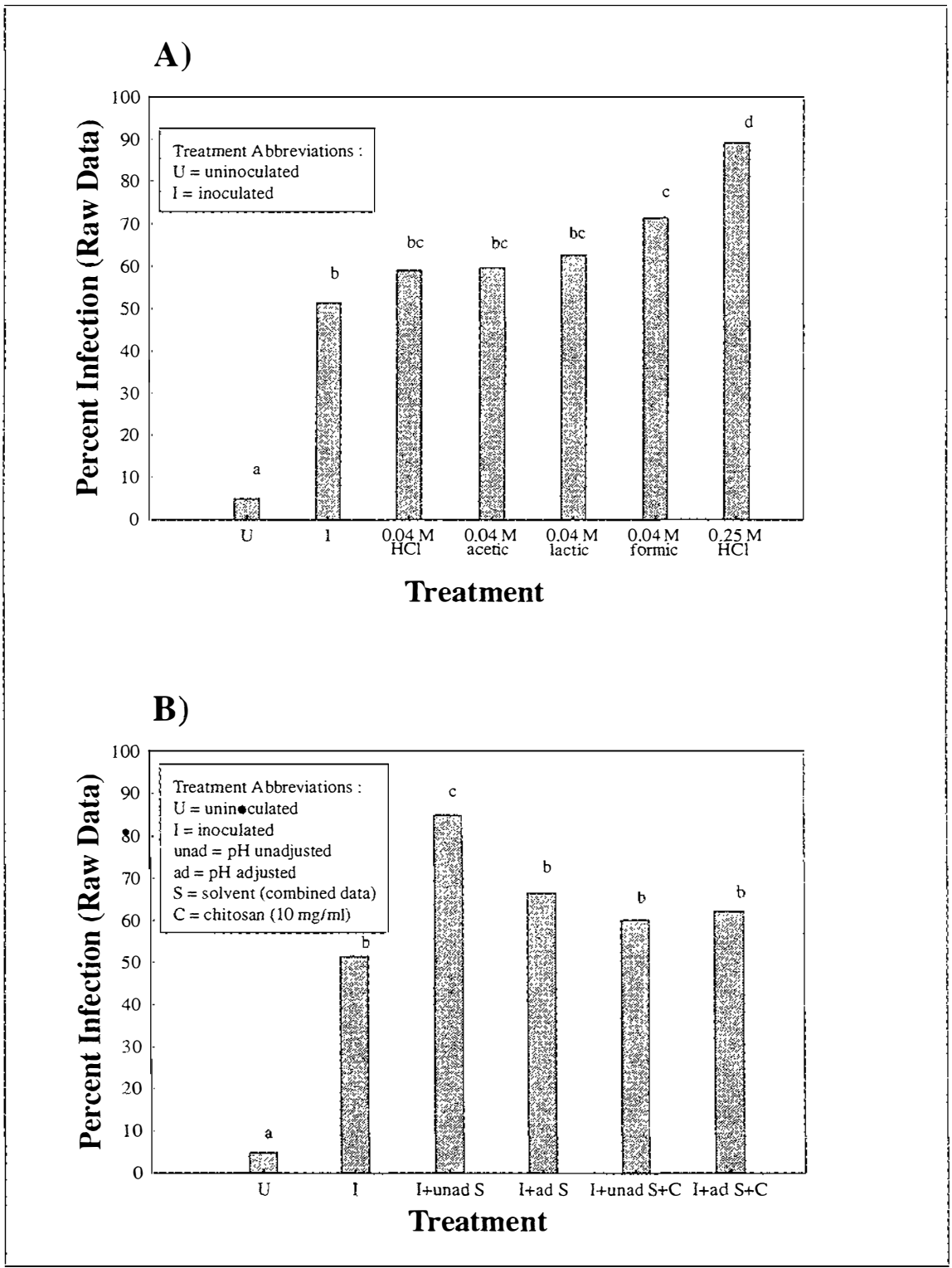


Figure 6-8. Percent infection (raw data) of fruit assessed after 12 wk storage at 0 ± 0.3°C in 1994, as influenced by A) solvent type (averaged over solvent pH adjustment and chitosan application), B) solvent pH adjustment and chitosan application (averaged over solvent type). a,b,c,d represent significant differences in Duncan's Multiple Range test (α=0.05).

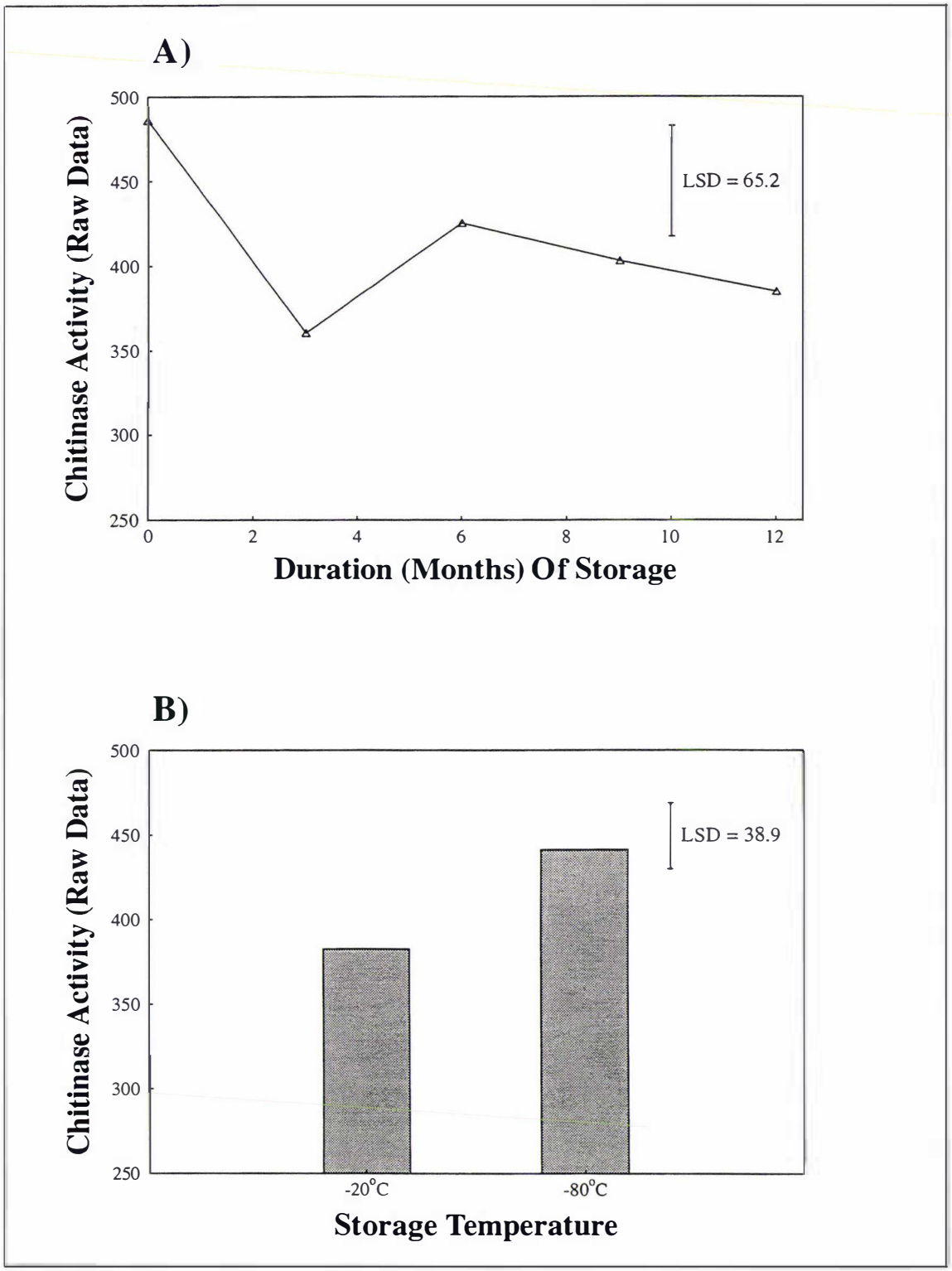


Figure 6-9. Chitinase activity (raw data) in cured, uninoculated, healthy 94/C/U/H kiwifruit stem plug extracts as influenced by A) storage duration (averaged over storage temperature), B) storage temperature (averaged over storage duration), assessed in 1994/1995. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of crude extract. LSD = least significant difference.

(P=0.0358 and Table 6-4).

Table 6-4: Chitinase activity (raw means ± SEM) in uncured, uninoculated, healthy 94/N/U/H kiwifruit stem plug extracts, as influenced by storage temperature (averaged over storage duration), assessed in 1994/1995.

STORAGE TEMPERATURE	CHITINASE ACTIVITY (ng/min/ml)*
-20°C	320.9 ± 21.4
-80°C	370.6 ± 16.9
LSD† = 43.5, n‡ = 20	

* ng of tritiated chitin solubilised per minute per ml of crude extract.
† least significant difference.
‡ sample number.

6.4.9 Experiment No. 9

There was no statistically significant interaction between storage duration and temperature (P>0.05).

The main effect of storage duration was significant (P=0.0071 and Fig. 6-9). Activity decreased over time, irrespective of the storage temperature, although the reading at three months was probably due to a low assay result rather than a real effect (Fig. 6-9, A).

Chitinase activity was higher in extracts stored at -80°C than at -20°C, regardless of the storage duration (P=0.0171 and Fig. 6-9, B).

6.5 DISCUSSION

The findings of this study suggest that in some situations chitinases may contribute to resistance in kiwifruit, since there was a correlation between reduced infection and increased chitinase activity in intact fruit during storage, particularly in late-harvested fruit, and the increased activity of a single chitinase isoform with curing.

Initial chitinase activities in all Experiment 1 fruit were similar at the point of harvest. However, biochemical changes accompanying ripening do not stop when the fruit is removed from the vine (Snowdon 1990). Postharvest increases in kiwifruit chitinase activity occurred during 12 wk storage at 0°C in Experiment 1, and these increases were accentuated in the later-harvested fruit which were less prone to storage rots (Chapter 3, Section 3.4.2). Increases in chitinase activity during prolonged storage of intact fruit were also observed in Experiment 6. Sharrock & Hallet (1992a) observed that pericarp tissue extracts from kiwifruit stored at 0°C for five months were able to inhibit *B. cinerea* spore germination, whereas pericarp extracts from freshly harvested fruit promoted spore germination. Chitinase appears to be a component of stem plug antifungal activity after prolonged storage, and could be partly responsible for the effect reported in Experiment 4, Chapter 3 (Section 3.4.4), where fruit were virtually impossible to infect after six weeks, unless the stem plug and associated antifungal contents were partially removed with a 5 mm diameter drill bit.

Most fruit become more susceptible to infection as they mature (Brown & Swinburne 1980; Sommer 1982; Lavy-Meir et al. 1989; Prusky et al. 1985), and this is often attributed to a weakening of host cell walls, to a reduced ability to synthesize antifungal substances and/or increased proteolytic degradation of existing antifungal compounds (Prusky et al. 1985; Snowdon 1990; Alonso et al. 1992).

In certain crops such as tobacco (Wyatt et al. 1991), and kiwifruit (Pennycook & Manning 1992; Pyke et al. 1993b; Poole & McLeod 1994), resistance improves with age and can be positively correlated with an increase of specific antifungal compounds. Age-related resistance to blue mould in tobacco was correlated with increases in β-1,3-glucanase, chitinase and peroxidase activities (Wyatt et al. 1991). Mauch et al. (1988a) also observed that endochitinase and β-1,3-glucanase activities increased in maturing pea pods, and proposed that this might protect the growing seeds from bacteria, fungi and insects. To our knowledge, the current study is the first to provide data suggesting that a similar relationship between chitinase and maturation also occurs in kiwifruit. Unlike many fruit, kiwifruit can be maintained in coolstorage for up to one year without unacceptable decrease in quality, and in general there is a paucity of literature examining

biochemical changes in defence compounds of intact fruit after prolonged storage.

In Experiments 1 and 6, increases of chitinase activity during 12 wk storage at 0°C occurred regardless of fruit disease status, although activity was significantly higher in the diseased fruit from Experiment 6. Increased activity in diseased stem plugs was either due to additional stimulation of chitinase activity by infection, as observed by McLeod & Poole (1994), or to the presence of pathogen exochitinases which are not distinguished by the Molano et al. (1977) chitinase assay. There are many reports of chitinase induction by pathogenesis and abiotic stress, but more recent literature has shown that chitinases can be regulated developmentally, irrespective of microbial attack or other stress. In tobacco, chitinases accumulate during normal flower development (Lotan et al. 1989), and in non-senescent healthy petunia flowers chitinase activity is localised in the stigma and increases five-fold following anther dehiscence (Leung 1992). Leung (1992) proposed that chitinase could play a role in pollen recognition and/or germination. (In this hypothesis, the substrate is unlikely to be chitin.) Swegle et al. (1992) demonstrated that chitinase mRNA accumulated during barley seed development, with a decrease at the very end of growth. Increases in chitinase activity with fruit maturation in healthy tissue provides another example of developmental regulation of chitinase activity. The role of ubiquitous PR-proteins such as chitinase may be much wider than first thought.

An increase in total protein content was associated with curing in Experiment 3, and the results of Experiment 2 suggest that a chitinase isozyme may be among the proteins stimulated. Curing did not induce new chitinase isoforms, since patterns of chitinase activity were identical for cured and uncured extracts, and most of the existing isoforms were also unaffected, as indicated by the total chitinase activity measurements (Experiment 2). However, ELISA and Western blot results showed that CH4 antibody bound to a single ≈30 kDa protein which increased with curing. It was not possible to determine whether this protein was a chitinase by direct alignment with a Calcofluor activity stain, because attempts to renature proteins after SDS electrophoresis by addition of 1% Triton X-100 (Trudel & Asselin 1989) were unsuccessful. In addition, poor resolution prevented the use of native instead of SDS electrophoresis in the Western blot

system. Nevertheless, the high specificity of CH4 binding suggests that this protein is a chitinase. CH4 antibody was raised to a sugar beet basic class IV chitinase (see Chapter 1, Section 1.8.1, Part B, (iv)). The antibody is highly specific since it reacts with class IV chitinases, which have only been observed in three species of plants thus far (Margis-Pinheiro et al. 1991; Mikkelsen et al. 1992; Rasmussen et al. 1992), but does not cross react with chitinases from other classes, both in sugar beet and in a diverse range of other plants including bean, tobacco, wheat, barley and pea (Mikkelsen et al. 1992). Moreover, the antibody has a very low background and can be used at dilutions as high as 1:10,000 (Mikkelsen, pers comm). In our study, CH4 binding appeared to be highly specific, since the background in the "no antibody" control (refer Chapter 2, Section 2.2.9, Part A) was very low (Fig. 6-3, A), the antibody bound to only one protein in the crude kiwifruit extracts which would have contained a diverse range of compounds, and it did not cross react with bean chitinase. This putative chitinase, which is bound by CH4, appears to be part of the curing induced response in kiwifruit, may play a role in defence against *B. cinerea* and is particularly worthy of purification and characterisation. Other proteins induced by curing (Experiment 3) also deserve further investigation.

Curing reduces infection in several crops including citrus fruits (Ben-Yehoshua et al. 1987), and potatoes (Stewart et al. 1983), and concurrently stimulates plant defence mechanisms. Brown & Barmore (1983) found that curing increased phenols and lignins in citrus, and Ben-Yehoshua et al. (1988) recorded coumarin accumulation in the flavedo of pummelo fruit in response to curing. As discussed previously (see Chapter 3, Section 3.5), physical barriers may contribute to curing-mediated reduced infection in kiwifruit, but are not the sole cause. Induction of hydrolytic enzymes may also be important, as suggested by correlative evidence in the current study and by Sharrock & Hallet (1993), who reported that stem plug endochitinase activity increased significantly (several-fold) in fruit that had been cured for seven days at 20°C, compared to initial activity at harvest. The stem plug tissues in their experiment were removed and assayed for chitinase activity immediately after curing, whereas the fruit in Experiment 2 spent a further nine weeks in the coolstore before tissues were assayed. This may account for the larger effect of curing on chitinase reported by these authors than was observed in

Experiment 2. Their study did distinguish which isoform(s) were induced by curing.

Curing generally reduces *B. cinerea* infections in kiwifruit (Pennycook & Manning 1992; Poole & McLeod 1994), but the success of this treatment can be somewhat variable (Beever 1992). Inadequate control of temperature and relative humidity during curing may explain capricious results. Morris et al. (1989) found that the efficacy of curing in potato was highly dependent on these two factors. Temperature affected enzymic processes such as lipid and lignin deposition, whilst infection severity increased at low humidity, possibly due to cell dehydration and a subsequent reduced ability to manufacture antifungal compounds. More research is required to determine the role of kiwifruit chitinases in curing under controlled temperature and relative humidity.

Timing of chitinase induction appears to influence its effectiveness as a defence mechanism. The rapidity and extent of a defence response typically establishes the difference between a resistant and sensitive interaction (Bowles 1990; Sequeira 1990). For example, Joosten & de Wit (1989) found that chitinases accumulated four days earlier in an incompatible compared to a compatible interaction between tomato and *Cladosporium fulvum*. Rasmussen et al. (1992) observed rapid and large increases in chitinase activity of oilseed rape cultivars resistant to *Phoma lingam*, compared with susceptible plants where an increase in activity was delayed until 24 h after infection. Broglie & Broglie (1994) eliminated the temporal factor in chitinase production by creating transgenic tobacco plants with strong constitutive chitinase expression. These plants showed increased resistance to *Rhizoctonia solani*. Chitinases appear to play a role in kiwifruit defence, provided endogenous levels are elevated prior to establishment of infection. For example, enhanced chitinase activity and resistance were found in kiwifruit subjected to prolonged storage at 0 ± 0.3°C. Chitinases are also stimulated by inoculation (McLeod & Poole 1994), but without prior induction they may not accumulate in time to prevent infection. In such cases, defence responses which occur more rapidly, such as production of phenylpropanoids, may be more important (refer Chapter 4, Section 4.5).

Experiments 4, 5 and 7 demonstrated that pH of acidic solvents had a significant effect

on disease incidence. In these experiments, the addition of pH unadjusted acidic solvent significantly increased infection relative to the inoculated control. Experiment 7 demonstrated that the low pH (pH 1-3) of the unadjusted solvent was largely responsible for raised infection. The findings of the current study are supported by those of Stössel & Leuba (1984), who noted a stimulation in the growth of some fungi when chitosan was applied in a low pH environment. The low pH of the unadjusted solvents may have damaged fruit tissue, thereby facilitating entry of the fungus. It is unlikely that a low pH favoured *B. cinerea* growth, since Qadir (1994) found no correlation between mycelial dry weight of *B. cinerea* grown in basal medium and pH of the medium, over the range 2.5-8.5.

Chitosan application significantly lowered infection when the host tissue had been predisposed to high levels of disease by the addition of pH unadjusted solvent (Experiment 7). However, this effect was not observed with pH adjusted solvent (Experiment 7), and chitosan did not reduce disease incidence below the level found in inoculated but otherwise untreated fruit (Experiments 4 and 7). Consequently, there appears to be no practical benefit in the kiwifruit industry adopting this control technique. The use of water-soluble chitosan oligomers or soluble chitosan derivatives, such as glycol chitosan and carboxymethyl chitosan, requires investigation before the commercial use of chitosan for control of *B. cinerea* on kiwifruit can be disregarded. These methods were not considered to be within the scope of this project because of time and financial constraints. There is some disparity in the literature regarding efficacy of the various soluble chitosan oligomers and derivatives. Kendra & Hadwiger (1984) reported that high concentrations of trimer, tetramer and pentamer units derived from crab chitosan were antifungal against *Fusarium solani* and induced phytoalexin production in pea, with the hexamer having maximal antifungal and pisatin-inducing activity. These researchers also noted that glycol chitosan exhibited antifungal and pisatin-eliciting properties. Hirano et al. (1990a) reported that coating radish and soybean seeds with carboxymethyl chitosan weakly stimulated chitinase activity in the resultant seedlings, whilst glycol chitosan coatings were ineffective. White & Gadd (1983) demonstrated that glucosamine (monomer of chitosan) reduced the growth of *Verticillium albo-atrum*. Liénart et al. (1993) found that chitosan tetramers induced β-1,3-glucanase

activity in *Rubus fruticosus* protoplasts. Kauss et al. (1989) found that chitosan oligomers caused only slight or in some cases no callose induction in *Catharanthus roseus*. Chitosan elicited lignification in wounded wheat leaves (Pearce & Ride 1982), but the monomeric-tetrameric oligomers were ineffective (Barber et al. 1989). Efficacy of a particular chitosan oligomer or derivative appears to vary with the type of defence response being studied and the host.

The results of Experiment 6 suggest that the ability of chitosan to reduce *B. cinerea* infection in acid-stressed kiwifruit tissue is related to its fungistatic action rather than its ability to elicit chitinases, since there was no strong correlation between chitosan-elicited chitinase activity levels and reduced infection. It remains possible that chitosan elicited other kiwifruit defences, but in general it appears that induced host resistance is not considered to be the major factor behind chitosan-mediated disease control. Chitosan is fungistatic against a wide range of fungi (Allan & Hadwiger 1979) including *B. cinerea* (El Ghaouth et al. 1992b). In agreement with the findings of the current study, El Ghaouth et al. (1992c) proposed that the mechanism by which chitosan reduced decay in strawberries was related to its fungistatic property rather than its ability to induce chitinases, because chitinase activity was only stimulated on cut (wounded) surfaces and the extracts from cut strawberries treated with chitosan were unable to inhibit *B. cinerea*. Reduction of *B. cinerea* decay in bell pepper fruit was attributed mainly to the antifungal effects of chitosan, whilst plant structural barriers elicited by chitosan application were thought to play a supporting rather than a primary role in preventing spread of the pathogen (El Ghaouth et al. 1994b).

Proteins are fragile molecules whose functions can be disrupted by numerous factors such as aggregation, extremes of heat and pH, and proteolysis by contaminant microbes or enzymes in the extract. Minimizing protein inactivation is crucial to both purification and storage (Volkin & Klibanov 1989). Long term storage of enzyme solutions is normally maximised at temperatures below -70°C, providing that cycles of freezing and thawing are minimised (Ersson et al. 1989). This was borne out by the findings of this study (Experiments 8 and 9), where enzyme activity of extracts tended to decline more quickly at -20°C than at -80°C. In Experiment 9, activity was significantly reduced by

nine months storage (ignoring the anomalous result at three months), and extracts should be used well in advance of this time.

The correlative evidence of the role of chitinases in kiwifruit defence presented in the findings of this study justifies the need for enzyme purification to provide further more direct evidence of their function in the kiwifruit defence arsenal.

7 PROTEIN PURIFICATION

Proteins require special care during purification to retain activity. Successful isolation requires that factors such as dilution, proteolysis, change in physical state (eg. freezing and thawing), exposure to heavy metal ions, and extremes of heat and pH which denature proteins are minimised (Ersson et al. 1989; Volkin & Klibanov 1989; Deutscher 1990). Perhaps the most vital preliminary stage in protein purification is the development of appropriate assays to monitor the protein of interest eg. enzyme activity (Ersson et al. 1989; Linn 1990). This proved the greatest challenge in the current study, but realisation of this task meant that protein purification was now possible.

7.1 PART 1 - PRELIMINARY EXPERIMENTS

7.1.1 Introduction

A Optimisation of the extraction step

The process of protein purification begins with extraction of the desired activity from the plant tissue. Optimisation of this step is necessary to maximise release of the desired protein whilst minimizing liberation of contaminants (Errson et al. 1989).

i Choice of extraction buffer

Inclusion of buffers in the extraction medium prevents damage from pH extremes. The influence of buffer species on extraction efficiency and stability of kiwifruit chitinases was not known. Sharrock & Hallet (1992b) used sodium acetate, and this buffer was also used in the previous experiments of the present study, but acetate can inhibit enzymes which have a similar mode of action to chitinases (Van Noorden & Frederiks 1992). Ideally, buffer capacity should encompass the optimal pH for chitinase activity. Plant chitinases usually have a broad pH optimum around pH 6 (Collinge et al. 1993) and therefore buffer species with a pKa in the 5-7 range were considered for further study.

ii Importance of protease inhibition

Plant tissues are rich in proteases (Gegenheimer 1990) which pose a serious threat to

protein stability (Ersson et al. 1989). Wegrzyn & MacRae (1992) found it necessary to inhibit actinidin with sodium tetrathionate when extracting proteins from kiwifruit flesh. The role of protease inhibitors in extracts from stem plugs remains to be established.

iii Efficiency of protein extraction

Maintaining a high protein concentration (> 1 mg/ml) contributes to enzyme stability (Deutscher 1990). A high initial concentration is also important because losses and dilution of activity can occur at each purification step. In this study, the concentration of protein in cured, uninoculated, healthy kiwifruit stem plugs was extremely low: 0.10 ± 0.01 mg/ml. Since chitinase activity may be associated with the insoluble material discarded during extraction, the effect of leaving extracts as a slurry for an extended period before removal of the precipitate and the inclusion of a mild, non-denaturing detergent in the buffer was investigated to determine whether protein content of the extract could be elevated.

B Subsequent purification steps

Treatments which can be used to remove the bulk of contaminant proteins during the early stages of purification include ammonium sulphate precipitation, heat treatment and ion exchange chromatography (Boller et al. 1988; Ersson et al. 1989). Ammonium sulphate precipitation and heat treatments in the current study were ineffective, so ion exchange was considered. The choice of ion exchange column and buffers depends on the pI of the protein(s) of interest. The objective of the next section of work was to determine the pI values of kiwifruit chitinase isoforms.

7.1.2 Materials and Methods

Unless otherwise stated, 94/C/U/H extract (Chapter 5, Section 5.1.2, Part B (i)) was used in all the experiments.

Chitinase activity was quantified by the radioactive assay of Molano et al. (1977), as described in Chapter 2, Section 2.2.4, Part E. Extracts were diluted until the activity was in the linear range of the reaction curve. The Bradford (1976) protein assay (see Chapter 2, Section 2.2.5) was used to measure total protein content. Specific activity (ng of

solubilised tritiated chitin/min/μg) was calculated by total chitinase activity (ng/min) per μg total protein.

General measures to minimize protein losses which were adopted in this study included carrying out all procedures on ice, and inclusion of 1 mM dithiothreitol (DTT) and 1% (w/v) polyvinylpolypyrrolidone (PVPP) in the extraction buffer to reduce oxidation, particularly of cysteine residues, and to bind polyphenols respectively (Gegenheimer 1990).

A Optimisation of the extraction step

i Choice of extraction buffer

Samples (10 g) of stem plugs, were ground with liquid nitrogen in a cryomill (Franz Morat) for 1 min. Batches (2 g) of the resultant powder were homogenized for 1 min with 6 ml of appropriate buffer containing PVPP and DTT, centrifuged 15 min at 14,500*g*, and filtered through Whatman No. 1 filter paper. Four different buffers were used:

1) 0.1 M NaOAc, pH 5.8
2) 0.1 M MES (2-(N-Morpholino)ethanesulphonic acid, BDH chemicals), pH 5.8
3) 0.1/0.2 M citrate/phosphate (McIlvaine), pH 5.8
4) 0.1 M citric acid/sodium citrate, pH 5.8

Chitinase activity and total protein content of the resultant extracts were measured and the specific activity calculated.

ii Importance of protease inhibition

Stem plugs and pericarp (from a cross-sectional slice 1 cm below the stem plug) were ground to a powder in the cryomill (1 min), then extracted using 1:3 w/v ratio in 0.1 M NaOAc extraction buffer, pH 5.8, both with and without 10 mM sodium tetrathionate (NaTT). Total proteins, chitinase activities and specific activities of the four resultant extracts were determined.

Protease activity in the extracts was also measured using the (unpublished) actinidin assay of Teresa Wegrzyn (HortResearch, Mt Albert). Enzyme extract (3 ml) was mixed with 1.5 ml 2% azocasein (Sigma), 1.5 ml Tris pH 7.8 and 1.5 ml Milli-Q water. A 750 µl aliquot of the mix was placed into 750 µl of 12% trichloracetic acid after 0, 2, 4, 6 and 8 min. Samples were centrifuged at 11,000*g* for 5 min, and the absorbance of 1 ml of each supernatant was read at 340 nm on a spectrophotometer (Pye Unicam, PU 8600 UV/VIS, Philips). The assay was performed twice in succession and data were analysed as a split plot design. A $\log_e$ data transformation was required. "Least squares" means were used to make specific comparisons between pairs of means.

iii Efficiency of protein extraction

Uninoculated, healthy, cured stem plugs (12 g) were ground with liquid nitrogen in the cryomill (1 min), and 1.5 g samples of the resultant powder homogenized (1 min) in an Ultra-Turrax with a 1:3 w/v ratio of NaOAc extraction buffer, both with and without 0.02% Triton. Extracts were either centrifuged (15 min at 14,500*g*) immediately and filtered through Whatman No. 1, or left on a magnetic stirrer at 20°C for 3 or 24 h prior to centrifugation and filtration as above.

Total protein, chitinase activities and specific activities of the extracts were determined.

B Subsequent purification steps

Kiwifruit chitinase pI values were estimated by isoelectric focusing (IEF). A bulk extract of uncured, uninoculated, healthy kiwifruit stem plugs (92/N/U/H extract - refer Chapter 5, Section 5.1.2, Part A) was dialysed against Milli-Q water overnight and centrifuged 5 min at 1,000*g* prior to addition of 2% Bio-Rad pH 3-10 ampholytes and 4 h isoelectrofocusing in the Rotofor chamber, as detailed in Chapter 2, Section 2.2.6, Part F. The twenty fractions collected from the Rotofor run were numbered sequentially from the anode end. Aliquots (10 µl) of representative Rotofor fractions and 1 µl of Serva pI 3-10 protein test mixture were applied to lanes on a silicone applicator strip centred on a precast 150 µm, 125 x 125 mm pH 3-10 Servalyt Precote IEF gel, and focused for 2 h, with running conditions as described in Chapter 2, Section 2.2.6, Part D. A 0.01% glycol chitin gel (Chapter 2, Section 2.2.6, Part B) was then overlaid on the IEF gel.

After 45 min, the overlay gel was placed in a humid chamber at 37°C for 4 h, then stained with Calcofluor white. The IEF gel was silver stained. (See Chapter 2, Section 2.2.6, Part E and Appendix V for staining protocols.) The entire process was repeated using a bulk extract (92/N/I/D) of uncured, inoculated, diseased kiwifruit stem plugs (refer Chapter 4, Section 4.3.1, Part B), except that the Rotofor and IEF gel runs lasted 5.5 h and 3.7 h respectively, until focusing was complete, as indicated by constant voltage (hence the different focusing times for the 92/N/U/H and 92/N/I/D extracts).

7.1.3 Results

A Optimisation of the extraction step

Chitinase activity in Section 7.1.3, Part A is expressed as ng of tritiated chitin solubilised per min per ml of crude extract.

i Choice of extraction buffer

The MES buffer extract contained the least protein and the lowest total chitinase activity (approximately 10% less than that of the other extracts), but the highest chitinase specific activity (Table 7-1). In contrast, a higher proportion of extraneous protein was extracted with citrate buffer. NaOAc had the second highest specific activity and did not appear to inhibit chitinase activity (Table 7-1).

ii Importance of protease inhibition

More chitinase activity (ng/min/ml) was associated with stem plug than with pericarp extracts (Table 7-2). The "stem plug + NaTT" extract had the highest chitinase specific activity (Table 7-2).

Actinidin activities in various kiwifruit crude extracts are plotted in Fig. 7-1. There is no LSD bar on Fig. 7-1 because data were unbalanced, and also Duncan's Multiple Range test is not applicable to "interactions" (refer Chapter 2, Section 2.3.4, Part C). Consequently, "least squares means" P-values (given in the text below) were used to determine significant differences between the $\log_e$ transformed data of Fig. 7-1. Absorbances in both pericarp extracts were higher than in the stem plug extracts and had

Table 7-1: Efficiency of kiwifruit stem plug extraction as measured by specific activity of chitinase for four different extraction buffers in 1994.

BUFFER*	PROTEIN (μg/ml)	CHITINASE ACTIVITY (ng/min/ml)†	SPECIFIC CHITINASE ACTIVITY (ng/min/μg)
Na acetate	85	1665	20[b‡]
MES	64	1530	24[a]
McIlvaine	94	1739	19[b]
Citrate	179	1702	10[c]

* All buffers were at pH 5.8 and 0.1 M as described in Methods.
† ng of tritiated chitin solubilised per minute per ml of crude extract.
‡ a,b,c represent significant differences in Duncan's Multiple Range test (α=0.05).

Table 7-2: Specific activity of chitinase in kiwifruit stem plug and pericarp extracts as affected by sodium tetrathionate (NaTT) inhibition of actinidin, measured in 1994.

TREATMENT	PROTEIN (μg/ml)	CHITINASE ACTIVITY (ng/min/ml)*	SPECIFIC CHITINASE ACTIVITY (ng/min/μg)
Stem plug + NaTT	122	2755	23[a†]
Stem plug - NaTT	151	2005	13[b]
Pericarp + NaTT	145	1309	9[c]
Pericarp - NaTT	108	1588	15[b]

* ng of tritiated chitin solubilised per minute per ml of crude extract.
† a,b,c represent significant differences in Duncan's Multiple Range test (α=0.05).

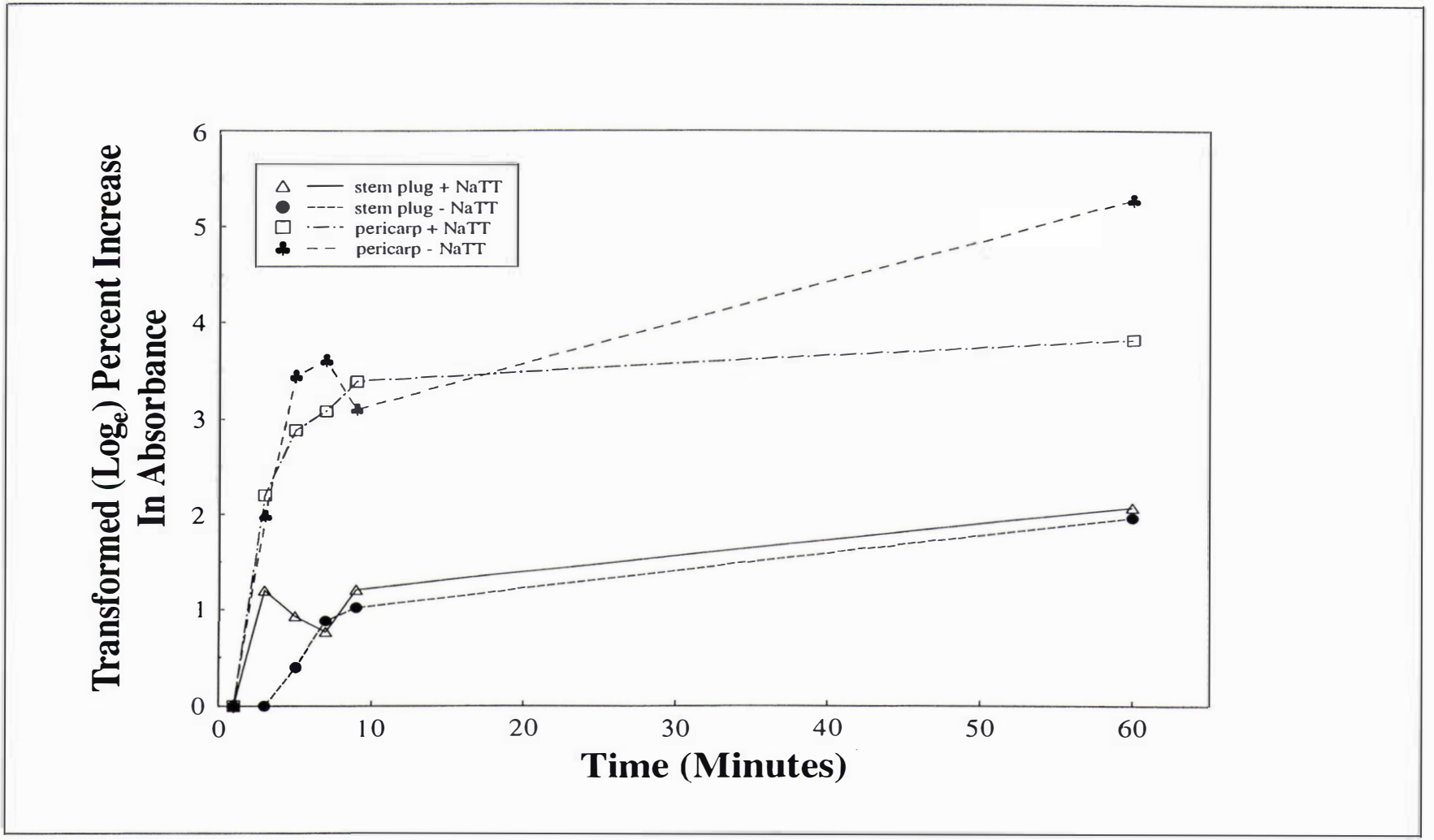


Figure 7-1. Actinidin activity of cured 94/C/U/H kiwifruit pericarp and stem plug extracts with and without sodium tetrathionate (NaTT) protease inhibitor, as measured by percentage increase in absorbance at 340 nm over time, in 1994.

increased significantly relative to the initial absorbance by the first three minutes (LSMeans P=0.0063 for "Peri+NaTT" and P=0.0121 for "Peri-NaTT"). In contrast, increases in absorbance over time for both stem plug extracts were not significant (P>0.05). After prolonged incubation, actinidin activity was highest in the pericarp extract without protease inhibitor (Fig. 7-1, LSMeans not estimable because of missing data), but inclusion of NaTT had no effect on actinidin activity at earlier stages (P>0.05).

iii Efficiency of protein extraction

Addition of 0.02% Triton significantly increased the proportion of chitinase in extracts (Table 7-3).

Leaving extracts as slurries for an extended period before removal of the particulate matter did not increase chitinase specific activity in the supernatant (Table 7-3).

Chitinase activity was markedly higher than in previous trials where stem plugs from cured, uninoculated, healthy fruit were also used (Table 7-3 compared with Tables 7-1 and 7-2).

B Subsequent purification steps

Silver staining of proteins in the IEF gel of healthy (92/N/U/H) Rotofor fractions was unsuccessful, despite strict adherence to the Bio-Rad staining protocol. However, omission of the recommended wash step between addition of the silver reagent and developer enabled the successful detection of proteins in the IEF gel of diseased (92/N/I/D) Rotofor fractions (Plate 7-1). Proteins migrated horizontally through the pH 3-10 gradient in this IEF gel until reaching a pH where they had no net charge (protein pI). Serva pI 3-10 protein test mixture ("Std", Plate 7-1) was used to locate specific pI values. Fractions applied to the IEF gel that had been in close proximity to the Rotofor anode (fractions 1, 2, 4 and 5, Plate 7-1) contained proteins with acidic pI's, whilst proteins with basic pI values were found in fractions that had been closer to the Rotofor cathode (fractions 18, 19 and 20, Plate 7-1). In contrast, a sample of unfocused crude 92/N/I/D extract ("C", Plate 7-1) contained proteins with both basic and acidic pI's.

Table 7-3: Total chitinase activity, chitinase specific activity and total protein content of kiwifruit stem plug extracts after varying time lapses before removal of particulate matter, measured in 1994.

TREATMENT	PROTEIN (μg/ml)	CHITINASE ACTIVITY (ng/min/ml)*	SPECIFIC CHITINASE ACTIVITY (ng/min/μg)
0 h + Triton	89	31215	351[a†]
3 h + Triton	108	35050	325[a]
24 h + Triton	-‡	-	-
0 h - Triton	83	21125	255[b]
3 h - Triton	111	25133	226[b]
24 h - Triton	-	33356	-

* ng of tritiated chitin solubilised per minute per ml of crude extract.
† a,b represent significant differences in Duncan's Multiple Comparison test (α=0.05).
‡ Not measured due to spillage.

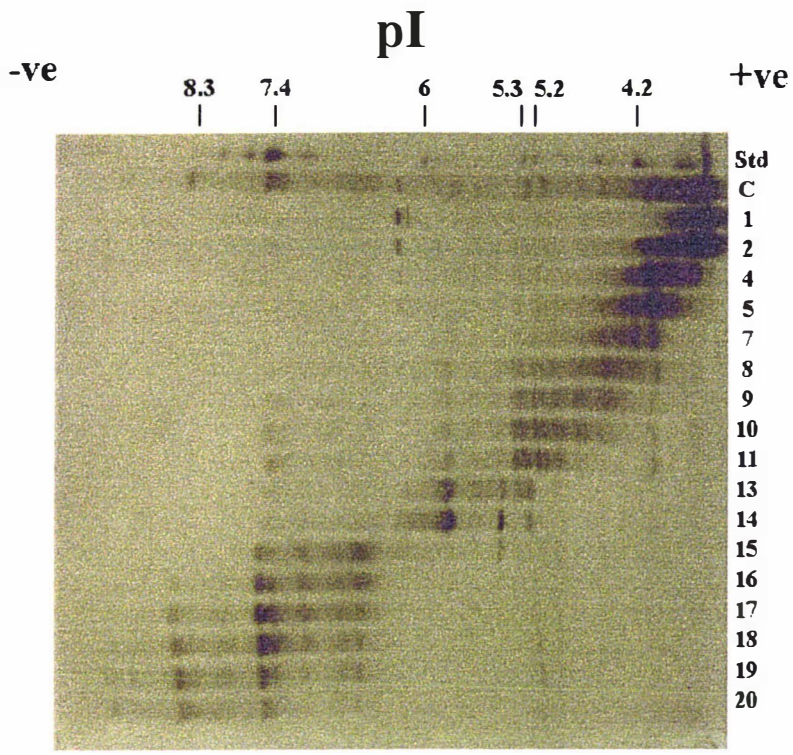


Plate 7-1. Silver stained pH 3-10 IEF gel of total proteins in Rotofor fractions of diseased 92/N/I/D kiwifruit stem plug extract. Std, pI standards (1 µl); C, crude unfocused 92/N/I/D extract; 1, 2, 4 ... 18, 19, 20, fractions collected after electrofocusing in the Rotofor, where fraction 1 was closest 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.

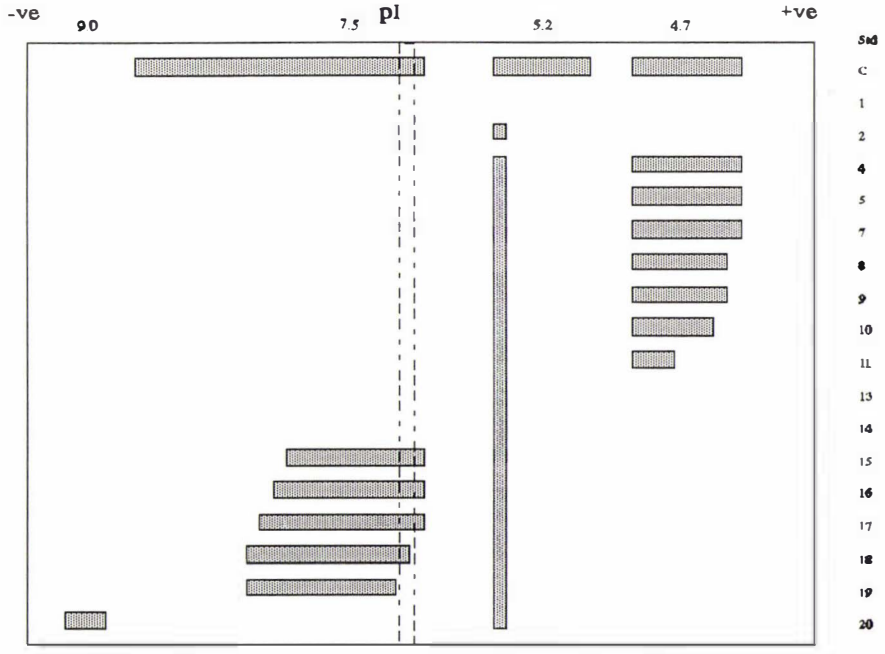


Figure 7-2. Schematic diagram of corresponding chitinase activities (shaded areas) in the Rotofor fractions of diseased 92/N/I/D kiwifruit stem plug extract, as detected by Calcofluor staining of an overlay gel with a glycol chitin substrate. Dashed line indicates position of applicator strip.

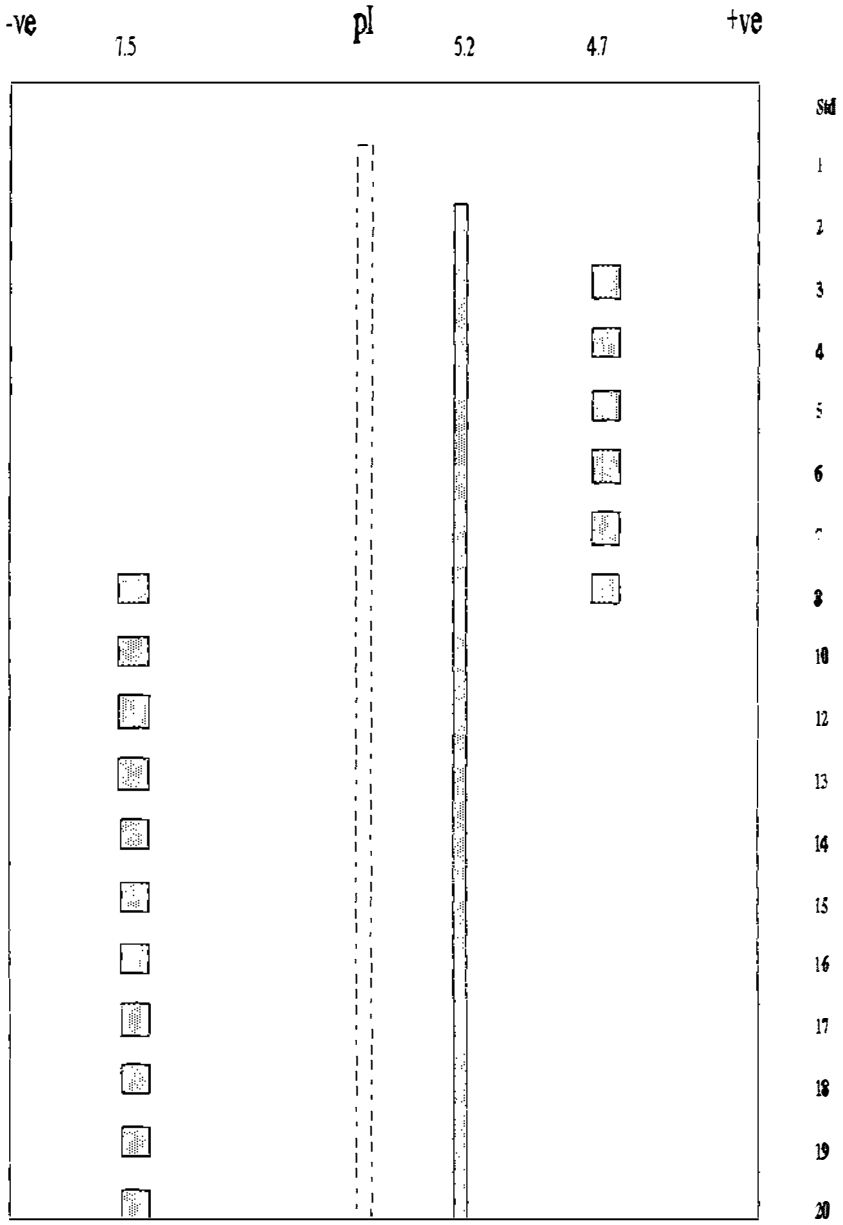


Figure 7-3. Schematic diagram of chitinase activity (shaded areas) in Rotofor fractions of healthy 92/N/U/H kiwifruit stem plug extract, as detected by Calcofluor staining of an overlay gel with glycol chitin as a substrate. Std, pI standards (1 µl); 1, 2, 3 ... 18, 19, 20, fractions collected after 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. Dashed line indicates position of applicator strip.

The 92/N/I/D glycol chitin overlay (Fig. 7-2) was a replica of the IEF gel of 92/N/I/D Rotofor fractions (Plate 7-1), but stained only those proteins with chitinase activity. Four areas of chitinase activity, with average pI's of 9, 7.5, 5.2 and 4.7 were observed in this 92/N/I/D glycol chitin overlay (Fig. 7-2). The bands of activity were markedly broader than in the 92/N/U/H overlay which had three areas of chitinase activity with pI's of 4.7, 5.2 and 7.5 (Fig. 7-3). Chitinase activity was absent from fraction 1 in the 92/N/I/D overlay (Fig. 7-2). Fractions 2-11 contained chitinases with acidic pI's (4.7 and 5.2 respectively), whilst basic pI chitinases were only found in fractions 15-20. The unfocused 92/N/I/D crude extract ("C") contained both acidic and basic chitinases (Fig. 7-2). Small amounts of pI 5.2 chitinase activity were also detected in fractions 15-20 (Fig. 7-2), but little protein was present in this region as shown by the corresponding protein stain (Plate 7-1). Areas of chitinase activity coincided with some major protein bands in the corresponding IEF gel, but there were many other non-chitinolytic proteins present in the fractions.

7.2 PART 2 - PROTEIN PURIFICATION

7.2.1 Introduction

Chitinases have been isolated from many different plants (Graham & Sticklen 1994), but there is no record in the literature of purification of a kiwifruit chitinase. Separation of chitinases from other components in the fruit is a key step to elucidating their role in the kiwifruit defence arsenal. Optimisation of extraction and knowledge of pI's associated with kiwifruit chitinases meant that protein purification could now proceed.

7.2.2 Materials And Methods

Cured, uninoculated, healthy 94/C/U/H extract (refer Section 7.1.2) was used for all protein purification. The NaOAc extraction buffer contained PVPP, DTT, Triton and NaTT, as described in Section 7.1.2. All buffers used in protein purification were degassed and filtered (0.45 μm) and contained 0.02% sodium azide. Chitinase stability in all these buffers was established prior to commencing purification. Refer Chapter 2, Section 2.2.8, Part A for further general protein purification details.

Optimising yield from the extraction step was not sufficient on its own to overcome the problem of low protein concentration. Since enzyme activity was unaffected by lyophilisation (see Table 7-4), proteins were conveniently concentrated by freeze drying (Chapter 2, Section 2.2.7) between each purification step.

A First Run

Extract (32 ml) was dialysed against approximately 250 ml of 20 mM NaOAc, pH 5 for 12 h at 5°C with stirring. It was then lyophilised for 24 h, reconstituted in 5 ml of Milli-Q water, filtered through a 0.45 μm Sartorius disposable filter and exchanged in 20 mM NaOAc buffer, pH 5 using a Bio-Rad Econo-Pac P6 cartridge, molecular weight cut-off 6,000 (Chapter 2, Section 2.2.8, Part B). A 2 ml aliquot of the resultant eluate was injected onto a Bio-Rad Econo-Pac S cation exchange column equilibrated with 20 mM NaOAc, pH 5. Adsorbed protein fractions were eluted after 15 min washing with a 0-1 M linear gradient of NaCl in 20 mM NaOAc over 50 min. Protein elution was monitored by absorbance at 280 nm. The flow rate was 1 ml/min and fractions were collected every 2 min. The Calcofluor petri dish assay (Chapter 2, Section 2.2.4, Part C) was used to identify fractions containing chitinase activity. Active fractions which had bound to the ion exchange column were pooled (5.9 ml), freeze dried (25 h), reconstituted in Milli-Q water (250 μl) and centrifuged at 13,000*g* for 5 min. A 50 μl aliquot of this concentrated, active, pooled material was then applied to a Phenomenex BioSep SEC-S3000 (250 x 10 mm) HPLC gel filtration column, equilibrated with 50 mM NaOAc buffer, pH 5. Fractions, collected at 1 min intervals, were eluted with 50 mM NaOAc, pH 5 at a rate of 0.5 ml/min. Proteins in the HPLC fractions were monitored at 218 and 280 nm by a Hewlett Packard Multiple Wavelength detector. Active fractions eluted from the SEC-S3000 column were pooled (0.9 ml), concentrated by freeze drying (5 h), redissolved in 0.15 ml Milli-Q water, then centrifuged, as described previously, and 80 μl applied to a 1 ml (bed volume) high resolution Resource S cation exchange column (Pharmacia) equilibrated with 50 mM NaOAc, pH 5. A 0-0.5 M salt gradient was used to elute 0.5 ml fractions at a rate of 1 ml/min.

At each purification step, samples were removed for quantitative measurement of protein and chitinase activity by the Bradford (1976) and Molano et al. (1977) assays

respectively. Results were used to compile a purification table. Purification progress was also monitored by applying samples to IEF, glycol chitin overlay and SDS gels, as described in Chapter 2, Section 2.2.6. Samples often needed to be concentrated by lyophilisation before they could be applied to gels, due to the low protein content of fractions.

B Second Run

Since negligible protein and chitinase activity levels were obtained in fractions from the final stages of the first purification, a second purification was attempted. Steps were identical to that described previously, except that the original extract was reconstituted into a smaller volume of Milli-Q water (3.3 ml), aliquots were not removed for quantification of chitinases and proteins, overnight dialysis at 4°C into 20 mM NaOAc, pH 5 using a "Slide-a-Lyzer" dialysis cassette (10,000 MWCO, Labsupply Pierce) was substituted for the Econo-Pac P6 buffer exchange step, and a larger volume (3 ml) was injected onto the Econo-Pac S cation exchange column in an attempt to further improve chitinase yield. Protein content in the ion exchange fractions was monitored by transmittance instead of absorbance, and fractions were collected at 1 min intervals. Active fractions which had bound to the Econo-Pac S column were divided into two groups instead of just one. They were freeze dried, reconstituted in 100 µl and 170 µl of Milli-Q water respectively, and centrifuged as described previously. Each pooled bound sample (50 µl of CHa, and 75 µl of CHb) was then separately passed through the SEC-S3000 gel filtration column equilibrated with 20 mM NaOAc, pH 5. Fractions, collected at 0.5 min intervals, were eluted with the same buffer at 1 ml/min. Chitinase activity in the fractions was identified by the Calcofluor petri dish assay. The final HPLC cation exchange step was excluded. Purity of fractions was tested by Coomassie and silver stained IEF and SDS-PAGE gels, and in Calcofluor-stained glycol chitin overlay gels, as described above.

Approximately 20 µg of lyophilised protein from fraction CHb (ii) was electroblotted, and N-terminally sequenced by Catriona Knight of the Auckland University School of Biological Sciences, using an Applied Biosystems 470A Sequencer. The sequence was compared with other N-terminal amino acid sequences by the Basic Local Alignment

Search Tool (BLAST) on the Internet.

Purity of the N-terminal sequenced sample was reassessed with Western blots to increase sensitivity of chitinase detection. CH2, CH4 and SP sugar beet chitinase antibodies and the blotting protocol described in Chapter 2, Section 2.2.9, Part B were used. Approximately 5 μg of test protein was applied to the SDS-PAGE gel and 500-fold dilutions of the primary antibodies were used instead of the normal 1000-fold dilution.

7.2.3 Results

A First Run

Total enzyme activity in the freeze dried sample was higher than in the original. This anomaly was caused by variability in the Molano et al. (1977) assay, as there was no significant difference between the two values ($P>0.05$). Yield and specific activity values for the freeze dried sample were consequently overestimates (Table 7-4).

Activity losses occurred at each major purification step. A 21% loss in yield and a 33% loss of protein occurred during buffer exchange (Table 7-4). Losses at this step were caused by unexpected prolonged retention of material on the P6 column. (The remaining 33% of protein was eluted when the P6 column was washed after use.) Chitinase activity was detected in both bound and unbound fractions eluted from the S cation exchange column (Fig. 7-4, A), but the process of ion exchange caused a further 51% loss in yield (Table 7-4). Removal of non-chitinolytic proteins during ion exchange decreased total protein, but increased specific activity of the pooled bound sample, compared with previous purification steps (Table 7-4). As a result, the bound sample became the focus of subsequent purification efforts (Fig. 7-4, B). Chitinase activity and proteins were also lost during gel filtration, as shown by the reduced areas under the protein and activity curves (Fig. 7-4, B). By the final stage of purification, accumulated losses were so great that levels of total protein and chitinase activity in fractions eluted from the HPLC S cation exchange column were too small to measure, and consequently could not be presented.

Since protein and chitinase banding patterns on gels were similar for both purification

Table 7-4: Purification of kiwifruit chitinase activity in 1994.

STAGE IN PURIFICATION	VOLUME (ml)	TOTAL PROTEIN (μg)	TOTAL CHITINASE ACTIVITY (ng/min)*	SPECIFIC CHITINASE ACTIVITY (ng/min/μg)	YIELD (%)
Crude extract	30	3828	1534600	401	100
Freeze dried	5	3071	1910000	622	>100
P6 buffer exchange eluate	3	2065	1210800	586	79
Cation S, unbound CH eluate	10	447	119700	268	8
Cation S, bound CH eluate	8	239	303400	1269	20
SEC S3000, bound CH eluate	1.5	-†	96400	-	6

* ng of tritiated chitin solubilised per minute.
† Incalculable due to insufficient sample material.

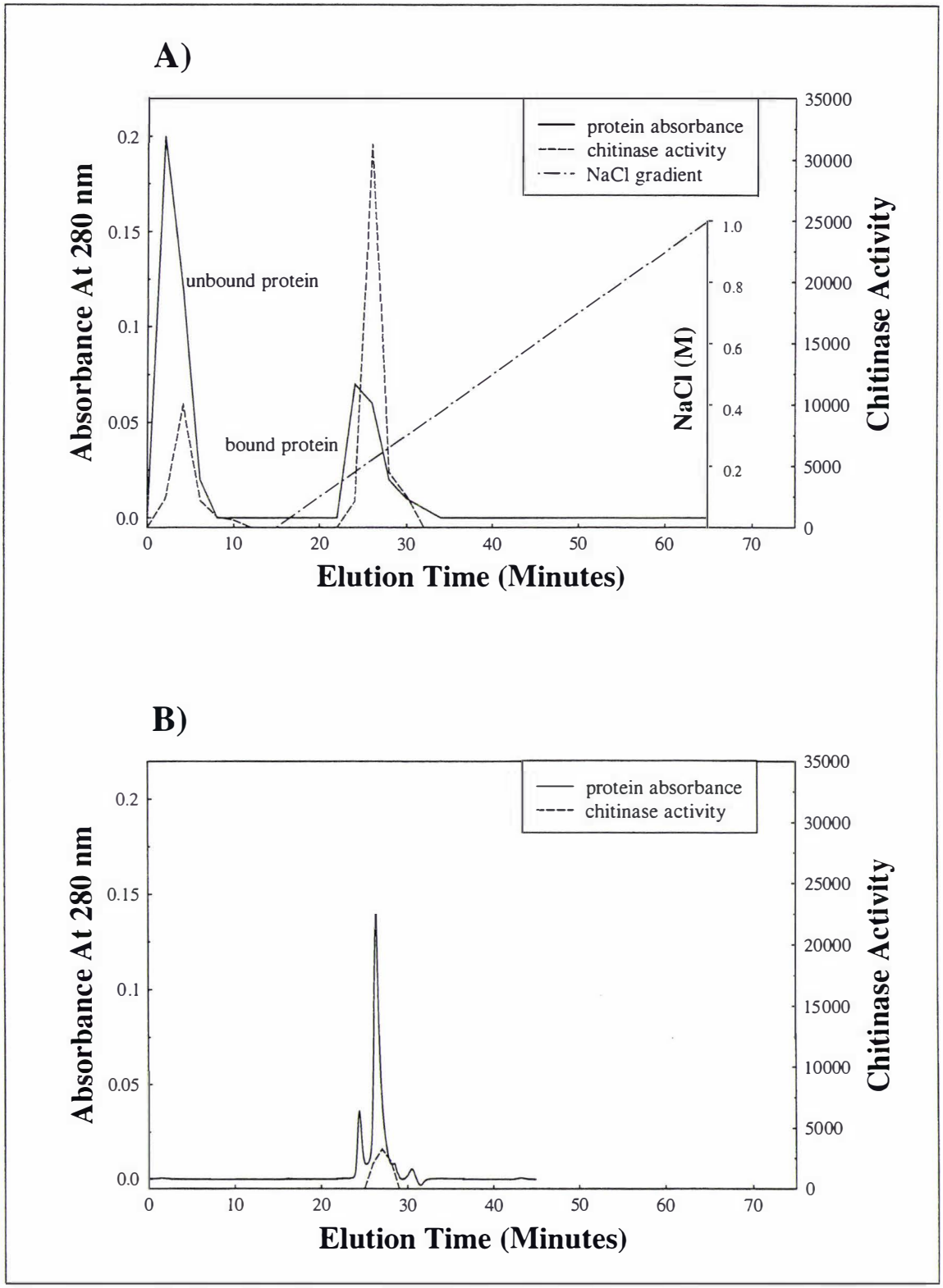


Figure 7-4. Elution profiles and chitinolytic activity of chromatography fractions of cured 94/C/U/H kiwifruit stem plug extract. A) Econo-Pac S cation exchange, B) SEC-S3000 gel filtration. Chitinase activity is expressed as ng of tritiated chitin solubilised per minute per ml of extract.

attempts, only the gels with greater clarity, from the second purification run will be presented.

B Second Run

The majority of proteins did not bind to the cation exchange column and were eluted within the first 20 min (Fig. 7-5, A). Adsorbed proteins were eluted with less than 0.5 M NaCl (Fig. 7-5, A). The peak associated with adsorbed proteins had a distinct shoulder at elution time = 30 min (Fig. 7-5, A). Active bound fractions were pooled into two separate groups on the basis of this shoulder:

CHa - bound fractions eluted at 27-30 min, and
CHb - bound fractions encompassing the shoulder, eluted at 31-36 min.

Gel filtration further separated proteins in the CHa and CHb pooled samples into several peaks. In both cases, chitinase activity was associated with the tallest central peak in the gel filtration elution profile (Fig. 7-5, B and C). Fractions from these active SEC-S3000 peaks were not pooled. The active peak in Fig. 7-5, B comprised three fractions labelled in ascending elution time CHa (i), CHa (ii) and CHa (iii), and the two active fractions in Fig. 7-5, C were named CHb (i) and (ii). High salt in Econo-Pac S cation exchange fractions caused band distortion on IEF gels. Hence samples were applied to IEF gels once salt had been removed by SEC-S3000 gel filtration. Most proteins and chitinases in the crude 94/C/U/H extract had acidic pI values, apart from one basic chitinase with an approximate pI of 9 (lane 2, Plate 7-2, A and B). Individual acidic isoforms were difficult to distinguish because bands on the activity stain had merged (lane 2 and 3, Plate 7-2, B). Several acidic isoforms (pI 4.2-5.5) and one basic (pI 9) isoform were also observed in the gels from the first attempt at purification. Ion exchange separated proteins into unbound acidic (lane 3) and bound basic (lanes 4-6) proteins (Plate 7-2, A), and the proportion of 20-25 kDa proteins increased in the more basic fractions (lanes 3 and 4, Plate 7-3). High specific activity in the bound fractions was predominantly associated with the pI 9 chitinase (Table 7-4 and lanes 4-6, Plate 7-2, B).

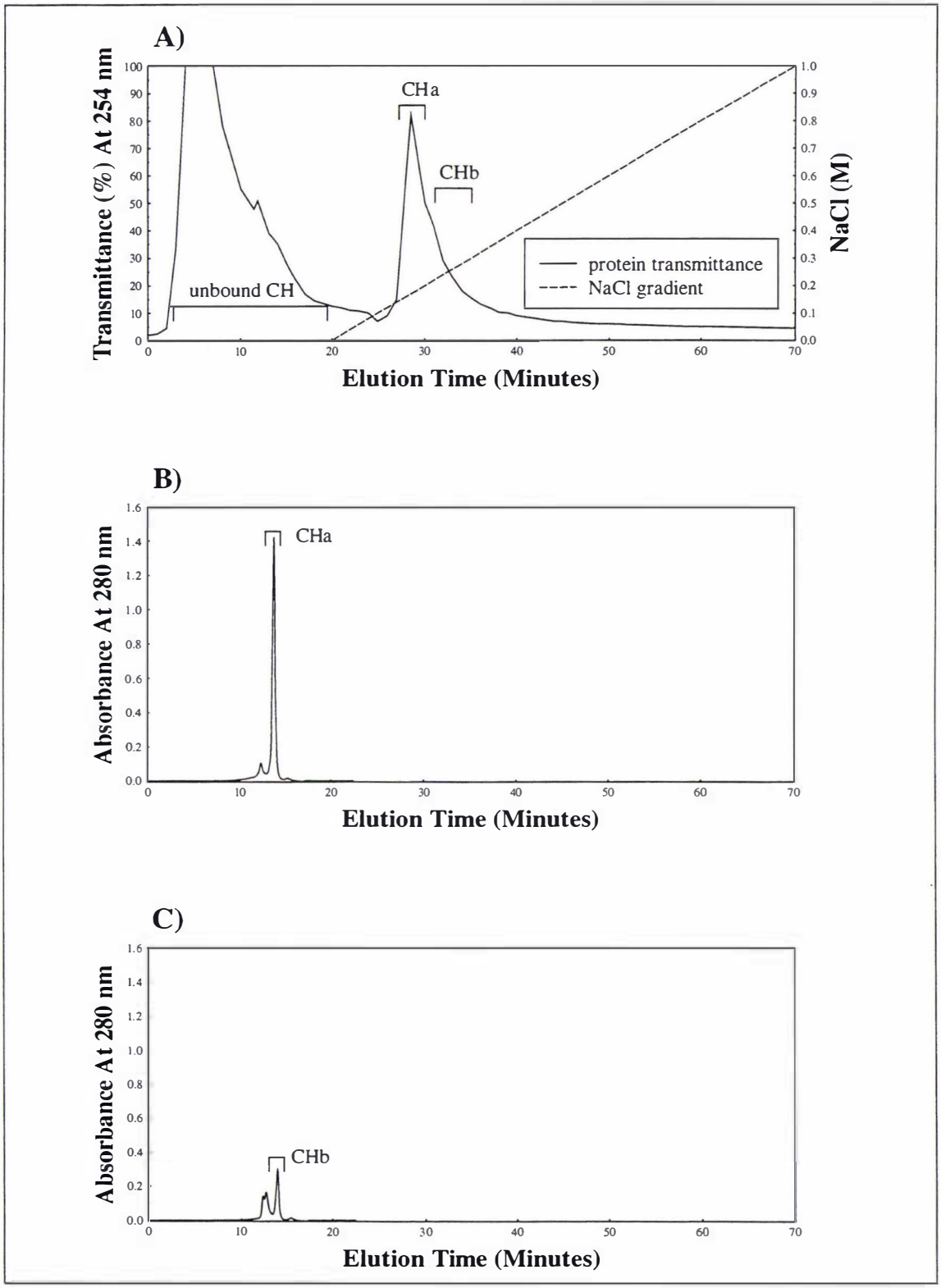


Figure 7-5. Elution profiles from various stages of protein purification using cured 94/C/U/H kiwifruit stem plug extract. Fractions with chitinase activity were identified by the Calcofluor petri dish assay. A) Econo-Pac S cation exchange, B) SEC-S3000 gel filtration of "CHa", C) SEC-S3000 gel filtration of "CHb".

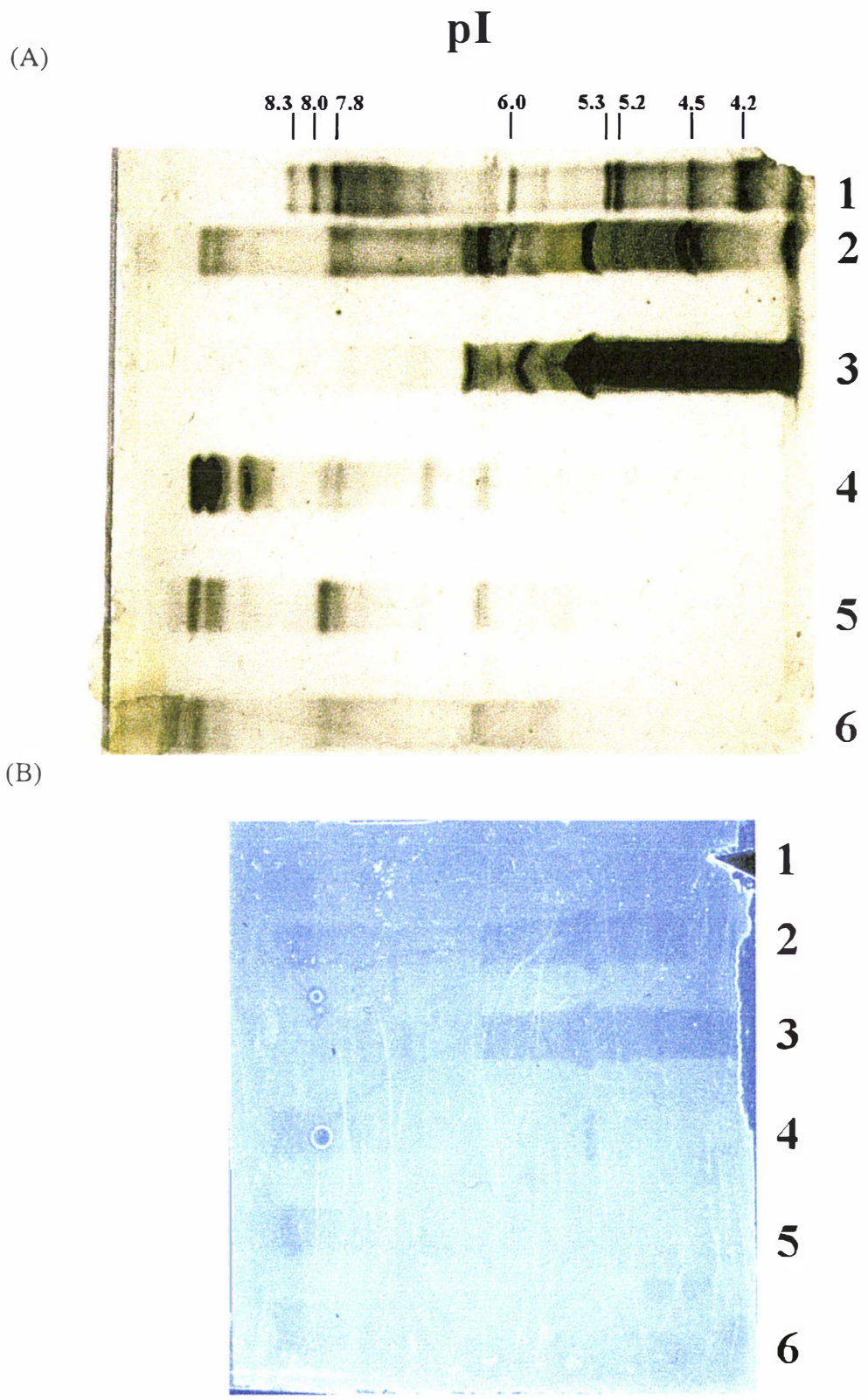


Plate 7-2. Total protein and corresponding chitinase activities of cured 94/C/U/H kiwifruit stem plug extract after fractionation during protein purification. Proteins on a pH 3-10 IEF polyacrylamide gel were silver stained (A) and chitinase activity in the glycol chitin overlay gel was detected by Calcofluor 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).

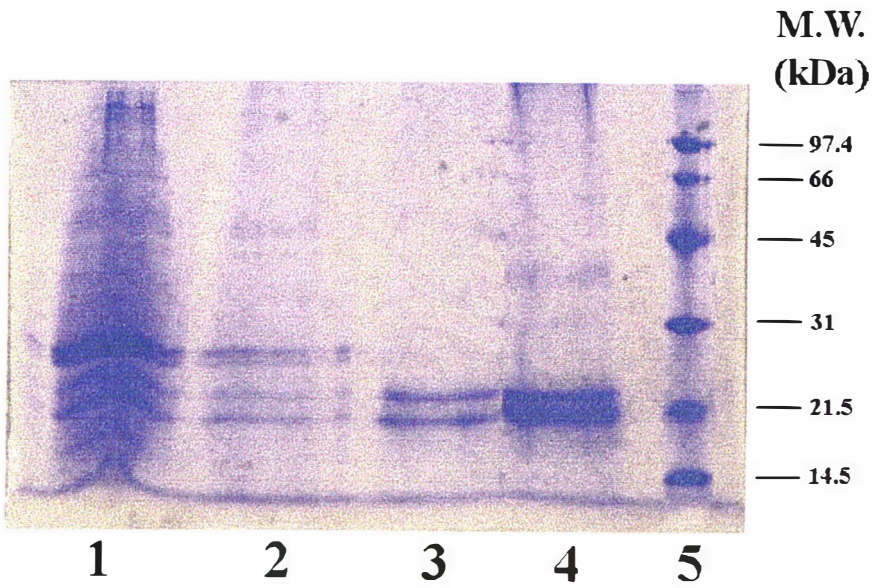


Plate 7-3. Coomassie stained SDS-PAGE of total proteins in cured 94/C/U/H kiwifruit stem plug extract after fractionation by HPLC on an Econo-Pac S cation exchange column. Lane 1, unbound chitinase (CH) eluted at 3-10 min; lane 2, unbound CH eluted at 11-20 min; lane 3, bound CHa eluted at 27-30 min; lane 4, bound CHb eluted at 31-36 min; lane 5, molecular weight standards.

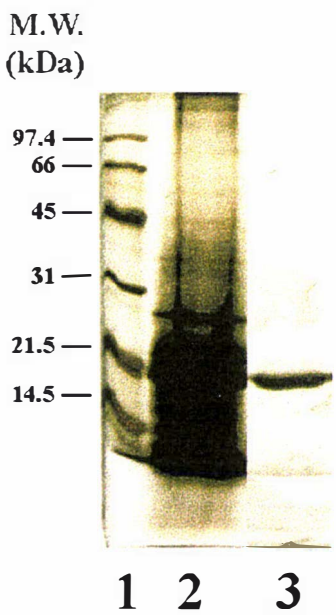


Plate 7-4. Silver stained SDS-PAGE of total proteins in crude and purified cured 94/C/U/H kiwifruit stem plug extract. Lane 1, molecular weight standards; lane 2, crude extract; lane 3, ≈ 5 μg of fraction CHb (ii) recovered from the central peak (14 min) of gel filtration chromatography on a SEC-S3000 HPLC column following adsorption to an Econo-Pac S cation exchange column.

SDS electrophoresis suggested that the CHb (ii) fraction contained only one protein (lane 3, Plate 7-4). However, two other distinct bands were also present in the IEF gel (left hand side of lane 6, Plate 7-2, A), indicating that this fraction was not homogenous. (The "band" in the centre of lane 6, Plate 7-2, A was created by residual protein that had not diffused out of the applicator strip.) A single band of basic chitinase activity in lane 6, Plate 7-2, B aligned directly with the predominant CHb (ii) protein in the corresponding IEF gel (lane 6, Plate 7-2, A). N-terminal sequencing of this predominant protein produced a single clean sequence free of background contamination. The N-terminal amino acid sequence was:

A-T-F-N-I-I-N-N-C-P-F-T-V-W-A-A-A-V-P-G-G-G-K-R-L-D-R-G-Q

This sequence did not bear any resemblance to known chitinase sequences, but was highly homologous with pathogenesis related thaumatin-like proteins (Table 7-5 and Fig. 7-6).

Initial Western blot results (not shown here) revealed that the CH4 sugar beet antibody did not react with the semi-purified CHb (ii) fraction, whilst the CH2 and SP antibodies appeared to bind to different proteins in this fraction. Bands in these original blots were very faint, so more protein was loaded onto the next gel, dilutions of the CH2 and SP antibodies were reduced, and both antibodies were applied simultaneously to the blotted membrane. The antibodies reacted with a protein in CHb (ii) which was also present in crude extracts of cured and uncured fruit, but at least one other band was also present in the sample (Plate 7-5).

Table 7-5: Sequence homology between a kiwifruit pathogenesis-related (PR) protein and other members of the PR-5 family.

PROTEIN	SOURCE	ACCESSION NUMBER	SEQUENCE HOMOLOGY WITH CHb (ii)	REFERENCE
Thaumatin-like protein?	Kiwifruit	-*	100%	Chapter 7, this thesis
Thaumatin-like protein	Tobacco	p13046	72%	Payne et al. 1988
Thaumatin-like protein	Tobacco	p07052	65%	Cornelissen et al. 1986
Thaumatin-like protein	Barley	p33044	65%	Hejgaard et al. 1991
Zeamatin fragment	Maize	p33679	66%	Roberts & Selitrennikoff 1990
AP24	Tomato	pq0170	66%	Woloshuk et al. 1991

* kiwifruit TL protein does not currently have an accession number.

CHb(ii)	A	T	F	N	I	I	N	N	C	P	F	T	V	W	A	A	A	V	P	G	G	G	K	R	L	D	R	G	Q
PRR1	A	T	F	D	I	V	N	K	C	T	Y	T	V	W	A	A	A	S	P	G	G	G	R	R	L	D	S	G	Q
PRR2	A	T	F	D	I	V	N	Q	C	T	Y	T	V	W	A	A	A	S	P	G	G	G	R	Q	L	N	S	G	Q
THHR	A	T	I	T	V	V	N	R	C	S	Y	T	V	W	P	G	A	L	P	G	G	G	V	R	L	D	P	G	Q
ZEAM	A	V	F	T	V	V	N	Q	C	P	F	T	V	W	A	A	S	V	P	V	G	G	G	R					
AP24	A	T	F	E	V	R	N	N	C	P	Y	T	V	W	A	A	S	T	P	I	G	G	G	R					
	*				+		*		*		+	*	*	*	+	+	+		*		*	*		+	*	+		*	*

Figure 7-6. N-terminal amino acid sequence comparisons between a kiwifruit thaumatin-like (TL) protein and other TL proteins. CHb (ii), thaumatin-like protein from kiwifruit stem plug extract; PRR1, tobacco pathogenesis-related protein PRR1; PRR2, tobacco pathogenesis-related protein PRR2; THHR, barley pathogenesis-related protein fragment THHR; ZEAM, maize zeamatin fragment; AP24, tomato pathogenesis-related protein fragment AP24. Amino acids from position 1 up to a maximum position of 29 in CHb(ii), THHR, ZEAM and AP24 are aligned with amino acids in positions 26 to 54 in PRR1 and PRR2. Positions with identical residues in all proteins are marked with asterisks, and conserved positions (amino acid substitutions that are unlikely to alter function) with + signs.

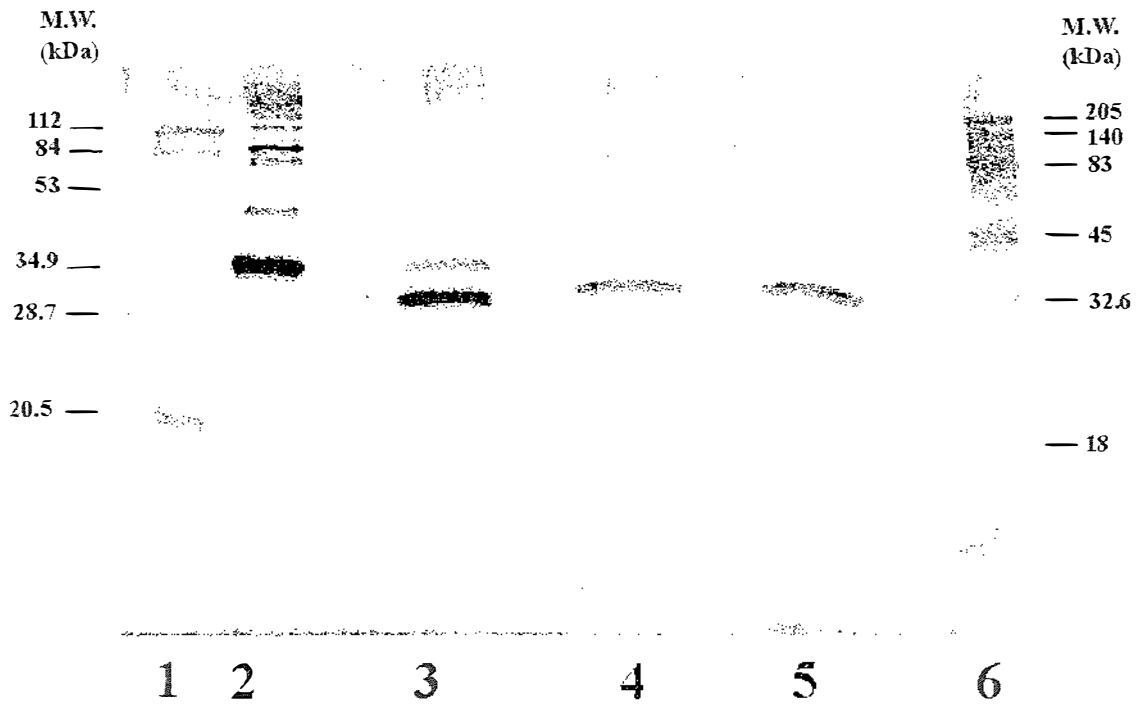


Plate 7-5. Western analysis of proteins in various chitinase preparations treated with CH2 and SP antibodies raised against sugar beet basic and acidic chitinase. Lanes 1 and 6, molecular weight standards; lane 2, partially purified bean chitinase; lane 3, purified kiwifruit chitinase = fraction CHb (ii); lane 4, uncured 94/N/U/H crude extract; lane 5, cured 94/C/U/H crude extract. Each lane contained ≈ 5 μg of protein. CH2 and SP antibodies were diluted 500-fold in PBS-Tween.

7.3 DISCUSSION

The results of enzyme and protein assays, electrophoresis and antibody-antigen reactions indicate that further optimisation of the purification protocol is required, since no homogeneous products were obtained.

Extremely low endogenous kiwifruit chitinase levels posed the greatest limitation to purification. Final chitinase activities and total proteins became too low to measure due to losses in consecutive purification stages (Section 7.2.3, Part A). The problem was partially alleviated by optimising extraction yield. Sodium acetate extraction buffer was used in preference to MES because it provided the best compromise between high specific activity and high total chitinase activity (Section 7.1.3, Part A, (i)). Stem plug extracts were the preferred source for protein purification, since chitinase activity was higher than in the pericarp, there was no actinidin activity (Section 7.1.3, Part A, (ii)), and stem plugs are the site of infection and initial growth. In support of the findings of this study, Sharrock & Hallet (1992b) reported that actinidin was present in high levels in the pericarp but not in stem plug extracts. These authors stated that actinidin is the predominant endogenous protease in kiwifruit. In the current study, NaTT had no apparent inhibitory effect on actinidin activity in the pericarp except after 60 min, possibly because prolonged storage of NaTT had diminished its inhibitory activity. Chitinase specific activities were markedly higher in cured, healthy, uninoculated extracts from Section 7.1.3, Part A, (iii) compared to those in Parts (i) and (ii). This difference is difficult to explain given that all stem plugs were stored at -80°C, and results from Chapter 6, Sections 6.4.8 and 6.4.9 showed that chitinase activities in extracts stored at this temperature tended to remain constant or decrease slowly over time.

Results in Section 7.1.3, Part B indicated that electrofocusing in the Rotofor had separated proteins according to charge, and that kiwifruit produces at least five predominantly acidic, chitinolytic activities. Most plant chitinases are basic proteins (Boller 1985; Mauch et al. 1988a), but acidic chitinases have been found in several plants including orange (Osswald et al. 1994), yam (Tsukamoto et al. 1984), cucumber (Métraux et al. 1988; Zhang & Punja 1994), sugar beet (Nielsen et al. 1993), wheat

(Ride & Barber 1990) and oat (Fink et al. 1988). Poole et al. (1993), in an unpublished report, noted the presence of at least five isoforms with basic and acidic pI values in healthy kiwifruit stem plug extracts after anion exchange chromatography. The broad activity zones in the glycol chitin overlay of diseased 92/N/I/D extract (Fig. 7-2) indicate that even greater numbers of isoforms with similar pI values could exist, or, alternatively, that focusing was incomplete, although the latter explanation is unlikely given the separation of proteins with different pI values in the corresponding silver stain (Plate 7-1). At least one new isoform with a pI of 9 was present in diseased 92/N/I/D extract compared to the healthy 92/N/U/H extract. (The healthy and diseased extracts were directly comparable because they were sourced from the same original experiment - Chapter 3, Experiment 1, Section 3.3.1). In contrast, a different pattern of chitinase induction occurs in response to curing, as shown in Chapter 6, Experiment 2 results (Section 6.4.2) where curing increased activity of one existing isoform rather than eliciting production of new forms. Chitinase activity (of a pI 5.2 isoform) was sometimes detected in areas on the 92/N/I/D overlay gel where there was virtually no protein on the corresponding silver stained gel (Section 7.1.3, Part B), owing to the extreme sensitivity of the Calcofluor staining method (refer Chapter 5, Section 5.1.3, Part B (ii)). This isoform could have been present in trace quantities in most fractions, due to a peculiar tendency to bind to ampholytes over-riding pH and charge effects.

In the current study, substantial losses of chitinase activity occurred during purification. In Section 7.2.3, Part A, ion exchange resulted in a 51% yield reduction. Losses of similar magnitude are relatively common in the literature (Deutscher 1990). Giordani et al. (1992) and Yamagami & Funatsu (1993) recorded 45% yield losses with ion exchange, whilst Kunz et al. (1992) found that activity decreased 70% after anion exchange. Losses could not be attributed to inactivation at room temperature, since kiwifruit chitinases activity was unaltered even at 50°C (results not shown). Other workers have also found that plant chitinases are thermally stable (Boller et al. 1988; Kunz et al. 1992). Freeze drying the crude extract did not reduce activity, but the effect of repeated lyophilisation was not established and could reduce activity. It is also possible that the various isoforms/classes of chitinase are synergistic, hence separating them could have a greater than expected effect on their activity, and consequently on

apparent yield.

Many researchers have used chitin affinity columns to purify chitinases because of their high specificity (Mauch et al. 1988a; Verburg & Huynh 1991; Balasubramanian & Manocha 1992; Rasmussen et al. 1992). However, affinity chromatography was not utilised in the current study because it can produce >70% yield losses (Molano et al. 1979; Wadsworth & Zikakis 1984) due to irreversible protein binding (Flach et al. 1992). Column shrinkage at acid pH, leading to decreased flow, may also create problems. An alternative approach, which may overcome these problems, involves using a batch-wise procedure in which chitinase is adsorbed to a chitin at a pH where the chitinolytic enzyme is inactive. Unbound components are subsequently removed by filtration, then the pH is changed to allow substrate digestion and release of the enzyme. Chitinase can then be separated from the hydrolysed chitin by dialysis. Cabib (1988) successfully used this system to obtain a semi-pure chitinase preparation from *Serratia marcescens*.

Future attempts at chitinase purification require higher initial levels of total protein and chitinase activity. Viscosity constraints limit the concentration increase that can be achieved by dissolving a large amount of lyophilized protein in a small volume of buffer. This problem could be overcome by separating strongly negatively charged mucilaginous compounds such as pectins from chitinase fractions in the Rotofor prior to lyophilisation.

The silver stained SDS gel in Section 7.2.3, Part B suggested that a homogeneous product had been obtained by cation exchange and gel filtration. However, IEF results (Plate 7-2) indicated that although fraction CHb (ii) appeared to contain one predominant species with basic chitinase activity, at least two other contaminant proteins were also present. When the predominant protein from fraction CHb (ii) was sequenced, a strong signal free of any background was obtained, and the staff at the School of Biological Sciences, Auckland University, noted that it was the cleanest sample they had sequenced from an electroblot. In the process of sequencing, the contents of fraction CHb (ii) were electrophoretically separated and blotted onto a membrane, and only the portion of the

blot which contained the 21.5 kDa protein was sequenced. This process appeared to remove all contaminants. An alternative explanation for the purity of the sequence was that any contaminants remaining in the sample were N-terminally blocked. The N-terminal sequence obtained bore no resemblance to chitinases, but was homologous with thaumatin-like (TL) proteins of the PR-5 family (Ruiz-Medrano et al. 1992). These proteins are characterized by low molecular weights (20-25 kDa) and basic pI values (Cornelissen et al. 1986; Hejgaard et al. 1991; Borgmeyer et al. 1992; Huynh et al. 1992), although some acidic isoforms do exist (Bowles 1990; Jung et al. 1993). The TL protein isolated from kiwifruit appeared to exhibit similar properties (pI=9, molecular weight = 21.5 kDa).

Most TL proteins are thought to be antifungal, but their function has not been clearly elucidated (Rebmann et al. 1991; Ruiz-Medrano et al. 1992). Inhibition of pathogen enzyme systems has been suggested as a mode of action, since many TL proteins are highly analogous to a protein which inhibits both trypsin and α-amylase, although not all members of the PR-5 family exhibit such enzyme-inhibitory activity despite sequence homology (Pierpoint et al. 1987; Hejgaard et al. 1991). A more likely mechanism for fungistasis is non-enzymic membrane permeabilization, since maize zeamatin and several other TL proteins induce hyphal rupture of various fungi (Richardson et al. 1987; Roberts & Selitrennikoff 1990; Woloshuk et al. 1991).

Direct alignment of the Calcofluor-negative band in lane 6 of the glycol chitin overlay (Plate 7-2, B) with the most clearly defined protein in lane 6 of the IEF gel (Plate 7-2, A) suggests that the kiwifruit thaumatin-like protein in fraction CHb (ii) could have chitinase activity, assuming that the predominant band in the IEF gel was in fact the 21.5 kDa protein. However, other TL proteins do not exhibit chitinase activity (Roberts & Selitrennikoff 1990; Hejgaard et al. 1991; Woloshuk et al. 1991), and the CH4 and CH2 sugar beet chitinase antibodies did not bind to the 21.5 kDa protein. (SP binding was observed on occasion, but the binding was weak and inconsistent; results not shown.) Moreover, Western analysis of the CHb (ii) fraction revealed the presence of at least two bands (with molecular weights of around 29 and 34 kDa) which were probably chitinases, given the specificity of the sugar beet antibodies. In the light of

these results, it seems more likely that CHb (ii) chitinase activity is associated with small amounts of contaminants rather than with the 21.5 kDa protein, although a definitive answer awaits the purification and activity staining of the kiwifruit TL protein. Many TL protein purification procedures include specific steps to exclude chitinases (Roberts & Selitrennikoff 1990; Borgmeyer et al. 1992; Huynh et al. 1992), suggesting that chitinase contaminants are common. This is probably due to co-purification of TL proteins and plant chitinases during cation exchange because of similar basic pI values. TL proteins can be separated from chitinases by hydrophobic chromatography (Roberts & Selitrennikoff 1990; Woloshuk et al. 1991). This technique should facilitate purification of the kiwifruit TL protein.

All the sugar beet antibodies recognized antigens in crude, cured and uncured kiwifruit stem plug extracts, and SP and CH2 (but not CH4) reacted with proteins in CHb (ii) and with antigens in a partially purified chitinase extract from bean (Section 7.2.3, Part B). Cross reactivity is a relatively common phenomenon because chitinases from different plant families are often serologically closely related (Broekaert et al. 1988; Joosten & de Wit 1989), although those in different genetic classes (refer Chapter 1, Section 1.8.1, Part B) may not cross react. For example, this study and the findings of Mikkelsen et al. (1992) both demonstrated that the CH4 antibody did not cross react with bean chitinases, possibly because sugar beet chitinases bound by CH4 are in a different class (Class IV) from those of bean.

This study provides the first record of a PR-5 protein in kiwifruit. This exciting find merits further investigation to establish whether this compound influences resistance and taste in kiwifruit. Thaumatins are often sweet tasting, although not all TL proteins share this property (Cornelissen et al. 1986; Richardson et al. 1987; Pierpoint et al. 1987). Antigenic sites in thaumatin in some cases appear to be the same sites which interact with human sweet taste receptors (Pierpoint et al. 1987). Cross reactivity with thaumatin antibody could therefore provide a simple test for sweetness in the kiwifruit TL protein. It would be interesting to see if TL-levels vary in response to development and/or external influences and, if so, whether sweetness and resistance levels are correlated. This study has only demonstrated the presence of a TL protein in kiwifruit stem plug

tissue. The influence that kiwifruit thaumatin has on flavour will also depend on whether this protein is found in the pericarp, so localization will also be an important aspect of any future research.

8 GENERAL DISCUSSION

Kiwifruit are one of New Zealand's most significant horticultural export earners (Evans 1995) and any constraints on this industry may have an adverse economic impact on the whole nation. In recent years, competition from countries, such as Chile, which have lower fruit production and transport costs, has made it increasingly important for New Zealand to minimise losses and produce high quality fruit in order to retain its place in the foreign market. *Botrytis* storage rot, the major postharvest disease of kiwifruit (Brook 1990a), represents a serious threat to the continued success of this industry. In addition to spoilage of diseased tissue, ethylene produced by infected fruit may lead to premature softening and reduced storage life of other kiwifruit within close proximity (Qadir 1994). Furthermore, high costs are associated with manual inspection and repackaging of infected lines (Evans 1992). The appearance of rots in some of our export fruit has also damaged our image to the point where some international markets now refer to the problem as the "New Zealand disease" (Tapper 1994). The seriousness of the storage rot problem is compounded by the fact that current control measures are inadequate. Pre-harvest sprays are applied to reduce the level of inoculum in the orchard, but the sprays do not make direct contact with the site of infection, the stem wound, which is created by removal of the pedicel at harvest (Pyke et al. 1994). Moreover, an increase of the numbers of low-level resistant strains in grape has been associated with a reduction in fungicide efficacy (Pak et al. 1990). Even greater losses from this disease could be expected in the future, given that environmental concerns have prompted calls to reduce and/or eliminate the use of certain pesticides. Consequently, there is an urgent need to seek alternative, more sustainable forms of control. One of the most promising areas in this regard is that of host resistance. This area was largely ignored until recently because the absence of *Botrytis*-tolerant cultivars was considered to mean that kiwifruit exhibited little or no resistance to *B. cinerea* (Brook 1990a). However, there is some preliminary evidence to suggest that kiwifruit resistance to *B. cinerea* varies with tissue type (Poole & McLeod 1992), developmental stage (Brook 1990a), and external factors which appear to activate the defence armoury (Poole & McLeod 1991).

An important pre-requisite of this study on host resistance was to confirm the existence of this phenomenon in kiwifruit. Several inoculum levels were used in artificial inoculation trials to examine the degree of resistance under a range of disease pressures. Host resistance was observed as a reduction in the total number of infected fruit and an increase in the time taken for symptoms to manifest. Resistance was inducible by wounding, and the defence response was more rapid when wounds were made directly at the stem scar, as opposed to a more distant site at the end of the pedicel. Similar findings were reported by Hallet & Sharrock (1993), who also observed that fungal penetration occurs through the vasculature of the stem plug. Poole & McLeod (1992) noted that *B. cinerea* conidia were more likely to cause rots if inoculated in the pericarp than in the stem zone. All of these results suggest that resistance is site-specific and appears to be centred in the stem plug. Production of defence mechanisms is an energy-consuming process which occurs often at the expense of yield (Sequeria 1983). Directing resources to a small area where infection will most probably occur could conserve energy, hence site-specific resistance is less likely to cause an unacceptable loss in yield, making it a particularly attractive candidate for further study.

Data from two maturity experiments support the hypothesis that age-related resistance occurs in kiwifruit. Although fruit resistance increased with ageing, this response was overcome by the pathogen at high inoculum loads. Unknown factors appeared to influence the cut-off point at which inoculum load overwhelmed host defences, both in these maturity trials and in experiments by other workers (Brook 1990a; Pyke et al. 1993a). More research is required to identify and standardize these factors, and thereby reduce inherent variability in artificial inoculation experiments. In particular, the hypothesis that epiphytic microflora on the picking scar may limit the success of *B. cinerea* establishment through active competition for nutrients should be examined. In the current study, amending inoculum with yeast extract significantly increased infection. Sharrock & Hallet (1992b) demonstrated that microbial antagonists isolated from the stem scar inhibited the development of *B. cinerea.* It would be interesting to determine whether the numbers of these inhibitory microorganisms are correlated with nutrient levels on the stem scar surface. Overall, results from these maturity experiments should remind growers that the industry guideline of harvesting once fruit reach 6.2% soluble

solids represents the minimum acceptable maturity. Whilst it is commercially appropriate to harvest some fruit early in the season to obtain price premiums overseas, delaying harvest can result in the added benefits of increased resistance, and improved storage and eating quality (Hopkirk 1992). In the long run it is these factors that are important if we are to retain our reputation for producing quality fruit.

Findings from this work indicate that resistance develops on a more rapid time-scale than previously thought. Sharrock & Hallet (1992b), Pennycook & Manning (1992), and Hallet & Sharrock (1993) measured a reduction in infection after one or more days' curing. However, the current study shows that curing can result in a significant decrease in infection within 24 hours, and this finding has been subsequently confirmed by Poole & McLeod (1994). The practical application of this observation is that for experimental work, fruit should always be harvested with pedicels attached, and these should be removed immediately prior to treatment application. This delays the onset of host resistance and should ensure that curing remains constant across different treatments, making it possible to attain higher more consistent levels of infection in artificial inoculation (AI) experiments. In summary, results from the AI trials indicate that host resistance does occur in kiwifruit and is one of the factors which determines the success or failure of infection.

The next step was to select an area of host resistance to study in detail. A survey of current literature (Chapter 1) indicated that kiwifruit antifungal compounds and enzymes with putative defence functions were two areas warranting further investigation. Chitinase enzymes were selected as the project focus for several reasons. Chitin is one of the most ubiquitous substances on this planet, forming a major component of the cell walls of fungi and some algae, and the exoskeleton of many arthropods (Cabib 1987). The large amounts of chitin continuously generated in nature require recycling on a formidable scale to prevent a sink in the global carbon and nitrogen (Gooday, 1990). Hence, hydrolysis by chitinases is a process of vital importance in the biosphere. These enzymes are also involved in fungal morphogenesis (Manocha & Balasubramanian 1994), insect moulting (Cabib 1987), and are thought to form part of a plant's defence against pathogens (Punja & Zhang 1993). Use of chitinases may even have implications

for human health. Renkema et al. (1995) isolated a chitinolytic enzyme from the spleen of patients with Gaucher disease and hypothesized that, since chitin is not found in humans (a situation analogous to that in plants), this enzyme could be involved in defence against chitin-containing pathogens, or play a role in morphogenic processes. Moreover, *Plasmodium* chitinase appears to play an important role in malaria transmission, and inhibition of this enzyme may represent a potent control measure of this devastating disease (Shahabuddin & Kaslow 1994). Any information which adds to our knowledge of chitinolytic enzymes, therefore, is likely to have important practical applications.

An examination of the role of kiwifruit chitinases requires reliable measurements of chitinase activity. The search to find a suitable assay was particularly challenging because activity levels were low and induced to a much smaller extent in kiwifruit (McLeod & Poole 1994) than in other plants (Métraux & Boller 1986; Mauch et al. 1988a). The overall shape of most assay progress curves, in both this study and others, was quadratic with an initial linear stage. Variability was high due to the insoluble and heterogeneous nature of chitin substrates (Cabib 1987). Despite high variation (SEM's were 15-59% of the mean), results from the Molano et al. (1977) radioassay were repeatable. Under optimal incubation conditions (18 h at 25°C), the assay could resolve two-fold or greater concentration differences, and chitinase concentrations above 200 ng/min/ml were significantly different from enzyme-free controls. This level of sensitivity and resolution made the Molano et al. (1977) assay the best quantitative method for this project. The major drawback of this assay is that it is does not distinguish exo- and endochitinase activity. To overcome this problem, these activities were measured separately when required, using the Roberts & Selitrennikoff (1988) exochitinase assay and a viscometric endochitinase assay, or by use of the Boller et al. (1983) assay which can reputedly measure both activities. However, results from the Boller et al. (1983) assay were less reliable due to non-specific detection of the end product (Domard & Vasseur 1991) coupled with its instability (Reissig et al. 1955) and, in addition, many samples were close to the detection limit of this assay. The poor resolution of the Calcofluor petri dish assay meant it was unsuitable for quantitative measurements, but its high sensitivity, rapidity and ease of use made it an excellent

candidate for qualitatively monitoring chitinase activity in the large numbers of samples generated by protein purification. An important practical consideration was to de-sensitize this assay so that it would only detect activity which was sufficient to purify (allowing for inevitable losses during the purification process), and to subsequently characterise using less sensitive quantitative assays. This was achieved using 0.01% glycol chitin substrate and a 5 h incubation at 37°C. Successfully overcoming the problem of how to accurately measure small amounts of chitinase activity meant that it was now possible to examine the role of this enzyme in kiwifruit

The next step was to distinguish kiwifruit and *B. cinerea* chitinases. Fungal chitinases can have exo- or endo- modes of substrate cleavage (Sahai & Manocha 1993; Hodge et al. 1995), whilst plants chitinases are predominantly endochitinases (Graham & Sticklen 1994). Differences in the kiwifruit/*B. cinerea* system were evaluated by measuring exo- and endochitinase activities in healthy and diseased regions of live and autoclaved leaves, and in kiwifruit stem plugs. Measurements using a range of assays showed that significant amounts of exochitinase activity were detected only in tissue with visible signs of infection, as found by Sharrock & Hallet (1992a). Moreover, exochitinase levels in diseased tissue were the same regardless of whether live leaves or those which had been autoclaved prior to inoculation were used. Thus exochitinases in diseased, live tissue were not a plant response to infection and must be of fungal origin. In contrast, McLeod & Poole (1994) observed exochitinase activity in uninoculated tissue, but the amounts measured were small and highly variable, and subject to the uncertainties of interpretation associated with use of the Boller et al. (1983) assay for this purpose. Endochitinase activity in the current study was only detected in live tissue of both healthy and diseased samples, indicating that it was a plant product. Studies on the role of chitinases in kiwifruit defence against *B. cinerea* should therefore focus on endo- rather than on exochitinase activity. Although the Molano et al. (1977) assay does not distinguish between the two, it can still be used for specific endochitinase measurements on healthy fruit or inoculated fruit without visible signs of infection (which have no appreciable exochitinase activity).

Chitinases have been extensively studied in other plants, but not in kiwifruit where their

role in defence is not known. Sharrock & Hallet (1992b) observed that exochitinase levels were unaffected by curing, but increased four-fold upon *B. cinerea* infection. It has now been shown that these increases were almost certainly of fungal origin. Subsequently, McLeod & Poole (1994) demonstrated that plant endochitinases increased three-fold in response to inoculation with *B. cinerea.* In Experiment 6, Chapter 6, activity was also fractionally higher in inoculated verses uninoculated tissue, within the first 7 days of harvest. (Although the Molano et al. (1977) assay was used to measure chitinase activity in this experiment, there was no sign of infection within the first week of harvest, and the current study showed that exochitinase activity was only detected in visibly decayed tissue, as discussed in Chapter 4, Section 4.5)). A profile of endochitinase isoforms in uninoculated, healthy (92/N/U/H) and inoculated, diseased (92/N/I/D) tissue was obtained using Calcofluor-stained glycol chitin overlay gels. Results showed that there were at least two acidic and one basic isoform in healthy tissue, with respective pI values of 4.7, 5.2 and 7.5. One new basic isoform with a pI of 9 was clearly present in diseased tissue, and the horizontal spread of the remaining activity stains suggested that multiple acidic and basic isoforms were also induced by inoculation and/or infection. Similar levels of endochitinase activity, as measured by the viscometric assay, were found in healthy and infected regions of kiwifruit leaves. However, inoculation could have induced endochitinase activity throughout the leaf, and future studies should also measure chitinase levels in uninoculated leaves. Activity levels in "resistant" stem plug tissue, which had been inoculated but had not developed infection, were also compared with that in inoculated diseased tissue. Analysis of inoculated, healthy and diseased stem plugs in Experiment 6, Chapter 4, using the Boller et al. (1983) assay, demonstrated that endochitinase activity was higher in "resistant" stem plugs. (The presence of fungal exochitinases in the diseased tissue of Experiments 1 and 6 (Chapter 6) confounds any comparisons and these results are consequently not discussed here.) On the basis of all these results, it appears that kiwifruit responds to *B. cinerea* by increasing total chitinase activity and producing multiple new basic and acidic isoforms.

The effect of curing on stem scar chitinases was evaluated. There was a slight but non-significant increase in total endochitinase activity in cured fruit, and no new isoforms

were induced. However, the activity of an existing ≈30 kDa protein, which bound to sugar beet antibody "CH4", increased with curing. This protein was most probably a chitinase, given the binding specificity of the antibody. In addition, a small but significant increase in total proteins suggested that other compounds are involved in the curing response. Sharrock & Hallet (1993) reported that stem plug endochitinase activity increased significantly in kiwifruit that had been cured for seven days at 20°C, but did not determine which isoforms had been induced. Disparity between their results and mine could be attributable to the different time lapses between curing and sampling of stem plugs. In their study, samples were taken at the end of the seven day curing period, while fruit in this study remained in coolstore for a further nine weeks before sampling. Increases in chitinase which can occur during prolonged storage (Chapter 6, Section 6.5) may have masked increases due to curing. Inadequate control of environmental factors, such as humidity and temperature may also contribute to the variable effect of curing. Morris et al. (1989) found that curing efficacy in potato was highly dependent on temperature and relative humidity. Bautista-Baños (1995) found that over three seasons, the greatest curing effect in kiwifruit was obtained at 10°C and that this effect diminished at higher temperatures. Disease incidence was also reduced when fruit was cured at high relative humidities (89-95%), possibly because high humidities are necessary to prevent weight loss and help retain wounded tissue in a state where it can actively carry out repair work and stimulate the development of defence mechanisms. These studies emphasize the need to carry out further curing investigations under controlled temperature and humidity to determine the role of chitinases in curing under standardized conditions.

In general, there was a variable response of kiwifruit chitinase to stress. The pathogen induced both new acidic and basic isoforms, compared with the increase of a single existing isoform in response to curing. Brederode et al. (1991) observed that chitinase isoforms in tobacco responded differently to various stresses. They proposed that acidic but not basic forms were involved in resistance, since induction of acidic chitinases only correlated with diminished infection. In contrast, Bol et al. (1990) stated that tobacco basic chitinases were more antifungal than acidic isoforms. In kiwifruit, acidic isoforms predominate over basic forms but it appears that both may be involved in defence.

There is also increasing evidence to suggest that chitinases may be involved in plant morphogenesis (De Jong et al. 1992; Leung 1992). Current research on the role of chitinases in kiwifruit could be extended by examining the pattern of isoforms in relation to physiological events such as flowering. In this study, the role of chitinases in one such developmental process, fruit maturation, was examined. Harvest maturity had little effect on endochitinase levels, but increases during postharvest storage were accentuated in the later-harvested fruit, which were also less prone to stem-end rot. Resistance of many fruit and vegetables typically diminishes with age, and this is frequently linked to a reduction in antifungal compounds (Prusky et al. 1985; Snowden 1990; Alonso et al. 1992). Conversely, Wyatt et al. (1991) found an improved resistance with age in tobacco, which correlated with increases in β-1,3-glucanase, chitinase and peroxidase activities. The current study is the first to suggest that a similar situation applies in kiwifruit. Further research is required to identify the specific isoforms which change with maturation and the timing of their induction.

A 1.5-4.0 fold increase in endochitinase activity in fruit stored for twelve weeks at 0 ± 0.3°C was a common feature of experiments in this study. Unlike many other fruit, kiwifruit can be maintained in coolstorage for at least six months (McDonald 1990) without unacceptable decrease in quality. Results from Experiment 4, Chapter 3 demonstrate that fruit stored for such prolonged periods exhibit increased resistance. Prolonged storage allows more time for wound repair and fruit maturation. Biochemical changes which accompany these processes (including an increase in chitinase activity) therefore appear partially responsible for the long storage life of this fruit.

In certain experimental situations, for example when assaying a large number of samples over an extended period of time, it may be important to inhibit time-based increases in chitinases so that endogenous activity levels are not changed. This can be achieved by storage at sub-zero temperatures, preferably -80°C. In this frozen state, chitinase activity in extracts remains constant for at least six months.

The potential of the natural compound chitosan to induce chitinases and other defence mechanisms (El Ghaouth et al. 1994a; Hadwiger et al. 1984) made it a useful tool for

elucidating the response of kiwifruit chitinases to an elicitor. In addition to its effect on host resistance, this compound is able to directly inhibit fungal growth (Allan & Hadwiger 1979) and act as a coating that reduces weight loss and respiration, thereby prolonging shelf life (El Ghaouth et al. 1992a). Solubilization of chitosan requires an acidic solvent, and it was found that use of this solvent without pH adjustment predisposed host tissue to disease. Although chitosan significantly reduced infection in the presence of pH unadjusted solvent, disease incidence was never reduced below the level found in the inoculated control. Therefore, from an industrial perspective, chitosan application appears an unsuitable method of controlling *B. cinerea* storage rot of kiwifruit. Experiment 6, Chapter 6 results showed that there was no strong correlation between chitosan-elicited chitinase activity and reduced infection, which suggests that chitosan-mediated disease reduction at unadjusted pH (Experiment 7, Chapter 6) was attributable to antifungal properties of this compound, rather than its ability to elicit chitinase. El Ghaouth et al. (1992c, 1994b) proposed that host elicitation in general plays a supporting rather than a primary role in the control afforded by chitosan.

Induction of kiwifruit chitinases appears to be a relatively slow response. Increases in chitinase activity in response to inoculation and/or chitosan application were not significant until one day or more after harvest, and they continued to rise slowly over several weeks in storage (Experiment 6, Chapter 6). In contrast, Poole & McLeod (1994) found that resistance occurred on a much more rapid time scale. Their measurements of various enzyme activities after harvest led them to postulate that early resistance involves phenylpropanoid metabolites, whereas endochitinases were more likely to contribute to the late phases of resistance development, since activities in inoculated fruits did not increase significantly until three days or more after harvest.

Correlations between high chitinase activity and low infection do not in themselves provide definitive evidence of the role of these enzymes in host resistance, because other antifungal compounds may be simultaneously induced. Consequently, there was a need to separate chitinases from other plant components. A partially purified fraction, containing one predominant 21.5 kDa protein with apparent chitinase activity, plus small amounts of contaminants, was obtained by cation exchange and gel filtration.

Comparisons of the N-terminal amino acid sequence of the 21.5 kDa protein with other sequences indicated that this protein was a member of the thaumatin-like (TL) pathogenesis related family. This novel finding opens a whole host of possibilities for future research, including whether this compound influences resistance and taste in kiwifruit. An important first step would be to find a suitable method of assaying this protein. In other studies this has been achieved by measuring its unusual property of acting synergistically with nikkomycin to inhibit the growth of *Candida albicans* (Roberts & Selitrennikoff 1990). Another logical step would be to complete purification, possibly by use of hydrophobic interaction chromatography, as this has been successfully used as a final purification step in other studies (Roberts & Selitrennikoff 1990; Woloshuk et al. 1991). This should clarify whether the chitinase activity detected in the partially-purified extract can be attributed to the kiwifruit TL protein, or to contaminants. Once a homogenous product has been obtained, assaying for enzyme inhibitory activities and membrane permeabilization, as well as chitinase activity, would also help resolve this question and possibly establish the mode of action of the TL protein. Antifungal and taste properties of the purified protein could also be ascertained using in vitro assays and taste panels. Antibodies could be raised for use in immunolabelling studies to visualise location of the protein within the fruit. This would be an important aspect of any future research, as the TL protein would need to be present in the pericarp tissue for it to have any influence on flavour. Another interesting study could be to determine the effect of curing on endogenous levels of this protein.

There are several aspects of research on kiwifruit chitinases that require more study. In particular, further optimisation of the purification protocol and confirmation of the identity of proteins bound by the sugar beet antibodies is required. A major limitation to purification was the low initial level of protein. Electrophoretic separation of mucilaginous compounds in the Rotofor may surmount this problem by enabling larger amounts of lyophilized protein to be dissolved in small buffer volumes, thereby effectively increasing the concentration. Use of a batch-wise affinity procedure could also prove to be a useful step, as this method avoids the column blockage problems which are commonly associated with affinity chromatography. Once isolated, antifungal and/or anti-bacterial properties of kiwifruit chitinases both alone and in concert with

other peptides could be investigated. In particular, the putative kiwifruit chitinase bound by sugar beet CH4 antibody warrants further study. This protein was lost in the current purification protocol. The key to its successful isolation may be to use CH4 antibody to screen fractions, since the CH4-bound protein did not appear to constitute the bulk of chitinase activity, and therefore might be difficult to locate if fractions are monitored on the basis of total chitinase activity.

Specificity of sugar beet antibody binding suggested attachment to chitinases. However, affirmation of this would require an activity stain. Attempts to link antibody binding with chitinase activity in a native electrophoresis/Western blot system were unsuccessful. Western blots were obtained following SDS-PAGE, but incubation of gels in buffer containing Triton X-100, as described by Trudel & Asselin (1989), did not restore enzyme activity. However, a longer Triton X-100 incubation (Marek et al. 1995) or use of isopropanol (Blank et al. 1982) might facilitate protein renaturation. Alternatively, the Western blot protocol could be applied to an IEF system which allows proteins to retain their activity. Once it has been established that the sugar beet antibodies are binding to kiwifruit chitinases, they could then be used for chitinase purification and in immunolocalization studies.

Another important question which remains to be answered concerns the location of kiwifruit chitinases, as this may provide further clues to their roles in defence. For example, vacuolar forms are thought to act as a last line of defence at a late stage of infection when they are released upon cell lysis (Mauch & Staehelin 1989), whereas it has been suggested that extracellular enzymes either limit pathogen growth upon entry into the host, and/or release elicitors, thereby inducing other defence mechanisms (Graham & Sticklen 1994). An observation that *Nectria hematococca* developed resistance to antifungal hydrolases when exposed continually to these enzymes led Boller (1993) to propose that antifungal hydrolases are more likely to be effective in defence when suddenly brought into contact with invading fungi by release from an intracellular compartment, as opposed to being present constitutively in the extracellular space. However, this hypothesis does not necessarily apply if the main function of extracellular isoforms is elicitor release rather than direct inhibition, as suggested by Graham &

Sticklen (1994). Approaches to localizing kiwifruit isoforms could include comparing chitinase activity levels in intercellular wash fluids and intact tissues, and immunohistology as described by Keefe et al. (1990)

Although the story is not yet complete, successes in the current study have included adapting chitinase assays for use in a system with low enzyme activity, distinguishing host and fungal chitinases, and examining the changes in activity in response to development, curing, infection and elicitor application. They have made it possible to draw several conclusions about the significance of chitinases in kiwifruit defence against *B. cinerea*. Most importantly, the relatively low levels of induction relative to other crops, coupled with slow timing of the response, suggest that chitinase expression alone may be insufficient to prevent infection. This conclusion does not mean that chitinases are unimportant in resistance, since they could be directed to a very small area where they could delay fungal development while other defence materials accumulate. In many cases, the antifungal activity of chitinase requires the co-presence of β-1,3-glucanases (Mauch et al. 1988b). Van Den Elzen et al. (1993) found that concurrent expression of tobacco Class I chitinases and Class I β-1,3-glucanase genes in tomato elevated resistance to *Fusarium oxysporum* f.sp. *lycopersici*, whereas tomato plants expressing either gene alone were not significantly protected. Combining chitinases with other peptides such as lectins (Broekaert et al. 1989) can also enhance the fungistatic effect. Significant losses in chitinase activity at each step of the protein purification process suggests that the various kiwifruit isoforms are synergistic, hence separation would cause an underestimate of their activity. Exploring the potential of integrated gene expression in transgenics represents one of the most exciting areas for future research, since multi-component host resistance is likely to be more durable than chemical control. Success of this approach, however, will depend on balancing the trade-off between resistance and yield. Judicious selection of host resistance factors is also important to prevent the loss of useful microorganisms on the phylloplane.

A second conclusion about the role of chitinases in kiwifruit is that they are part of a general stress response, since they were induced by a number of different factors, including wounding and infection. Induction of chitinases by multiple stimuli is a

relatively common phenomenon (Métraux & Boller 1986; Brederode et al. 1991; Chang et al. 1995) and provides further testimony to the integral role of chitinases in plant responses to the environment.

Resistance to *B. cinerea* in kiwifruit involving chitinases appears to be A) "organ-specific", because chitinases were concentrated in the stem plug and this tissue was most resistant to disease, B) "inducible", since curing-mediated increases in a putative chitinase correlated with reduced infection, and C) "age-related", as mature fruit and those subjected to prolonged storage had highest levels of chitinase and were least susceptible to the pathogen.

Initially it was thought that kiwifruit had little resistance to *B. cinerea*, but this project has provided further evidence of the presence of putative antifungal defence compounds which appear to play a role in resistance of kiwifruit to *B. cinerea*. This should provide the industry with renewed confidence in further research to capitalize upon the natural defences of this plant for control of *B. cinerea*.

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APPENDIX I - BUFFER RECIPES

Milli-Q water was used in all recipes, unless otherwise stated. All chemicals were analytical or electrophoresis grade reagents purchased from either Sigma, BDH chemicals, May and Baker Ltd, Ajax Chemicals or Bio-Rad Laboratories Ltd.

(A) SODIUM ACETATE BUFFERS

(i) 0.1 M NaOAc Extraction Buffer, pH 5.8

[1] Glacial acetic acid (2.29 ml) was added to approximately 200 ml of water.

[2] A 0.08 g batch of sodium azide (NaN_3), and 0.06 g of dithiothreitol (or 0.8 g Na-diothionite) were dissolved in the acetic acid solution.

[3] In cases where NaTT and Triton X-100 were included in the buffer, 0.28 g NaTT and 80 µl of Triton were added.

[4] The pH was adjusted to 5.8 using concentrated NaOH (NaOH concentration was arbitrary).

[5] The final volume was adjusted to 400 ml with water and the buffer was stored at 4°C.

[6] PVPP, 1% (w/v) was sometimes added to buffer aliquots prior to stem plug extraction.

(ii) 1 M (and 0.1 M) NaOAc Buffer, pH 5.6

(This recipe is derived from the sodium acetate-acetic acid buffer system described by McKenzie & Dawson (1969).)

[1] Stock solutions of 1 M acetic acid (57.75 ml glacial acetic in 1 l of water) and 1 M Na-acetate (82 g of $C_2H_3O_2Na$ in 1 l of water) were prepared.

[2] A 4.8 ml aliquot of 1 M acetic acid and 45.2 ml of 1 M Na-acetate were added together.

[3] This buffer can be diluted 10x in water to produce 0.1 M NaOAc, pH 5.6.

(iii) 10 mM and 50 mM NaOAc Buffer, pH 5.6

[1] The 50 mM NaOAc buffer used in the Roberts & Selitrennikoff (1988) exochitinase assay was produced by a two-fold dilution of 0.1 M NaOAc buffer, pH 5.6 in water.

[2] A 10 mM NaOAc buffer was created by a 100-fold dilution of 1 M NaOAc buffer, pH 5.6 in water.

(iv) 20 mM NaOAc Buffer, ± 1 M NaCl, pH 5.0

[1] Glacial acetic acid (286 µl), 0.05 g NaN_3 and 14.61 g of NaCl (if required) were dissolved in ≈ 180 ml of water.

[2] The pH was adjusted to 5.0 using concentrated NaOH, and the final volume adjusted to 250 ml with water.

(v) 50 mM NaOAc Buffer, ± 0.5 M NaCl, pH 5.0

[1] Glacial acetic acid (2.884 ml), 0.2 g NaN_3 and 29.22 g of NaCl (if required) were dissolved in ≈ 800 ml of water.

[2] The pH was adjusted to 5.0 using concentrated NaOH, and the final volume adjusted to 1 l with water.

(B) McILVAINE CITRATE/PHOSPHATE BUFFERS

(i) McIlvaine, pH 5.0

(The recipe for this buffer is sourced from McKenzie & Dawson (1969).)

[1] A 0.1 M solution of citric acid monohydrate (21.01 g of $C_6H_8O_7.H_2O$/l of water) and 0.2 M solution of di-sodium hydrogen orthophosphate (28.40 g Na_2HPO_4/l of water) were prepared. (Both stock solutions also contained 0.02% NaN_3.)

[2] A total of 400 ml of pH 5.0 McIlvaine citrate-phosphate buffer was produced by adding together 194 ml of 0.1 M $C_6H_8O_7.H_2O$ and 206 ml of 0.2 M Na_2HPO_4.

[3] pH adjustment was usually unnecessary but, if required, was made with the appropriate stock solution.

(ii) McIlvaine, pH 5.8

[1] A 10 ml batch of this buffer, containing 1 mM dithiothreitol (DTT) and 0.02% sodium azide, was produced by dissolving 0.002 g DTT and 0.002 g of NaN_3 in 6 ml of 0.2 M Na_2HPO_4, followed by addition of 4 ml of 0.1 M citric acid.

[2] PVPP, 1% (w/v) was added prior to stem plug extraction.

(C) 0.5 M K-PHOSPHATE BUFFER, pH 7.1

(Recipe from McKenzie & Dawson (1969).)

[1] Stock solutions of 1 M sodium hydroxide (40 g NaOH/l of water) and 1 M potassium di-hydrogen orthophosphate (136.09 g KH_2PO_4/l of water) were produced.

[2] A 32.1 ml aliquot of 1 M NaOH was added to 50 ml of 1 M KH_2PO_4 and diluted to a final volume of 100 ml with water.

(D) 1 M BORATE BUFFER, pH 9.8

[1] A 4.95 g batch of boric acid was dissolved in 60 ml of water with gentle heat and magnetic stirring.

[2] The pH to was adjusted to 9.8 with concentrated NaOH.

[3] Water was used to bring the final volume to 80 ml.

(E) TRIS-HCl BUFFERS

(i) 500 mM Tris-HCl Buffer, pH 8.9

[1] Tris base (30.28 g), and 0.1 g of NaN_3 in 400 ml of water.

[2] The solution pH was adjusted to 8.9 with 1 M HCl.

[3] Water was added to bring the final volume to 500 ml.

(ii) 1.5 M Tris-HCl Buffer, pH 8.8

[1] Tris base (27.23 g) was dissolved in 80 ml water.

[2] The pH was adjusted to 8.8 with 1 M HCl.

[3] The final volume was brought to 150 ml with water and the buffer was stored at 4°C.

(iii) 0.5 M Tris-HCl Buffer, pH 6.8

[1] Tris base (6 g) was dissolved in 60 ml water.

[2] The pH was adjusted to 6.8 with 1 M HCl.

[3] The volume was made up to 100 ml with water and the solution was stored at 4°C.

(iv) 20 mM Tris-HCl Buffer, ± 1 M NaCl, pH 8.0

[1] Tris (0.6 g) and 0.05 g of NaN_3 was dissolved in 150 ml water.

[2] In cases where NaCl was included, 14.61 g was dissolved in the Tris solution.

[3] The pH was adjusted to 8.0 with 1 M HCl, and the final volume was made up to 250 ml with water.

(F) SDS SAMPLE BUFFER

(Recipe obtained from the Bio-Rad Mini-Protean II Dual Slab Cell instruction manual.)

[1] Water (4.4 ml), 1 ml of 0.5 M pH 6.8 Tris-HCl, 0.8 ml of glycerol, 1.6 ml of 10% (w/v) SDS (stored at room temperature), and 0.2 ml of 0.05% (w/v) bromophenol blue) were mixed together.

[2] The buffer was stored at 4°C.

(G) 5X, ELECTRODE (RUNNING) BUFFER, pH 8.3 FOR PAGE

[1] Tris (9 g), 43.2 g glycine and 3 g SDS were dissolved in water and the final volume brought to 600 ml.

[2] The buffer concentrate was stored at 4°C and warmed to 37°C if precipitation occurred.

[3] A 100 ml batch of 5X stock was diluted with 400 ml water for one electrophoretic run.

(H) BUFFERS FOR LOW pH DISCONTINUOUS NATIVE ELECTROPHORESIS

(i) Resolving Gel Buffer, pH 4.3

[1] A 9.6 ml aliquot of 1 M KOH was dissolved in water, and approximately 3.44 ml of glacial acetic acid was added until a pH of 4.3 was obtained.

[2] The final volume was adjusted to 20 ml with water and the buffer was stored at 4°C.

(ii) Stacking Gel Buffer, pH 6.8

[1] A 9.6 ml aliquot of 1 M KOH was dissolved in water, and approximately 0.58 ml of glacial acetic acid was added drop-wise until a pH of 6.8 was obtained.

[2] The volume was made up to 20 ml with water and the solution was stored at 4°C.

(iii) Reservoir (Running) Buffer, pH 4.5

[1] A 31.2 g batch of β-alanine was dissolved in water and the pH adjusted to 4.5 with 8 ml of glacial acetic acid.

[2] The final volume was brought to 1 l with water.

(I) ELISA AND WESTERN BLOT BUFFERS

(i) 0.1 M Sodium Carbonate Buffer, pH 9.4

[1] A 1.59 g batch of Na_2CO_3 and 2.93 g of $NaHCO_3$ were dissolved in water.

[2] The final volume was brought to 1 l with water.

[3] The resultant buffer was stored in a tightly capped bottle at room temperature.

(ii) PBS-Tween (Phosphate Buffered Saline-Tween), pH 7.5

[1] Sodium chloride (11.68 g), 23 g of Na_2HPO_4, 6.24 g of $NaH_2PO_4.2H_20$ and 1 g of pure Tween 20 were dissolved together in water.

[2] The pH was adjusted to 7.5 (using either HCl for acidic adjustments or NaOH to increase the pH).

[3] The final volume was brought to 2 l with water and the buffer was stored at 4°C.

(iii) 25 mM Tris, 192 mM Glycine, 20 % (w/v) Methanol Buffer, ≈pH 8.3

[1] Tris (3.03 g), 14.4 g glycine, and 200 ml of methanol were mixed together.

[2] The final volume was brought to 1 l with water. (Note: The pH of this buffer should not be adjusted.)

(J) 0.1 M MES Buffer, pH 5.8

[1] Sodium azide (0.002 g), 0.195 g of 2-(N-morpholino)ethanesulphonic acid (MES) and 0.002 g of DTT were dissolved in ≈8 ml of water.

[2] The pH was adjusted to 5.8 using concentrated NaOH, and the final volume brought to 10 ml using water.

[3] PVPP, 1% (w/v), was added prior to stem plug extraction.

(K) 0.1 M CITRIC ACID/SODIUM CITRATE BUFFER, pH 5.8

[1] A 0.1 M stock solution of tri-sodium citrate was prepared (2.94 g/100 ml of water).

[2] Sodium azide (0.002 g), and 0.002 g DTT were dissolved in 8.4 ml of 0.1 M tri-sodium citrate and 1.6 ml of 0.1 M citric acid was added.

[3] PVPP, 1% (w/v), was added to buffer aliquots prior to stem plug extraction.

APPENDIX II - ASSAY CALCULATIONS

(A) COLORIMETRIC ASSAY OF ROBERTS & SELITRENNIKOFF (1988)

This assay measured exochitinase activity, which was expressed as nanomoles of *p*-nitrophenol released per minute per ml of crude extract.

[1] A buffer mixture, comprising 50 parts 1 M Na-tetraborate and 100 parts 50 mM sodium acetate buffer, pH 5.6, was prepared to simulate the final environment in which the *p*-nitrophenol product is normally read. The absorbances of ten 150 µl samples of 0.1 mM *p*-nitrophenol in buffer mixture (10 mM p-nitrophenol was diluted 100-fold in the buffer mixture to produce a 0.1 mM solution) were measured and corrected for background absorbance due to the buffer mixture alone. The extinction coefficient (ϵ) gives the absorbance of a 1 M solution. Therefore, the mean corrected absorbance of the 0.1 mM solution was multiplied by 10^4 to obtain the extinction coefficient (ϵ) for a 1 M solution of *p*-nitrophenol. Using this method $\epsilon = 9310$.

[2] Beer's law states that concentration (moles/l) = absorbance/ϵ. Therefore exochitinase activity (nmol *p*-nitrophenol/min/ml of crude extract) in the Roberts & Selitrennikoff (1988) assay was calculated according to the following formula:

$$\textbf{absorbance}/\epsilon \quad \textbf{x } 10^6 \textbf{ /180 x 0.15/0.01}$$

where:

$\epsilon = 9310$

10^6 = conversion factor for changing mol/l to nmol/ml (where ml refers to total assay volume)

180 = incubation time (minutes)

0.15 = total assay volume (ml)

0.01 = volume of crude extract (ml)

(B) VISCOMETRIC ASSAY

In this assay, endochitinase activity was expressed as a percent decrease in viscosity relative to an enzyme-free control. The values obtained gave an indication of the relative activities of different samples rather than an absolute measure of activity. Validity of the calculations rested on the assumption that all samples, including the control, had approximately the same initial viscosity. Dynamic viscosity of the control had stabilized 15 minutes after the start of the assay, and decreases in dynamic viscosity at 15 minutes in samples containing chitinase were linearly related to $\log_{10}$ of enzyme concentration. [1] Dynamic viscosity (mPa/s) in samples was calculated using the following formula from the Haake Microviscometer instruction manual:

$$\eta = k \times (Q_1 - Q_2) \times t$$

where:

η = dynamic viscosity (mPa/s)

k = 0.03 mPa.s.cm^3/g.s (the value of this constant is determined by the size of the ball bearing and the reaction temperature)

Q_1 = 7.8 g/cm^3 (ball bearing density)

Q_2 = 1.069 g/cm^3 (sample density)

t = fall time (s) 15 minutes after the start of the assay

[2] the control was assigned a viscosity of 100%, and sample viscosities were expressed as a percentage of the control viscosity:

sample dynamic viscosity/control dynamic viscosity x 100/1 = A

[3] therefore:

percent decrease in sample viscosity relative to the control = 100 - A

(C) COLORIMETRIC ASSAY OF BOLLER ET AL. (1983)

The Boller et al. (1983) assay was a multiple step process i.e.

initial assay volume 0.3 ml (0.1 ml each of suspended substrate, buffer, and crude enzyme extract)

↓

1st incubation (2 h) to allow substrate digestion by chitinase

↓

boiling (3 min), followed by centrifugation to remove non-degraded substrate

↓

0.15 ml supernatant (from previous step) + 0.02 ml snail gut enzyme in K-phosphate buffer

↓

2nd incubation (1 h) to allow snail gut hydrolysis of oligomers

↓

addition of 0.035 ml of sodium borate buffer, followed by boiling (3 min)

↓

addition of dilute DMAB (1 ml)

↓

3rd incubation (20 min) to allow colour development

↓

final volume 1.205 ml (0.15 + 0.02 + 0.035 + 1), absorbance read at 585 nm

Calculation of chitinase activity (nmol of N-acetylglucosamine (GlcNAc) produced per minute per ml of crude enzyme extract) was consequently a fairly involved procedure, which is detailed as follows:

[1] A standard curve was produced by plotting absorbance against GlcNAc concentration (mmol/l of standard). The linear equation of this curve was rearranged to make GlcNAc concentration (mM) the subject:

GlcNAc concentration (mM) = (absorbance - y intercept)/slope

[2] Chitinase activity (nmol GlcNac/min/ml crude extract) was then calculated by the following formula:

0.17/0.15 x GlcNAc concentration x 10^6 x 0.15/1000 x 0.3/0.15 x 1/120 x 1/0.1

where:

0.17/0.15 = correction factor to account for the fact that 0.17 ml of each standard cf. a 0.15 ml sample of the first incubation mixture was used

GlcNAc (mM) = value obtained from the calculation in [1] (Note that the calculated concentration of GlcNac at the end of the first incubation included oligomeric GlcNAc residues which were subsequently separated into monomers by the snail gut enzyme hydrolysis step.)

10^6 = conversion factor for changing mM to nM

0.15/1000 = volume (l) of the sample of the first incubation mixture (i.e. volume containing potential GlcNAc)

0.3/0.15 = correction factor to account for the fact that the initial incubation volume (containing potential GlcNac) was 0.3 ml, but after centrifugation only 0.15 ml of supernatant was assayed for soluble GlcNAc content.

120 = first incubation time (minutes)

0.1 = volume (ml) of crude enzyme extract used in the assay

(D) RADIOASSAY OF MOLANO ET AL. (1977)

Chitinase activity was expressed as ng of tritiated chitin solubilised per minute per ml of enzyme extract.

[1] The number of disintegrations per minute (dpm) in each sample was corrected for background dpm due to the scintillant. (Alternatively, the effect of substrate leaching can be corrected for by subtracting the dpm of an enzyme-free blank from the sample dpm. Although substrate leaching was responsible for some radioactivity in enzyme-free blanks, overall its effect was not significant in this study.)

[2] The average dpm of three, 30 µl "substrate-only" replicates was used to calculate substrate specific activity:

substrate specific activity (dpm/mg) = dpm of 30 µl of substrate/0.2364

where:

30 µl = volume of substrate routinely used in the assay

0.2364 = mg of chitin in 30 µl of substrate (determined by dry weight measurements)

[3] chitinase activity (ng/min/ml) was calculated by:

corrected sample dpm/substrate specific activity x 0.3/0.2 x 1/1080 x 1/0.03 x 10^6

where:

corrected sample dpm = value calculated in [1]

substrate specific activity (dpm/mg) = value calculated in [2]

0.3/0.2 = correction factor to account for the fact that the total assay volume before centrifugation = 0.3 ml, but after centrifugation only 0.2 ml of supernatant was processed

1080 = incubation time (minutes)

0.03 = volume of enzyme extract (ml) used in the assay

10^6 = conversion factor for changing mg/min/ml to ng/min/ml

(E) BRADFORD (1976) PROTEIN ASSAY

Protein content was expressed as μg of total protein per ml of enzyme extract.

[1] A range of standards (160 μl) was prepared, and a standard curve was produced by plotting absorbance against μg of protein (absolute amount). The linear equation of this curve was rearranged to make protein (μg) the subject:

protein (μg) = (absorbance - y intercept)/slope

[2] Absorbance of samples (160 μl) was also determined and protein content (μg/ml) calculated by:

μg/160 x 1000

where:

μg = value determined in [1]

160 = extract volume (μl)

1000 = conversion factor for changing μg/μl to μg/ml

(F) SPECIFIC ACTIVITY OF ENZYME EXTRACTS

Specific activity of enzyme extracts was expressed as ng of tritiated chitin solubilised per minute per μg of total protein. This was calculated by:

chitinase activity/protein content

where:

chitinase activity (ng/min/ml) = value calculated in Appendix II, Part D, [3]

protein content (μg/ml) = value obtained in Appendix II, Part E, [2]

APPENDIX III - ADDITIONAL SUBSTRATE RECIPES

(A) PREPARATION OF 1% W/V GLYCOL CHITIN, pH 4

(A modification of the method of Trudel & Asselin (1989).)

[1] A 2.5 g sample of glycol chitosan was ground with 50 ml of 10% acetic acid in a mortar and pestle. The resulting solution was covered with parafilm and left overnight at 20°C to allow for complete dissolution.

[2] Following addition of 225 ml of methanol, the mix was vacuum filtered through Whatman (No. 1) filter paper. Acetic anhydride (3.75 ml) was added to the filtrate with magnetic stirring until the material gelled, bringing the flea to a standstill.

[3] After 50 minutes at 20°C, the gel was cut into small pieces, covered with methanol and homogenised for four minutes at top speed in a Warring blender.

[4] The homogenate was centrifuged at 27,000*g* for 30 min. The gelatinous pellet was resuspended in one volume of methanol, homogenised and recentrifuged as before.

[5] The pellet was resuspended in 250 ml of distilled water containing 0.02% (w/v) sodium azide and homogenised for four minutes in the Warring blender.

[6] Glycol chitin was stored at 4°C.

(B) PREPARATION OF COLLOIDAL CHITIN

(A modification of the method reported by Shimahara & Takiguchi (1988).)

[1] Chitin (0.5 g) was added to 10 ml of concentrated (85%) phosphoric acid and left at 4°C for 24 h.

[2] Milli-Q water (500 ml) was added, and the mixture was sonicated for 15 min, then filtered through a sheet each of glass fibre and hardened Whatman filter paper on a Buchner funnel under vacuum. A further 500 ml of water was passed through the funnel to wash the precipitate

[3] A 100 ml aliquot of 1 M NaOAc buffer, pH 5.6 was passed through the funnel and the precipitate was then homogenized with 60 ml of 0.05 M NaOAc, pH 5.6 and 40 mg sodium azide. The resultant suspension was stored at 4°C.

APPENDIX IV - GEL ELECTROPHORESIS RECIPES

(A) SDS-PAGE

(i) Polyacrylamide Resolving Gel (T=12%, C=2.7%)

[1] A 6 ml aliquot of 37.5:1 40% acrylamide/bisacrylamide (bis), 5 ml of 1.5 M pH 8.8 Tris-HCl, 0.2 ml of 10 % SDS, and 8.7 ml water were degassed together for 15 minutes in a stoppered Buchner flask with magnetic stirring under vacuum.

[2] TEMED (10 µl) and 100 µl of freshly made 10% (w/v) ammonium persulphate (APS) were added.

[3] The mix was immediately poured into both sides of the Mini-Protean, covered with water-saturated isobutanol and left at room temperature to polymerize.

(ii) Polyacrylamide Stacking Gel (T=4%, C=2.7%)

[1] A 1 ml aliquot of 37.5:1 40% acrylamide/bis, 2.5 ml of 0.5 M pH 6.8 Tris-HCl, 0.1 ml of 10 % SDS, and 6.4 ml of water were degassed together.

[2] TEMED (10 µl) and 50 µl 10% APS were added.

[3] The stacking gel mix was applied to the thoroughly water rinsed and dried surfaces of the two resolving gels.

[4] Plastic combs remained in the stacking gels throughout polymerization.

(B) LOW pH DISCONTINUOUS NATIVE GEL ELECTROPHORESIS

(Refer Blackshear 1984 for further details.)

(i) Acrylamide/Bisacrylamide (30:0.8)

(Gloves, a face mask and a laboratory coat were worn when handling these neurotoxins.)

[1] Acrylamide (14.55 g) and 0.46 g of bisacrylamide were dissolved in 20 ml water.

[2] The final volume was adjusted to 50 ml with water.

[3] The solution was filtered and stored in a foil covered glass jar at 4°C.

(ii) Resolving Gel (T=12.5%, C=2.7%)

[1] A 6.25 ml aliquot of 30:0.8 acrylamide/bisacrylamide, 1.875 ml of resolving gel buffer, pH 4.3 (Appendix I, (H), (i)), and 6.058 ml water were degassed together for 15 minutes in a stoppered Buchner flask with magnetic stirring under vacuum.

[2] TEMED (75 μl) and 750 μl of freshly made 1.5% (w/v) ammonium persulphate (APS) were added.

[3] The mix was poured into both sides of the Mini-Protean, covered with water-saturated isobutanol and left at room temperature to polymerize. (Polymerization is a slow process at acid pH.)

(iii) Stacking Gel

[1] A 1.25 ml aliquot of 30:0.8 acrylamide/bis, 2.5 ml of stacking gel buffer, pH 6.8 (Appendix I, (H), (ii)) and 4.93 ml of water were degassed together.

[2] TEMED (75 μl) and 1.25 ml of 0.004% (w/v) riboflavin were added.

[3] The stacking gel mix was applied to the thoroughly water rinsed and dried surfaces of the two resolving gels.

[4] The gels in the Mini Protean assemblies were placed approximately 10 cm away from a fluorescent tube in an enclosed box and left to polymerize.

[5] Plastic combs remained in the stacking gels throughout polymerization.

APPENDIX V - GEL STAINING PROTOCOLS

(A) SILVER STAINING OF PROTEINS IN PAGE AND IEF GELS

(The protocol is based on the method of Merril et al. (1981) using reagents from the Bio-Rad silver stain kit (No. 161-0443) and the accompanying Bio-Rad instructions.)

During each incubation step described below, the gel and its bathing solution were shaken gently in a glass container on an orbital shaker at ambient temperature. For IEF gels only, an extra preliminary 1 hour wash step in 30% (v/v) methanol, 10% (w/v) trichloroacetic acid, 3.5% (w/v) sulfosalicylic acid (May and Baker) was required to remove ampholytes before silver staining. An additional water wash step between silver reagent and developer addition (refer Bio-Rad 161-0443 silver stain kit instruction booklet) was omitted.

[1] The gel was placed in fixative (40% (v/v) methanol/10% (v/v) acetic acid) for at least 30 min. (On occasion, gels were left overnight in this fixative.)
[2] The gel received two 15 min washes in 10% (v/v) ethanol/5% (v/v) acetic acid.
[3] Bio-Rad oxidizer concentrate, diluted 10x in water, was applied for 3 min.
[4] Water washes (2 min each) were repeated (usually about 5x) until all orange coloration was removed from the gel.
[5] Bio-Rad silver reagent concentrate, diluted 10x in water, was applied for 15 minutes.
[6] Bio-Rad developer (11.2 g per 350 ml of water) was slowly poured over the vertically-held gel until all traces of brown/black precipitate had drained off into a sink.
[7] The gel was then bathed in fresh developer for ≈5 min intervals with agitation, until protein bands were evident.
[8] Colour development was halted by placing the gel in 5% (v/v) acetic acid.

(B) Coomassie Blue and Calcofluor White Stains

Coomassie blue was used to stain proteins, whilst Calcofluor white was used to detect the presence of chitinase activity. Details of both staining protocols are found in Chapter 2, Section 2.2.6, Part E.

APPENDIX VI - SAMPLE SAS PROGRAMMES AND OUTPUT

(A) SAS PROGRAMME AND OUTPUT FOR A SIMPLE EXPERIMENTAL DESIGN

The following SAS programme was written for Experiment 5 of Chapter 3 (Section 3.3.5). This experiment was carried out according to a completely randomized design (refer Chapter 2, Section 2.3.4, Part A). The dataset was "balanced" (Chapter 2, Section 2.3.2) and a transformation was not necessary because the requirements of ANOVA had already been satisfied (see Chapter 2, Section 2.3.1 for further explanation).

(i) SAS Programme

```
OPTIONS LS=78 PS=66 NOCENTER NODATE;
OPTIONS FORMDLIM=' ';
DATA NUTRI;
 DO TRT = 1 TO 3;
   DO REP = 1 TO 3;
      INPUT INFECT @@;
       OUTPUT;
    END;
  END;
CARDS;
41.7 38.9 44.4
72.2 69.4 80.6
47.2 50.0 58.3
;
RUN;

PROC FORMAT;
 VALUE TRE 1='SUCR' 2='YEAS' 3='WASH';
RUN;

TITLE 'ANALYSIS OF 12 WEEK 1992 SPORE NUTRIENT DATA - CRD';
TITLE2 'RAW DATA ANOVA AND MCPs';
PROC GLM DATA=NUTRI ORDER=DATA;
 FORMAT TRT TRE.;
 CLASS TRT;
 MODEL INFECT = TRT / SS1;
 MEANS TRT / LSD DUNCAN;
 OUTPUT OUT=NEW1 STUDENT=SRESD P=PRED;
RUN;

OPTIONS PS=55;
TITLE2 'RAW DATA RESIDUAL PLOT';
```

```
PROC PLOT DATA=NEW1;
 PLOT SRESD*PRED ;
RUN;

OPTIONS PS=66;
TITLE2 'ANALYSIS OF RAW DATA - MEANS etc';
PROC MEANS NOPRINT DATA=NUTRI NOPRINT;
 CLASS TRT;
 VAR INFECT;
 OUTPUT OUT=NEW2 MEAN=MINF STD=SINF STDERR=SEINF ;
RUN;
DATA NEW2; SET NEW2;
LMINF=LOG(MINF); LSINF=LOG(SINF);
MSINF=SINF/MINF; MSSINF=SINF/SQRT(MINF);
RUN;
PROC PRINT DATA=NEW2 UNIFORM;
RUN;

TITLE2 'REGRESSION TEST TO SEE IF A TRANS IS REQD';
PROC REG DATA=NEW2;
MODEL LSINF=LMINF;
RUN;
```

(ii) SAS Output

ANALYSIS OF 12 WEEK 1992 SPORE NUTRIENT DATA - CRD
RAW DATA ANOVA AND MCPs
General Linear Models Procedure
Class Level Information

Class	Levels	Values
TRT	3	SUCR YEAS WASH

Number of observations in data set = 9

Dependent Variable: INFECT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1647.442222	823.721111	33.01	0.0006
Error	6	149.720000	24.953333		
Corrected Total	8	1797.162222			

R-Square	C.V.	Root MSE	INFECT Mean
0.916691	8.943302	4.995331	55.85556

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRT	2	1647.442222	823.721111	33.01	0.0006

T tests (LSD) for variable: INFECT

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 6 MSE= 24.95333
Critical Value of T= 2.45
Least Significant Difference= 9.9801

Means with the same letter are not significantly different.

T Grouping	Mean	N	TRT
A	74.067	3	YEAS
B	51.833	3	WASH
C	41.667	3	SUCR

Duncan's Multiple Range Test for variable: INFECT

NOTE: This test controls the type I comparisonwise error rate, not the experimentwise error rate
Alpha= 0.05 df= 6 MSE= 24.95333

Number of Means 2 3
Critical Range 9.98 10.34

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	TRT
A	74.067	3	YEAS
B	51.833	3	WASH
C	41.667	3	SUCR

ANALYSIS OF RAW DATA - MEANS etc

OBS	TRT	_TYPE_	_FREQ_	MINF	SINF	SEINF	LMINF	LSINF	MSINF	MSSINF
1	.	0	9	55.8556	14.9882	4.99606	4.02277	2.70726	0.26834	2.00547
2	1	1	3	41.6667	2.7502	1.58780	3.72970	1.01166	0.06600	0.42605
3	2	1	3	74.0667	5.8287	3.36518	4.30497	1.76279	0.07869	0.67726
4	3	1	3	51.8333	5.7726	3.33283	3.94803	1.75313	0.11137	0.80181

REGRESSION TEST TO SEE IF A TRANS IS REQD

Model: MODEL1
Dependent Variable: LSINF

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	0.29850	0.29850	0.519	0.5460
Error	2	1.14939	0.57470		
Total	3	1.44789			

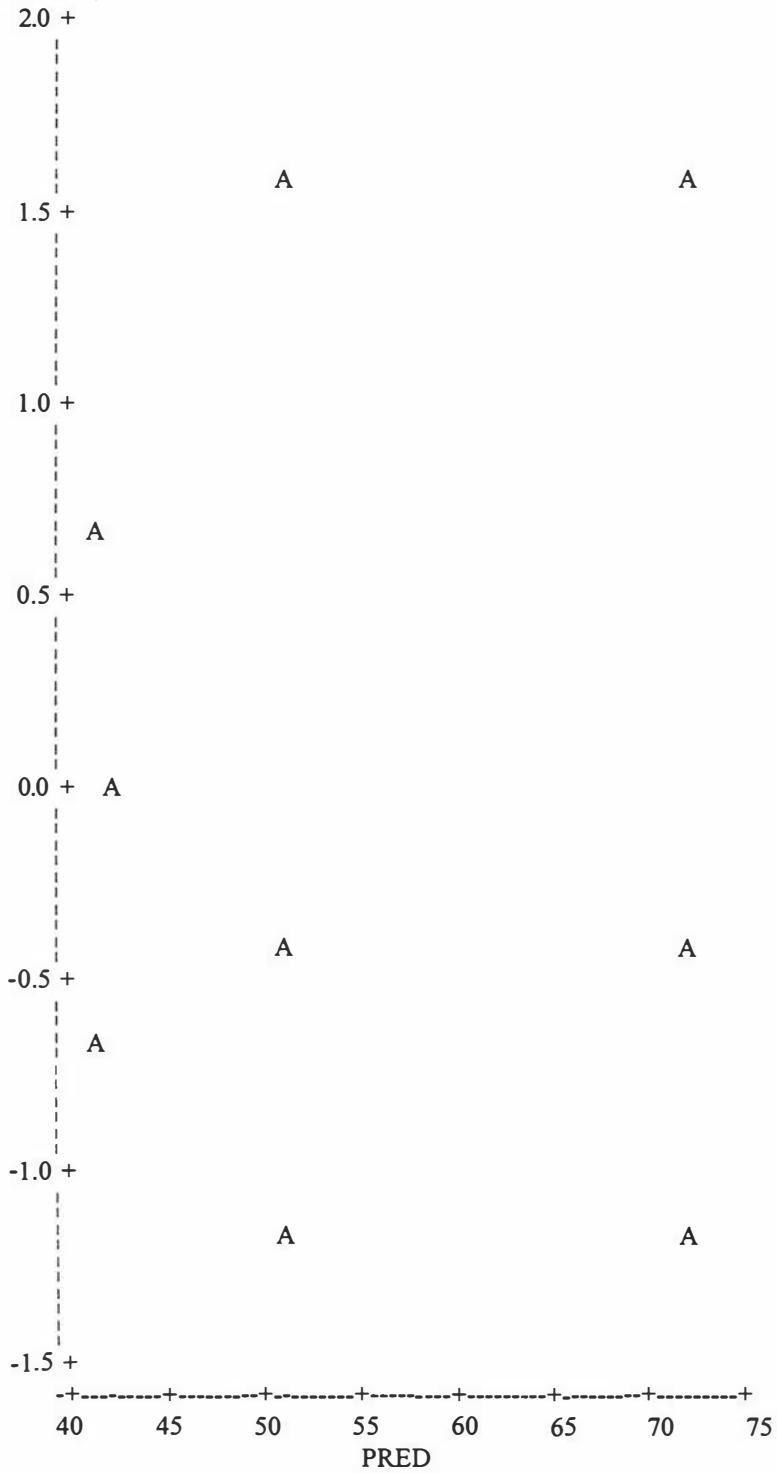
Root MSE	0.75809	R-square	0.2062
Dep Mean	1.80871	Adj R-sq	-0.1908
C.V.	41.91316		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	-3.504772	7.38247370	-0.475	0.6818
LMINF	1	1.327916	1.84255427	0.721	0.5460

RAW DATA RESIDUAL PLOT

Plot of SRESD*PRED. Legend: A = 1 obs, B = 2 obs, etc.



(B) SAS PROGRAMME AND OUTPUT FOR A MORE COMPLICATED EXPERIMENTAL DESIGN

The following SAS progamme was written for Experiment 2 of Chapter 3 (Section 3.3.2). This experiment was carried out according to a split plot design (Chapter 2, Section 2.3.4, Part D). The dataset was unbalanced and a square root transformation was required to satisfy the conditions of ANOVA (normality and homogeneity).

(i) SAS Programme

```
OPTIONS LS=78 PS=66;
OPTIONS FORMDLIM=' ';
TITLE '1993 MATURITY EXP - 12 WK DATA, SPLIT PLOT';
DATA MATURITY;
 DO MAT = 1 TO 4;
  DO INOC = 1 TO 3;
   DO BLOCK = 1 TO 4;
    INPUT INFECT @@;
     OUTPUT;
    END;
   END;
  END;
CARDS;
2.8 0 0 0
52.8 38.9 44.4 58.3
41.7 69.4 33.3 44.4
0 13.9 5.6 8.3
36.1 44.4 52.8 58.3
52.8 52.8 55.6 69.4
0 0 0 0
13.9 8.3 11.1 19.4
25 16.7 19.4 33.3
0 0 0 .
2.8 11.1 8.3 .
44.4 30.6 41.7 .
;
RUN;

PROC FORMAT;
 VALUE INO 1='0K' 2='25K' 3='125K';
 VALUE BLK 1='I' 2='II' 3='III' 4='IV';
RUN;

TITLE2 'ANALYSIS OF RAW DATA - FULLY FITTED ANOVA';
PROC GLM DATA=MATURITY ORDER=DATA;
  FORMAT INOC INO. BLOCK BLK.;
  CLASS BLOCK MAT INOC;
  MODEL INFECT = BLOCK MAT BLOCK*MAT INOC MAT*INOC
BLOCK*INOC(MAT) / SS1;
  TEST H = BLOCK MAT E = BLOCK*MAT / HTYPE=1 ETYPE=1;
```

```
  TEST H = INOC MAT*INOC E = BLOCK*INOC(MAT) / HTYPE=1 ETYPE=1;
  OUTPUT OUT=NEWMAT R=RINF P=PINF;
RUN;

TITLE2 'ANALYSIS OF RAW DATA - PARTIALLY FITTED ANOVA';
PROC GLM DATA=MATURITY ORDER=DATA;
  FORMAT INOC INO. BLOCK BLK.;
  CLASS BLOCK MAT INOC;
  MODEL INFECT = BLOCK MAT BLOCK*MAT INOC MAT*INOC / SS1;
  TEST H = BLOCK MAT E = BLOCK*MAT / HTYPE=1 ETYPE=1;
  OUTPUT OUT=NEWMAT R=RINF P=PINF;
RUN;

TITLE2 'ANALYSIS OF RAW DATA - PLOT OF THE SPLIT PLOT RESIDUALS';
PROC PLOT DATA=NEWMAT;
 PLOT RINF*PINF;
RUN;

TITLE2 'ANALYSIS OF RAW DATA - MEANS ETC';
PROC MEANS NOPRINT DATA=MATURITY ORDER=DATA;
 CLASS MAT INOC;
 VAR INFECT;
 OUTPUT OUT=TEMP MEAN=MINF STD=SINF STDERR=SEINF;
RUN;
DATA TEMP; SET TEMP;
SMINF=SINF/MINF;
SSMINF=SINF/SQRT(MINF);
RUN;
PROC PRINT DATA=TEMP UNIFORM;
WHERE _TYPE_=3;
RUN;

/* transforming the data */
DATA TRANSMAT; SET MATURITY;
SQINF = SQRT(INFECT+1);
RUN;

TITLE2 'ANALYSIS OF SQUARE ROOT DATA - FULLY FITTED ANOVA';
PROC GLM DATA=TRANSMAT ORDER=DATA;
  FORMAT INOC INO. BLOCK BLK.;
  CLASS BLOCK MAT INOC;
  MODEL SQINF = BLOCK MAT BLOCK*MAT INOC MAT*INOC BLOCK*INOC(MAT) / SS1;
  TEST H = BLOCK MAT E=BLOCK*MAT / HTYPE=1 ETYPE=1;
  TEST H = INOC MAT*INOC E=BLOCK*INOC(MAT) / HTYPE=1 ETYPE=1;
  CONTRAST 'CONTRL vs INOCULATED' INOC 6 -3 -3 / E=BLOCK*INOC(MAT)
ETYPE=1;
  MEANS MAT / DUNCAN LSD E=BLOCK*MAT ETYPE=1;
  MEANS INOC MAT*INOC / DUNCAN LSD E=BLOCK*INOC(MAT) ETYPE=1;
  LSMEANS  MAT / PDIFF STDERR E=BLOCK*MAT ETYPE=1;
  LSMEANS INOC MAT*INOC / PDIFF STDERR  E=BLOCK*INOC(MAT) ETYPE=1;
RUN;

TITLE2 'ANALYSIS OF SQUARE ROOT DATA - PARTIALLY FITTED ANOVA';
PROC GLM DATA=TRANSMAT ORDER=DATA;
  FORMAT INOC INO. BLOCK BLK.;
```

```
  CLASS BLOCK MAT INOC;
  MODEL SQINF = BLOCK MAT BLOCK*MAT INOC MAT*INOC / SS1;
  TEST H = BLOCK MAT E=BLOCK*MAT / HTYPE=1 ETYPE=1;
  OUTPUT OUT=NEWTRANS R=RSQINF P=PSQINF;
RUN;

TITLE2 'ANALYSIS OF SQUARE ROOT DATA - PLOT OF THE SPLIT PLOT
RESIDUALS';
PROC PLOT DATA=NEWTRANS;
 PLOT RSQINF*PSQINF;
RUN;

TITLE2 'ANALYSIS OF SQUARE ROOT DATA - MEANS ETC';
PROC MEANS NOPRINT DATA=TRANSMAT;
 CLASS MAT INOC;
 VAR SQINF;
 OUTPUT OUT=TEMP2 MEAN=MSQINF STD=SSQINF STDERR=SESQINF;
RUN;
PROC PRINT DATA=TEMP2 UNIFORM;
RUN;

TITLE2 'TWO-WAY TABLE OF SQUARE ROOT TRANSFORMED MEANS';
PROC TABULATE DATA=TRANSMAT F=7.2;
FORMAT INOC INO. BLOCK BLK.;
CLASS MAT INOC;
VAR SQINF;
TABLE (MAT ALL),(INOC ALL)*SQINF*(MEAN STDERR);
RUN;

OPTIONS PS=33;
TITLE2 'PROFILE PLOTS OF SQUARE ROOT TRANSFORMED MEANS';
PROC PLOT DATA=TEMP2;
FORMAT INOC INO.;
PLOT MSQINF*MAT=INOC;
PLOT MSQINF*INOC=MAT;
RUN;
```

(ii) SAS Output

1993 MATURITY EXP - 12 WK DATA, SPLIT PLOT

ANALYSIS OF RAW DATA - FULLY FITTED ANOVA

General Linear Models Procedure
Class Level Information

Class	Levels	Values
BLOCK	4	I II III IV
MAT	4	1 2 3 4
INOC	3	0K 25K 125K

Number of observations in data set = 48
NOTE: Due to missing values, only 45 observations can be used in this analysis.

Dependent Variable: INFECT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	44	22574.96000	513.06727	.	.
Error	0	.	.		
Corrected Total	44	22574.96000			

R-Square	C.V.	Root MSE	INFECT Mean
1.000000	0	0	24.93333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	634.12944	211.37648	.	.
MAT	3	4947.94315	1649.31438	.	.
BLOCK*MAT	8	386.61407	48.32676	.	.
INOC	2	12751.16133	6375.58067	.	.
MAT*INOC	6	2662.78700	443.79783	.	.
BLOCK*INOC(MAT)	22	1192.32500	54.19659	.	.

Tests of Hypotheses using the Type I MS for BLOCK*MAT as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	634.129444	211.376481	4.37	0.0422
MAT	3	4947.943148	1649.314383	34.13	0.0001

Tests of Hypotheses using the Type I MS for BLOCK*INOC(MAT) as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
INOC	2	12751.16133	6375.58067	117.64	0.0001
MAT*INOC	6	2662.78700	443.79783	8.19	0.0001

ANALYSIS OF RAW DATA - PARTIALLY FITTED ANOVA

General Linear Models Procedure
Class Level Information

Class	Levels	Values
BLOCK	4	I II III IV
MAT	4	1 2 3 4
INOC	3	0K 25K 125K

Number of observations in data set = 48
NOTE: Due to missing values, only 45 observations can be used in this analysis.

Dependent Variable: INFECT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	21382.63500	971.93795	17.93	0.0001
Error	22	1192.32500	54.19659		
Corrected Total	44	22574.96000			

R-Square	C.V.	Root MSE	INFECT Mean
0.947184	29.52607	7.361833	24.93333

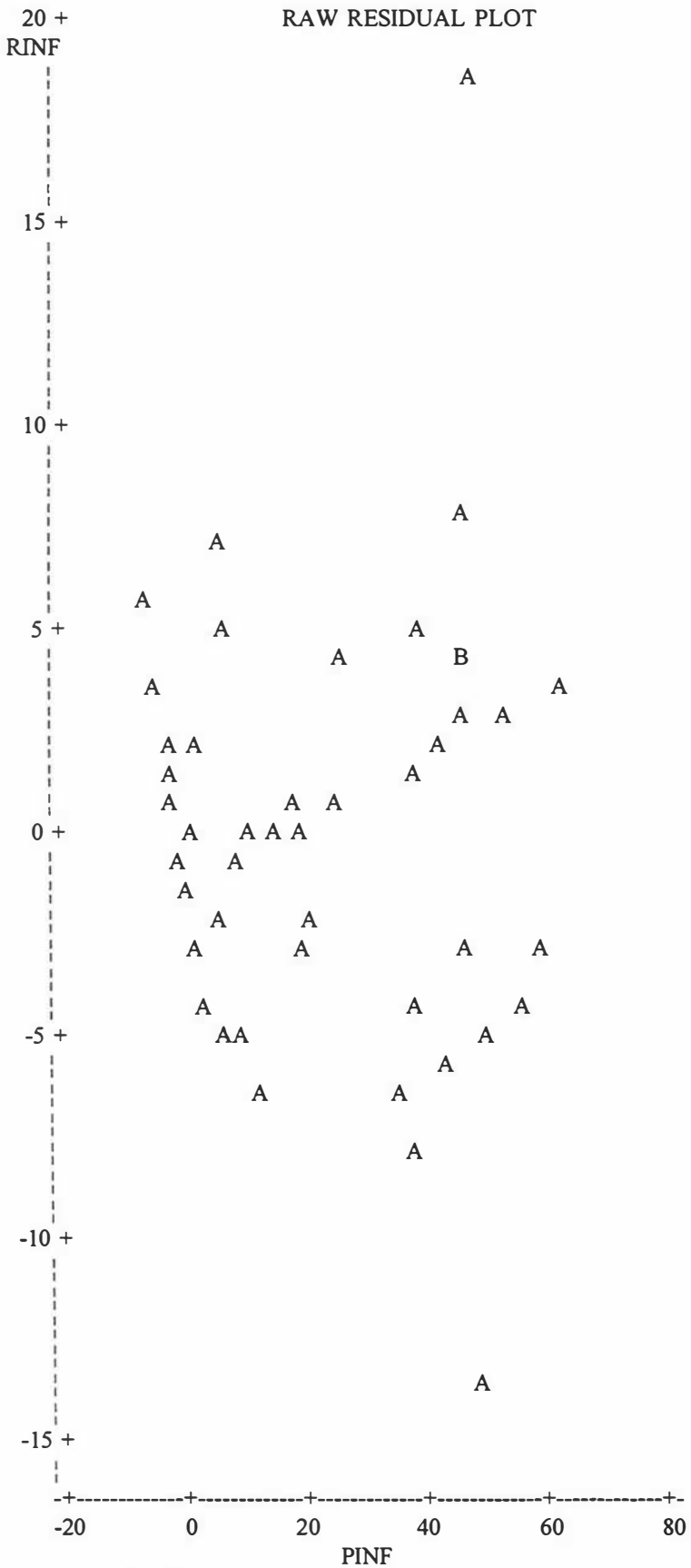
Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK*MAT	8	386.61407	48.32676	0.89	0.5395
INOC	2	12751.16133	6375.58067	117.64	0.0001
MAT*INOC	6	2662.78700	443.79783	8.19	0.0001

Tests of Hypotheses using the Type I MS for BLOCK*MAT as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	634.129444	211.376481	4.37	0.0422
MAT	3	4947.943148	1649.314383	34.13	0.0001

ANALYSIS OF RAW DATA - MEANS ETC

OBS	MAT	INOC	_TYPE_	_FREQ_	MINF	SINF	SEINF	SMINF	SSMINF
9	1	1	3	4	0.700	1.4000	0.70000	2.00000	1.67332
10	1	2	3	4	48.600	8.6306	4.31528	0.17758	1.23800
11	1	3	3	4	47.200	15.5364	7.76820	0.32916	2.26141
12	2	1	3	4	6.950	5.7807	2.89036	0.83176	2.19275
13	2	2	3	4	47.900	9.7239	4.86193	0.20300	1.40498
14	2	3	3	4	57.650	7.9438	3.97188	0.13779	1.04623
15	3	1	3	4	0.000	0.0000	0.00000	.	.
16	3	2	3	4	13.175	4.7381	2.36903	0.35962	1.30534
17	3	3	3	4	23.600	7.3326	3.66629	0.31070	1.50939
18	4	1	3	4	0.000	0.0000	0.00000	.	.
19	4	2	3	4	7.400	4.2226	2.43790	0.57062	1.55224
20	4	3	3	4	38.900	7.3137	4.22256	0.18801	1.17263



NOTE: 3 obs had missing values.

ANALYSIS OF SQUARE ROOT DATA - FULLY FITTED ANOVA

Class	Levels	Values
BLOCK	4	I II III IV
MAT	4	1 2 3 4
INOC	3	0K 25K 125K

Number of observations in data set = 48

NOTE: Due to missing values, only 45 observations can be used in this analysis.

Dependent Variable: SQINF

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	44	291.9138735	6.6344062	.	.
Error	0	.	.		
Corrected Total	44	291.9138735			

R-Square	C.V.	Root MSE	SQINF Mean
1.000000	0	0	4.409803

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	5.6415467	1.8805156	.	.
MAT	3	49.8325066	16.6108355	.	.
BLOCK*MAT	8	4.5629680	0.5703710	.	.
INOC	2	201.1570123	100.5785061	.	.
MAT*INOC	6	21.1879766	3.5313294	.	.
BLOCK*INOC(MAT)	22	9.5318632	0.4332665	.	.

Tests of Hypotheses using the Type I MS for BLOCK*MAT as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	5.64154674	1.88051558	3.30	0.0787
MAT	3	49.83250665	16.61083555	29.12	0.0001

Tests of Hypotheses using the Type I MS for BLOCK*INOC(MAT) as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
INOC	2	201.1570123	100.5785061	232.14	0.0001
MAT*INOC	6	21.1879766	3.5313294	8.15	0.0001

T tests (LSD) for variable: SQINF

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 8 MSE= 0.570371
Critical Value of T= 2.31
Least Significant Difference= 0.74
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 11.07692

Means with the same letter are not significantly different.

T Grouping	Mean	N	MAT
A	5.7439	12	2
A	5.0466	12	1
B	3.3746	9	4
B	3.2153	12	3

T tests (LSD) for variable: SQINF

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 22 MSE= 0.433267
Critical Value of T= 2.07
Least Significant Difference= 0.4985
Means with the same letter are not significantly different.

T Grouping	Mean	N	INOC
A	6.4451	15	125K
B	5.2891	15	25K
C	1.4952	15	0K

Duncan's Multiple Range Test for variable: SQINF
Alpha= 0.05 df= 8 MSE= 0.570371
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 11.07692

Number of Means 2 3 4
Critical Range .7400 .7712 .7886

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	MAT
A	5.7439	12	2
A			
A	5.0466	12	1
B	3.3746	9	4
B			
B	3.2153	12	3

Duncan's Multiple Range Test for variable: SQINF

Alpha= 0.05 df= 22 MSE= 0.433267

Number of Means 2 3
Critical Range .4985 .5234

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	INOC
A	6.4451	15	125K
B	5.2891	15	25K
C	1.4952	15	0K

Level of MAT	Level of INOC	N	SQINF Mean	SQINF SD
1	0K	4	1.23733972	0.47467943
1	25K	4	7.02252364	0.61553423
1	125K	4	6.87989172	1.07523015
2	0K	4	2.61967212	1.20405869
2	25K	4	6.96610670	0.70555648
2	125K	4	7.64586615	0.50428940
3	0K	4	1.00000000	0.00000000
3	25K	4	3.72619582	0.62232327
3	125K	4	4.91985310	0.72575979
4	0K	3	1.00000000	0.00000000
4	25K	3	2.82581814	0.78875074
4	125K	3	6.29795455	0.59468713

ANALYSIS OF SQUARE ROOT DATA - LSMeans

Standard Errors and Probabilities calculated using the Type I MS for BLOCK*MAT as an Error term

MAT	SQINF LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	5.04658502	0.21801586	0.0001	1
2	5.74388165	0.21801586	0.0001	2
3	3.21534964	0.21801586	0.0001	3
4	Non-est	.	.	4

Pr > |T| H0: LSMEAN(i)=LSMEAN(j)

i/j	1	2	3	4
1	.	0.0536	0.0003	.
2	0.0536	.	0.0001	.
3	0.0003	0.0001	.	.
4	.	.	.	.

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

INOC	SQINF LSMEAN
0K	Non-est
25K	Non-est
125K	Non-est

Standard Errors and Probabilities calculated using the Type I MS for BLOCK*INOC(MAT) as an Error term

MAT	INOC	SQINF LSMEAN	Std Err LSMEAN	Pr > |T| H0:LSMEAN=0	LSMEAN Number
1	0K	1.23733972	0.32911491	0.0011	1
1	25K	7.02252364	0.32911491	0.0001	2
1	125K	6.87989172	0.32911491	0.0001	3
2	0K	2.61967212	0.32911491	0.0001	4
2	25K	6.96610670	0.32911491	0.0001	5
2	125K	7.64586615	0.32911491	0.0001	6
3	0K	1.00000000	0.32911491	0.0060	7
3	25K	3.72619582	0.32911491	0.0001	8
3	125K	4.91985310	0.32911491	0.0001	9
4	0K	Non-est	.	.	10
4	25K	Non-est	.	.	11
4	125K	Non-est	.	.	12

LSMeans cont'd

Pr > |T| H0: LSMEAN(i)=LSMEAN(j)

i/j	1	2	3	4	5	6	7	8	9
1	.	0.0001	0.0001	0.0071	0.0001	0.0001	0.6152	0.0001	0.0001
2	0.0001	.	0.7621	0.0001	0.9046	0.1942	0.0001	0.0001	0.0002
3	0.0001	0.7621	.	0.0001	0.8547	0.1140	0.0001	0.0001	0.0004
4	0.0071	0.0001	0.0001	.	0.0001	0.0001	0.0021	0.0266	0.0001
5	0.0001	0.9046	0.8547	0.0001	.	0.1583	0.0001	0.0001	0.0002
6	0.0001	0.1942	0.1140	0.0001	0.1583	.	0.0001	0.0001	0.0001
7	0.6152	0.0001	0.0001	0.0021	0.0001	0.0001	.	0.0001	0.0001
8	0.0001	0.0001	0.0001	0.0266	0.0001	0.0001	0.0001	.	0.0177
9	0.0001	0.0002	0.0004	0.0001	0.0002	0.0001	0.0001	0.0177	.
10	.	.	.	.	.	.	.	.	.
11	.	.	.	.	.	.	.	.	.
12	.	.	.	.	.	.	.	.	.

Pr > |T| H0: LSMEAN(i)=LSMEAN(j)

i/j	10	11	12
1-12	.	.	.

ANALYSIS OF SQUARE ROOT DATA - PARTIALLY FITTED ANOVA

Class	Levels	Values
BLOCK	4	I II III IV
MAT	4	1 2 3 4
INOC	3	0K 25K 125K

Number of observations in data set = 48

NOTE: Due to missing values, only 45 observations can be used in this analysis.

Dependent Variable: SQINF

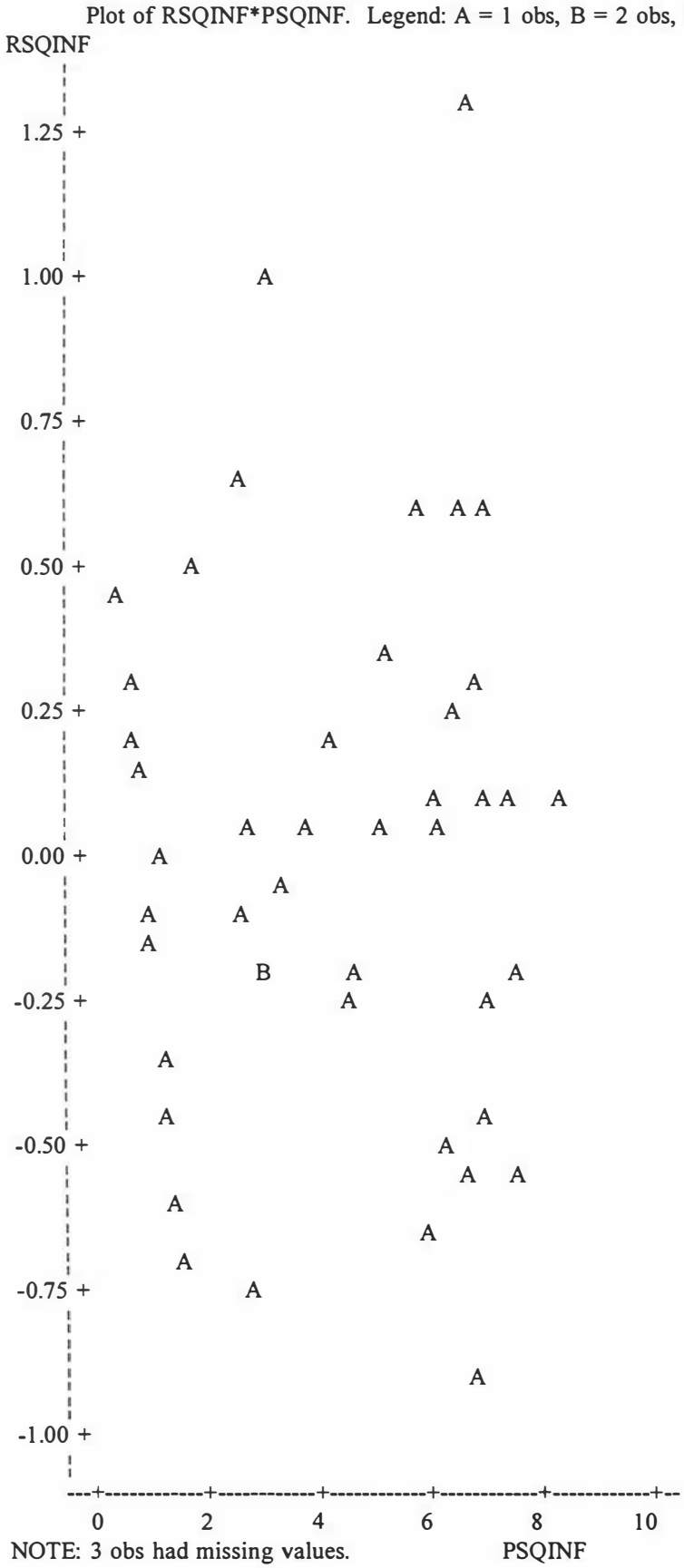
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	282.3820103	12.8355459	29.63	0.0001
Error	22	9.5318632	0.4332665		
Corrected Total	44	291.9138735			

R-Square	C.V.	Root MSE	SQINF Mean
0.967347	14.92651	0.658230	4.409803

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK*MAT	8	4.5629680	0.5703710	1.32	0.2866
INOC	2	201.1570123	100.5785061	232.14	0.0001
MAT*INOC	6	21.1879766	3.5313294	8.15	0.0001

Tests of Hypotheses using the Type I MS for BLOCK*MAT as an error term

Source	DF	Type I SS	Mean Square	F Value	Pr > F
BLOCK	3	5.64154674	1.88051558	3.30	0.0787
MAT	3	49.83250665	16.61083555	29.12	0.0001


Plot of RSQINF*PSQINF. Legend: A = 1 obs, B = 2 obs, etc.
RSQINF
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
0
2
4
6
8
10
NOTE: 3 obs had missing values.
PSQINF

ANALYSIS OF SQUARE ROOT DATA - MEANS ETC

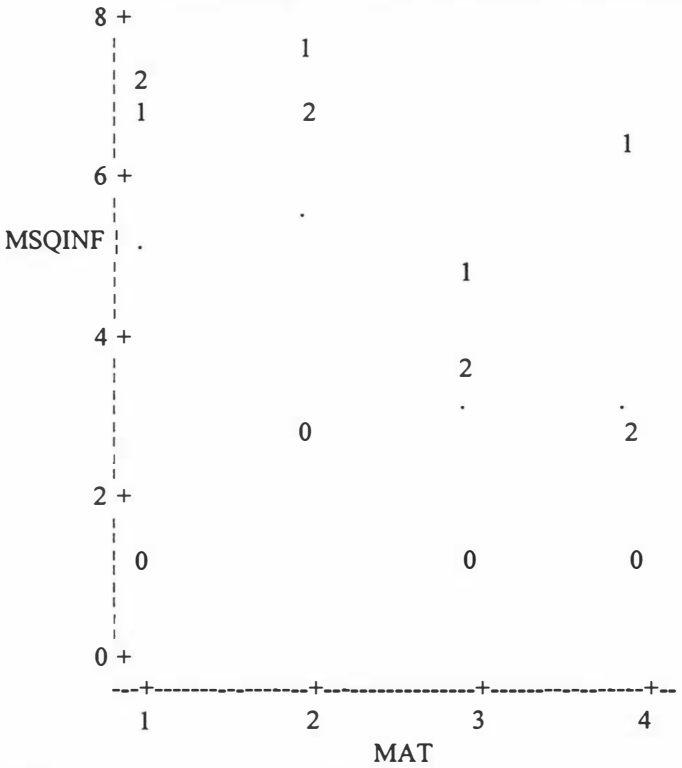
OBS	MAT	INOC	_TYPE_	_FREQ_	MSQINF	SSQINF	SESQINF
1	.	.	0	48	4.40980	2.57573	0.38397
2	.	1	1	16	1.49520	0.92834	0.23970
3	.	2	1	16	5.28912	2.00497	0.51768
4	.	3	1	16	6.44509	1.26527	0.32669
5	1	.	2	12	5.04659	2.89802	0.83659
6	2	.	2	12	5.74388	2.45123	0.70761
7	3	.	2	12	3.21535	1.78474	0.51521
8	4	.	2	12	3.37459	2.38247	0.79416
9	1	1	3	4	1.23734	0.47468	0.23734
10	1	2	3	4	7.02252	0.61553	0.30777
11	1	3	3	4	6.87989	1.07523	0.53762
12	2	1	3	4	2.61967	1.20406	0.60203
13	2	2	3	4	6.96611	0.70556	0.35278
14	2	3	3	4	7.64587	0.50429	0.25214
15	3	1	3	4	1.00000	0.00000	0.00000
16	3	2	3	4	3.72620	0.62232	0.31116
17	3	3	3	4	4.91985	0.72576	0.36288
18	4	1	3	4	1.00000	0.00000	0.00000
19	4	2	3	4	2.82582	0.78875	0.45539
20	4	3	3	4	6.29795	0.59469	0.34334

TWO-WAY TABLE OF SQUARE ROOT TRANSFORMED MEANS

MAT	INOC						ALL	
	0K		25K		125K			
	SQINF		SQINF		SQINF		SQINF	
	Mean	Stderr	Mean	Stderr	Mean	Stderr	Mean	Stderr
1	1.24	0.24	7.02	0.31	6.88	0.54	5.05	0.84
2	2.62	0.60	6.97	0.35	7.65	0.25	5.74	0.71
3	1.00	0.00	3.73	0.31	4.92	0.36	3.22	0.52
4	1.00	0.00	2.83	0.46	6.30	0.34	3.37	0.79
ALL	1.50	0.24	5.29	0.52	6.45	0.33	4.41	0.38

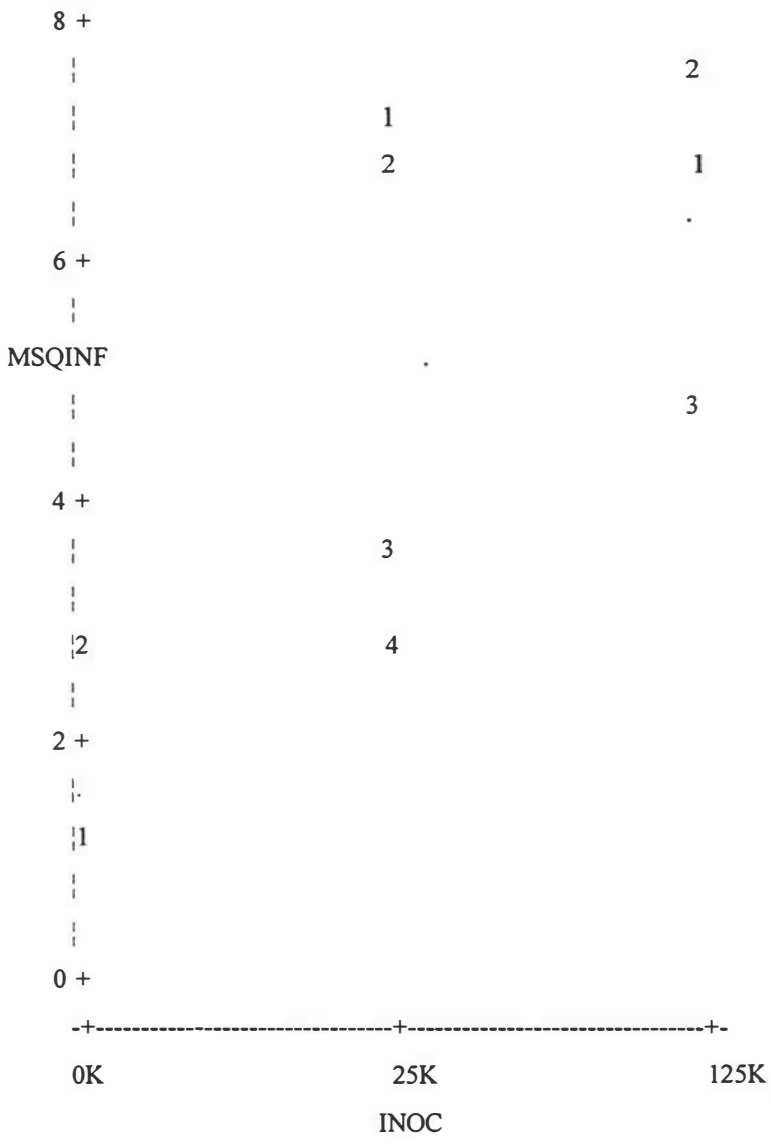
PROFILE PLOTS OF SQUARE ROOT TRANSFORMED MEANS

Plot of MSQINF*MAT. Symbol is value of INOC.



NOTE: 4 obs had missing values.

Plot of MSQINF*INOC. Symbol is value of MAT.



NOTE: 5 obs had missing values. 3 obs hidden.

APPENDIX VII - MEASUREMENT OF ENVIRONMENTAL PARAMETERS IN THE ARTIFICIAL INOCULATION EXPERIMENTS

Fruit temperature was measured using a temperature probe attached to a Grant Squirrel 1200 data logger. Air temperature and relative humidity were measured using a swinging hygrometer.

(A) MEASUREMENTS FOR EXPERIMENT 1 OF CHAPTER 3

There was no rain, and environmental conditions during harvest and inoculation were comparable at each harvest (VII-1). The afternoon of the first harvest was warmer than that of the others and fruit were just over 4°C warmer at coolstorage than those of the following harvest.

VII-1 : Timing, and temperature and relative humidity (rh) : Experiment 1, Chapter 3.

ENVIRONMENTAL CONDITIONS	HARVEST 1 15/4/92	HARVEST 2 18/5/92	HARVEST 3 25/5/92
Harvest-start			
Start time	10 am	8.35 am	8 am
Air temp.	11.5°C	11°C	8°C
rh	94 %	87 %	-[†]
Fruit temp.[*]	9.7 ± 0.1°C	9.7 ± 0.1°C	7.7 ± 0.1°C
Harvest-finish			
Finish time	10.35 am	9.20 am	8.25 am
Air temp.	11.5°C	12°C	8°C
rh	94 %	82 %	-
Fruit temp.	9.7 ± 0.1°C	10.3 ± 0.1°C	7.7 + 0.1°C
Inoculation-start			
Start time	3 pm	1.55 pm	3.50 pm
Air temp.	-	15°C	15.5°C
rh	-	63 %	-
Fruit temp.	-	13.2 ± 0.2°C	14.7 ± 0.4°C
Inoculation-finish			
Finish time	3.40 pm	2.10 pm	4.25 pm
Air temp.	18°C	15°C	15.5°C
rh	62 %	63 %	-
Fruit temp.	17.4 ± 0.0°C	13.2 ± 0.2°C	14.7 ± 0.4°C
Time into coolstore	5 pm	4.10 pm	5.50 pm

[*] mean ± SEM fruit temperature
[†] not measured

(B) MEASUREMENTS FOR EXPERIMENT 2 OF CHAPTER 3

At all four harvests the weather was fine, although on 17/5/93 it had rained overnight so the fruit was slightly wet when harvested. Temperatures varied by no more than 3°C across harvests at comparable stages, but relative humidities differed by up to 30 % at the start of harvests (VII-2).

VII-2 : Timing, temperature and relative humidity (rh) : Experiment 2, Chapter 3.

ENVIRONMENTAL CONDITIONS*	Harvest 1 30/4/93	Harvest 2 17/5/93	Harvest 3 3/6/93	Harvest 4 16/6/93
Harvest-start				
Start time	8.20 am	8.50 am	8.30 am	8.20 am
Air temp.	13°C	14°C	14°C	11°C
rh	85 %	72 %	52 %	81 %
Harvest-finish				
Finish time	8.40 am	9.15 am	9.35 am	8.50 am
Air temp.	13°C	14°C	11°C	11°C
rh	91 %	72 %	75 %	87 %
Inoculation-start				
Start time	9.30 am	10.10 am	9.55 am	9.25 am
Air temp.	-†	15°C	15.5°C	14°C
rh	73 %	68 %	66 %	72 %
Inoculation-finish				
Finish time	10.40 am	10.30 am	10.10 am	9.40 am
Air temp.	-	15°C	17°C	14°C
rh	73 %	68 %	65 %	72 %
Time into coolstore	12.30 pm	12.35 pm	12.55 am	12.05 pm

* Air temperature (Air temp.) and relative humidity (rh) at the start and finish of harvest were measured in the field. Measurement of the inoculation environmental conditions were made in the packing shed

† not measured

(C) MEASUREMENTS FOR EXPERIMENT 3 OF CHAPTER 3

Environmental conditions on the two harvest dates were very different (VII-3 and VII-4). On 13/5/92, the day began with a frost, then temperatures just above 0°C and moderate-low relative humidity (64%) by 8 am, with clear fine weather. The temperature rose throughout the day, peaking at 2 pm, then dropping 3°C over the next 6 h. On 19/5/92, the weather was overcast with occasional periods of drizzle. The cloud cover kept temperatures more constant throughout the day, although once again temperature peaked at 2 pm. Overall rh was higher than at the first harvest.

VII-3 : Time of harvest, temperature and relative humidity (rh) at the 13/5/92 harvest of Experiment 3 (Chapter 3).

CONDITIONS AT EACH TWO HOURLY HARVEST INTERVAL*	EXPERIMENTAL PHASE		
	Harvest Start	Harvest Finish	Inoculation
8 am	7.50 am	8.15 am	9.50 am
Air temp.	1°C	4°C	12°C
rh	64 %	75 %	48 %
Fruit temp.†	-0.9 ± 0.1°C	-‡	10.2 ± 0.4°C
10 am	9.55 am	10.10 am	10.25 am
Air temp.	9°C	9°C	13°C
rh	80 %	80 %	50 %
Fruit temp.	5.6 ± 0.0°C	6.1 ± 0.0°C	10.4 ± 0.2°C
12 noon	11.40 am	11.55 am	12.30 pm
Air temp.	11.5°C	11.5°C	13.5°C
rh	75.5 %	75.5 %	61 %
Fruit temp.	10.0 ± 0.1°C	10.0 ± 0.1°C	12.5 ± 0.3°C
2 pm	2.15 pm	2.30 pm	2.45 pm
Air temp.	13.5°C	14°C	15.5°C
rh	66.5 %	67 %	49 %
Fruit temp.	13.3 ± 0.0°C	12.6 ± 0.0°C	13.7 ± 0.2°C
4 pm	3.50 pm	4.05 pm	4.35 pm
Air temp.	12.5°C	12°C	16°C
rh	65.6 %	70 %	55.5 %
Fruit temp.	13.0 ± 0.0°C	13.0 ± 0.0°C	13.7 ± 0.5°C
6 pm	5.55 pm	6.10 pm	7.00 pm
Air temp.	12°C	12°C	13.5°C
rh	70 %	76 %	66.5%
Fruit temp.	9.6 ± 0.0°C	9.7 ± 0.0°C	12.7 ± 0.2°C
8 pm	8 pm	8.25 pm	9.35 pm
Air temp.	10.5°C	9.5°C	15.5°C
rh	74.5 %	86.5 %	58.5 %
Fruit temp.	9.0 ± 0.0°C	8.9 ± 0.0°C	11.8 ± 0.3°C

* environmental conditions at harvest were measured in the field and in the packing shed during inoculation

† mean ± SEM fruit temperature

‡ not measured

VII-4 : Time of harvest, temperature and relative humidity (rh) at the 19/5/92 harvest of Experiment 3 (Chapter 3).

CONDITIONS AT EACH TWO HOURLY HARVEST INTERVAL*	EXPERIMENTAL PHASE		
	Harvest Start	Harvest Finish	Inoculation
8 am	8.05 am	8.25 am	9.40 am
Air temp.	10.5°C	10.5°C	13.5°C
rh	87 %	87 %	66.5 %
Fruit temp.†	10.4 ± 0.0°C	10.5 ± 0.0°C	11.8 ± 0.2°C
10 am	9.55 am	10.05 am	10.50 am
Air temp.	13°C	13°C	15°C
rh	71 %	66 %	68 %
Fruit temp.	11.8 ± 0.0°C	11.8 ± 0.0°C	13.5 ± 0.1°C
12 noon	11.50 am	12.00 pm	12.50 pm
Air temp.	13.5°C	14.5°C	15.5°C
rh	71.5 %	67 %	63.5 %
Fruit temp.	13.4 ± 0.0°C	13.4 ± 0.0°C	14.4 ± 0.1°C
2 pm	1.50 pm	2.00 pm	2.35 pm
Air temp.	13.5°C	14°C	15°C
rh	71.5 %	67 %	63 %
Fruit temp.	14.2 ± 0.0°C	13.7 ± 0.0°C	14.7 ± 0.3°C
4 pm	3.55 pm	4.05 pm	4.35 pm
Air temp.	13°C	12.5°C	15°C
rh	77 %	88 %	73 %
Fruit temp.	11.9 ± 0.0°C	11.8 ± 0.0°C	13.3 ± 0.1°C
6 pm	6.10 pm	6.20 pm	7.00 pm
Air temp.	13°C	12.5°C	14°C
rh	71 %	70.5 %	72 %
Fruit temp.	11.3 ± 0.0°C	11.1 ± 0.0°C	12.5 ± 0.1°C
8 pm	7.50 pm	8.05 pm	8.45 pm
Air temp.	11.5°C	12°C	14.5°C
rh	87.5 %	82 %	67.5 %
Fruit temp.	10.2 ± 0.0°C	10.5 ± 0.0°C	12.2 ± 0.0°C

* environmental conditions at harvest were measured in the field and in the packing shed during inoculation

† mean ± SEM fruit temperature